UNIVERSITY OF SOUTHAMPTON

ALLOPATRIC SPECIATION IN THE LITTORAL GASTROPOD GENUS *OSILINUS* PHILIPPI, 1847 (GASTROPODA: TROCHIDAE) AT THE ATLANTIC/ MEDITERRANEAN INTERFACE

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ABSTRACT

FACULTY OF SCIENCE

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By Joanne Preston

This thesis addresses the problem of speciation in marine ecosystems using a phylogeographic approach. The overall aim was to present a model of speciation in the intertidal gastropod *Osilinus* Philippi, 1847 in the Northeast Atlantic and Mediterranean. The study of speciation in the natural environment is essentially the observation of patterns, such as geographic distribution, evolutionary relationships and taxonomic diversity. Hypotheses to explain these patterns are based largely on the allopatric model of speciation, and the processes of vicariance and dispersal.

Chapter 1 presents an overview of the taxonomic status and biogeography of *Osilinus* at the outset of the work to demonstrate the value of using this genus as a model organism for understanding speciation in the marine environment. The challenges faced by studying speciation in the marine environment are summarized. A case is made for using the Northeast Atlantic and Mediterranean as an ideal model system for furthering our understanding of this subject.

Current models of speciation are reviewed and placed in the context of marine ecosystems in chapter 2. Palaeoceanography can be used to contextualise temporal patterns of evolution, and infer processes that have resulted in the biogeography of a marine species. An overview of the palaeoceanography of the Northeast Atlantic and Mediterranean within timescales relevant to the speciation of *Osilinus* is also given.

A description of species and the geographic distribution of each species are presented in chapter 3 in light of taxonomic clarity (in part based on Chapter 6). Hypotheses are presented on the mechanisms (palaeoceanographic events) that may have caused the observed radiation of *Osilinus*, as suggested by their present distribution.

The molecular techniques and protocols, and phylogenetic analyses methods implemented in this thesis are outlined in chapter 4. In chapter 5 the partial nuclear 28S gene and partial mitochondrial 16S gene are used to construct a phylogeny of all *Osilinus* species and seven gibbuline outgroup taxa. The resultant phylogeny is used to identify the sister taxa to *Osilinus,* and thus the most appropriate outgroup for interspecific phylogenetic analyses.

The taxonomic status, and interspecific evolutionary relationships of species within *Osilinus* is clarified in chapter 6 using the partial mitochondrial genes 16S and COI. The geographic pattern of speciation is estimated from this species-level phylogeny. Allopatric speciation by vicariance, and peripatric speciation appear to have driven the simultaneous radiation of two main lineages in *Osilinus* within the Mediterranean and Northeast Atlantic. The speciation of *Osilinus* is discussed in context of the vicariant palaeoceanograhic events hypothesised in chapter 3.

A morphological cladistic approach is used to complement the molecular analyses of interspecific relationships of *Osilinus* and thereby provide a more robust phylogeny in chapter 7. The phylogenetic usefulness of radula characters in resolving evolutionary relationships within *Osilinus* and the selected outgroup taxa is investigated. The radula characters are superimposed on the molecular phylogeny from chapter 5, and the evolution of radula characters is discussed.

An interdisciplinary approach to understanding the evolution of *Osilinus* has been applied in this thesis. In the general discussion an overview of the phylogeography of *Osilinus* is given: the key results from this thesis are summarised and a model presented of allopatric speciation in *Osilinus* at the Mediterranean/ Atlantic interface.

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Table 7.3: Character state matrix for the cladistic analysis of Gibbulini species. For description of character states see Table 7.2. Unknown or ambiguous characters states are indicated by **?**

Chapter 1
General Introduction

1.1 Introduction

My thesis addresses the problem of speciation in marine ecosystems using a phylogeographic approach. The overall aim of my work is to present a model of speciation in the intertidal gastropod *Osilinus* (previously called *Monodonta*) in the Northeast Atlantic and Mediterranean. To achieve this, the evolutionary relationships of the species within the genus *Osilinus* are resolved using molecular and morphological phylogenetic analyses. The geographical distributions of these lineages are subsequently used to examine the mode of speciation that has led to the present radiation of *Osilinus* in the Northeast Atlantic and Mediterranean. Based on the inferred speciation mode, and interpreted in light of known historical events, hypotheses on the mechanisms and processes driving the evolutionary divergence of *Osilinus* will be considered.

This chapter will give a brief outline of the challenges faced by studying speciation in the marine environment, and why the Northeast Atlantic and Mediterranean is an ideal model system to use in furthering our understanding of this subject. An overview of the biology, ecology, taxonomic status and known distribution of *Osilinus* at the outset of this work is given to show the value of using species of this genus as model organisms for understanding speciation in the marine environment. The molecular and morphological systematic approaches implemented in this thesis are then summarised and their relevance to achieving the aims of the thesis justified. Finally the rationale of the thesis is presented, followed by the specific objectives of future chapters.

The present understanding of speciation in the marine environment is limited by several factors. The systematics of many cosmopolitan marine species is thought to be conservative (Klautau, *et al.,* 1999), thereby underestimating the number of species and impeding understanding of speciation patterns. Understanding of evolutionary relationships, particularly within apparent sibling species complexes is also limited (Knowlton, 1993). Knowledge of the life histories and dispersal abilities of marine organisms is inadequate (Palumbi, 1992), as is the recognition and discernment of barriers to gene flow in an essentially contiguous environment (Palumbi, 1994). These limitations to understanding speciation in the marine environment are compounded by the difficulties and incongruence of applying speciation models based on terrestrial environments in which high dispersal animal species are rare (Palumbi, 1992).

The Northeast Atlantic and Mediterranean is a tractable and interesting model system for the study of speciation in the marine environment (Krijgsman, 2002). Within this palaeoceanographically complex area, the intertidal trochid *Osilinus* has apparently undergone adaptive radiation (Hickman, 1996). There are several events that may have caused this adaptive divergence within *Osilinus.* Geographical vicariant events have separated previously continuous environments and their populations. The Mediterranean is a remnant of the ancient Tethys ocean that historically formed an extensive connection between the Atlantic and Eastern oceans (McKenzie, 1986). The

numerous Pleistocene ice ages have had a dramatic effect on North European climate, sea level and coastal topology (Zhang, 1996), leading to the extension and retraction of climate dependent ranges. The eastern and western Mediterranean basins have been desiccated and isolated from the Atlantic and each other at different stages during the Cenozoic era (Maldonado, 1985; Oliverio, 1996). Such catastrophic events are likely to have resulted in population bottlenecks or extinction of occupying fauna.

The oceanic islands of Azores, Madeira, Selvagens, Canaries and Cape Verde Islands arose by volcanic hotspot activity (Geldmacher, 2000; Juan *et al.*, 2000) and have never been connected to the continental landmasses (Nunn, 1994), providing new ecological niches at the periphery of species ranges. There have been successive fluctuations in the degree of isolation of these island habitats during the Pleistocene (Epp & Smoot, 1989). Seamounts have been periodically exposed and submerged during eustatic-glacial changes (Epp & Smoot, 1989), forming stepping-stones between the Iberian Peninsula and the Atlantic Islands (Madeira, Selvagens, Canaries, Azores and Cape Verde Islands). Changes in the presence and direction of oceanic currents within the Northeast Atlantic are also likely to have altered historical larval dispersal potential and direction (Benson *et al.*, 1991).

1.2 The taxonomic status and known distribution of *Osilinus* at the outset of this work

The taxonomic framework of *Osilinus*, is generally perceived as follows (Hickman & Mclean, 1990):

Kingdom – **Animalia**
- Phylum – **Mollusca**
  - Class – **Gastropoda**
    - Subclass – **Prosobranchia**
      - Order – **Archaeogastropoda**
        - Suborder – **Vestigastropoda**
          - Superfamily – **Trochoidea**
            - Family – **Trochidae**
              - Subfamily – **Trochinae**
                - Tribe – **Gibbulini**
                - Genus - ***Osilinus***

The evolutionary relationships of taxa within these groupings are confused, or at least debated below the class level (Hickman, 1996). A brief overview of the literature concerning the classification of groups within Gastropoda, is given below. A glossary of terms used within the text throughout the thesis to describe systematic relationships is given at the end of this chapter (see Table 1.3).

Gastropods are distinguished from other Mollusca by the event of torsion. Based on this synapomorphy, the Gastropoda are considered a monophyletic group (Fretter & Graham, 1994; Ponder & Lindberg, 1996). Traditional classification has used morphological characters to define the similarities and differences between organisms to estimate evolutionary relationships (Kitching *et al.,* 1998). Thiele (1925, 1929) published a handbook of systematics that definitively influenced the subsequent classification system for gastropods (Table 1.1).

Table 1.1. Early classification of Gastropoda. Taken from text in Fretter & Graham (1994).

Subclass	Order	Modifications
Prosobranchia	Archaeogastropoda	
	Mesogastropoda	Merged into the order Caenogastropoda by Cox (1960a).
	Stenoglossa (renamed Neogastropoda, Wenz, 1938).	
Opistobranchia		Merged into the subclass Euthyneura (Boettger, 1955) and later Pentaganglionata (Haszprunar, 1985b)
Pulmonata		

Since the Thiele-Wenz classification, there has been an extensive rearrangement of the classification of Gastropoda, particularly within Archaeogastropoda (Cox & Knight, 1960; Salvini-Plawen, 1980; Hazprunnar, 1988; Hickman, 1988; Hedegaard, 1990; Ponder & Lindberg, 1996, 1997; Sasaki, 1998). The Archaeogastropoda are now generally considered polyphyletic (Hazprunar, 1988). Hickman (1988) strived for a phylogenetic classification of Archaeogastropoda (but conceded this may not be feasible, leading to taxonomic inflation). In a systematic revision of the suprageneric classification of Trochoidean gastropods, 3 superfamilies were recognised within Archaeogastropoda: Trochacea (or Trochoidea), Pleurotomariacea and Fissurellacea (Hickman & McLean, 1990). The taxonomic revision by Sasaki (1998) incorporated new observations based on internal anatomy, hypotheses of character state transformation and the relationships between soft and shell parts, and used cladistic analysis to build on previous work (mainly Salvini-Plawen, 1980; Salvini-Plawen & Haszprunar, 1987; Haszprunar, 1988, 1993; Ponder & Lindberg, 1996, 1997). The resultant classification of Archaeogastropoda differed mainly in the definition of Caenogastropoda from Hazprunar (1988) and rejected the phylogenetic reorganization of Hickman & McLean (1990) which only included the three superfamilies: Pleurotomarioidea, Fissurelloidea, and Trochoidea. Hickman's narrow definition of the Archaeogastropoda phylogeny has not subsequently been adopted in the scientific literature (Sasaki, 1998).

Sasaki (1998) does, however, agree with Hickman on the status of families within Trochoidea. The Trochoidea is the most highly diverse group of marine gastropods, with more than 500 generic names. It has been represented in the marine fauna for 500Ma, providing a good model system for macroevolutionary studies and investigation of the biogeographic and ecological contexts of adaptive radiation (Hickman, 1996). There are three Trochoidean families: Turbinidae, Trochidae, and Skeneidae. The Trochidae are divided into 13 subfamilies, of which Trochinae contains three tribes: Gibbulini, Trochini and Cantharidini. The three Trochinae tribes form an unresolved trichotomy due to the distinct autapomorphies shown within each tribe and, despite shared

characteristics, the lack of morphological characters facilitating a branching order (Hickman, 1996). Therefore, Trochidea cannot be confidently considered a monophyletic group.

The evolutionary relationships between the genera of the tribe Gibbulini are not well understood, despite thorough biological knowledge of many individual species (e.g. *Osilinus lineatus* Fretter & Graham, 1994) and the ecology of their habitat, the rocky shore (Underwood, 1979). According to Hickman & Mclean (1990), the tribe Gibbulini Stoliczka, 1868 includes the following living genera: *Gibbula* Risso, 1826; *Monodonta* Lamarck, 1799; *Osilinus* Philippi, 1847; *Diloma (Oxystele)* Philippi, 1847; *Melagraphia* Gray, 1847; *Austrocochlea* Fischer, 1885; *Priotrochus* Fischer, 1879; *Chrysostoma* Swainson, 1840; *Cittarium* Philippi, 1847; *Margarella* Thiele, 1893; *Notogibbula* Iredale, 1924; *Cantharidella* Pilsbry, 1889; *Fossarina* Adams and Angas, 1864; *Calliotrochus,* Fisher, 1879; *Jujubinus* Monterosato, 1884; *Solariella* W. Wood, 1842; *Callumbonella* Thiele, 1924; *Phorcus* Risso, 1826; *Agagus* Jousseaume, 1894; *Nanula* Theile, 1924; *Notogibbula* Iredale, 1924; *Rubritrochus* Beck, 1995. Most genera within the Gibbulini exhibit restricted geographic patterns. The tribe is not represented in the tropical East Pacific, Northeast Pacific or Western Atlantic (except for the tropical species *Cittarium*) (Hickman & McLean, 1990). The cluster of *Gibbula* and *Osilinus* species in the Northeast Atlantic and Mediterranean are thought to form a `*prominent species complex*' (Hickman & Mclean, 1990:01).

Osilinus is the correct name for all European and Atlantic trochids previously included within *Monodonta* (BZN Opinion 1930). The genus has been considered to include the Indo-Pacific species *O. kotschyi* (Herbert, 1994). *Osilinus* is distinct from true *Monodonta (*type species *M.labio)* in its lack of the distinctive non-nacreous apertural ridge and its reduced columellar denticle (tooth) (Hickman & McLean, 1990). The history of the genus has been extensively reviewed (Hickman & Mclean, 1990; Gofas & Jabaud, 1997; Gofas & Herbert, 1998). There are many described species of *Osilinus.* Nordsieck (1982) lists 16 species; however, his revision can be considered as excessive (Beck, 1995). In his taxonomic revision of Nordsieck's classification, Beck (1995) considers the consensus species of *Osilinus* as listed in Table 1.2. Their distribution is summarised in Figure 1.1.

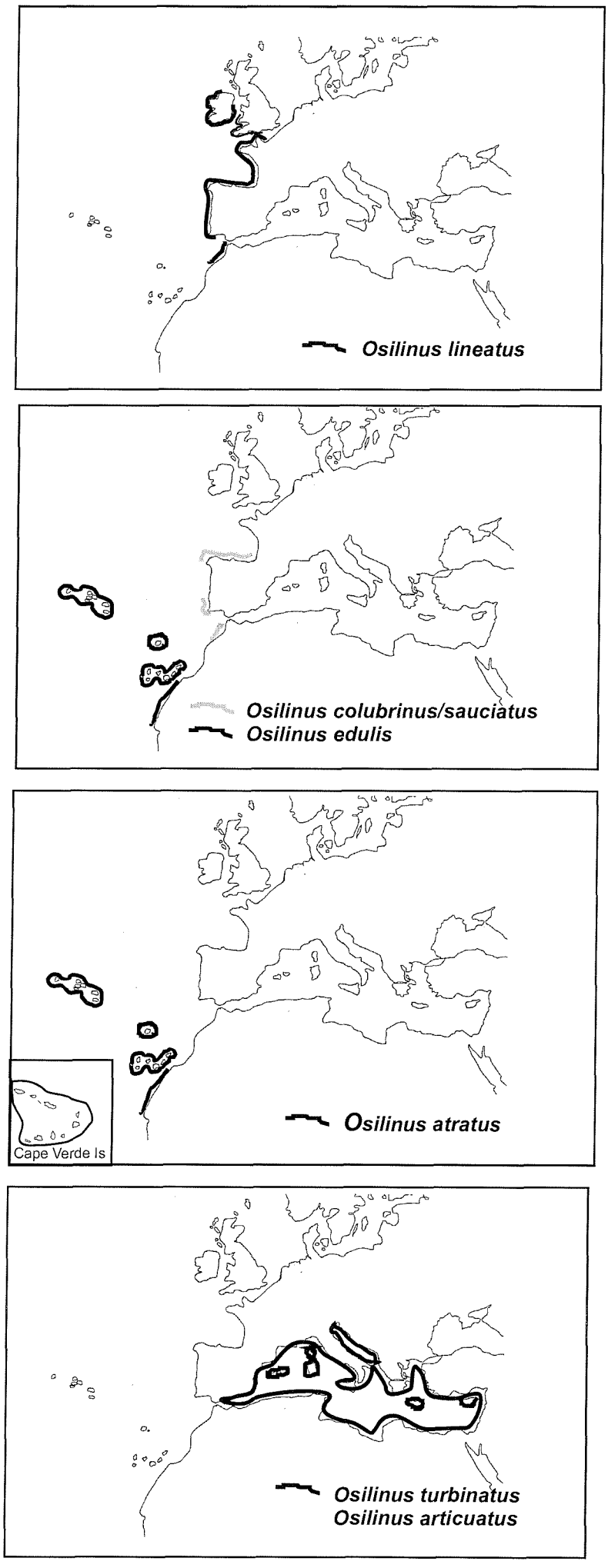


Figure 1.1. The distribution of the consensus species of *Osilinus* as known at the outset of this work (based on the literature reviewed in the text).

Table 1.2. The consensus species of *Osilinus* with synonyms, after Beck (1995).

Species	Synonyms
O. turbinatus (VON BORN, 1778)	*Trochus turbinatus* VON BORN, 1778 *T. tessulatus* VON BORN, 778 *T. zonatus* JEFFREYS, 1856 *Monodonta olivieri* PAYRAUDEAU, 1826 *Trochus elongatus* B.D.D., 1884 *Trochocochlea concava* MONTEROSATO, 1888 *Monodonta fragoroides* LAMARCK, 1822 *Gibbula serpa* NORDSIECK, 1982
O. sauciatus (KOCH, 1845)	*Trochus sauciatus* KOCH, 1845 *T. colubrinus* GOULD, 1849 *T. edulis* LOWE, 1842 *Osilinus colubrinus citrinus* NORDSIECK, 1982
O. articulatus (LAMARCK, 1822)	*Monodonta articulata* LAMARCK, 1822 *M. draparnaudii* PAYRAUDEAU, 1826 *M. sitis* RECLUZ, 1843 *Trochocochlea fulminea* MONTEROSATO, 1888 *Osilinus articulatus denudatus* NORDSIECK, 1982
O. richardi (PAYRAUDEAU, 1826)	*Monodonta richardi* PAYRAUDEAU, 1826 *Phorcus margaritacea* RISSO, 1826 *Trochus pallidus* B.D.D., 1884
O. mutabilis (PHILIPPI, 1846)	*Trochus mutabilis* PHILIPPI, 1846 *Osilinus mutabilis divaricatoides* NORDSIECK, 1982 *O. mutabilis farolito* NORDSIECK, 1982
O. lineatus (DA COSTA, 1778)	*Trochus lineatus* DA COSTA, 1778 *T. crassus* PULTENEY, 1799
O. atratus (WOOD, 1828)	*Trochus atratus* WOOD, 1828 *Osilinus atratus trappei* NORDSIECK, 1982 *O. atratus turbinoides* NORDSIECK, 1982
O. punctulatus (LAMARCK, 1821)	*Trochus punctulatus* LAMARCK, 1821
O. tamsi (DUNKER, 1858)	*Trochus tamsi* DUNKER, 1858 *Osilinus selvagens* TALAVERA 1978

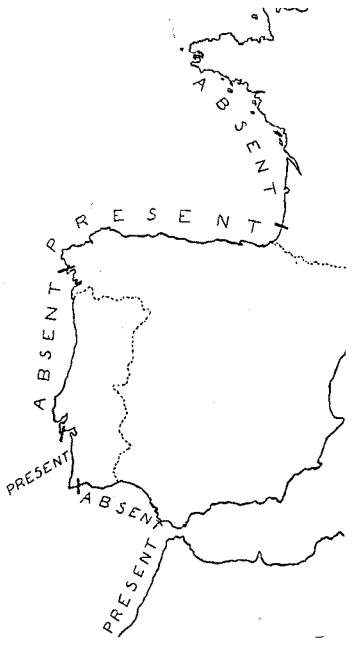
The species *O. richardi* and *O. mutabilis* have been subsequently reassigned to the genus *Phorcus* (Gofas & Jabaud, 1997). The species *O. lineatus, O. articulatus* and *O. turbinatus* are well described in the literature (e.g. Fretter & Graham, 1994; Giannuzzi-Savelli, 1994; Cesari & Pranovi, 1989 respectively). Neither their identification nor distributions are the subject of taxonomic confusion or ambiguity.

O. lineatus occurs in the upper eulittoral zone (Lewis, 1964) at less exposed locations from the British Isles, south along the French and Iberian Atlantic coasts until Morocco and Madeira (Crisp & Fisher-Piette, 1959). On the African coast it is found south to Asilah (150km south of Tangiers), at Cape Spartel, Tangiers, Cape Malabaton (where the Mediterranean meets the Atlantic) and further east (Fischer-Piette, 1959). *O. articulatus* and *O. turbinatus* are predominantly Mediterranean species just extending into the Atlantic beyond the straits of Gibraltar (Fischer-Piette, 1959).

At the outset of the study, the status of *O. atratus, O. punctulatus* and *O. tamsi* was ambiguous. *O. atratus* and *O. punctulatus* are probably the same species occurring on the Atlantic Islands (Madeira, Canaries and Cape Verde Islands) and West African continent respectively (Poppe & Guido, 1991). Burnay & Monteiro (1977), however, list *O. atratus* as the only *Osilinus* present on the Cape Verde Islands. *O. tamsi* is not in general current use; it is considered a synonym of *O.atratus* (Poppe & Guido, 1991). Nordsieck & Garcia-Talavera (1979) list the Madeira and Canary Island *Osilinus* species as *O.atratus, O. atratus Selvagensis, O. trappei* and *O.edulis*. *O. trappei* is also, however,

generally a listed synonym of *O. atratus* (Poppe & Guido, 1991; Beck, 1995). *O. atratus selvagensis* has been described as a separate species *Osilinus selvagensis* (García Talavera, 1978) endemic to the Selvagen Islands. But it has also been cited as a synonym of *O.atratus* (Poppe & Guido, 1991). In my work, *Osilinus* specimens collected from the Canary Islands or Madeira that are not *O. edulis* will be assigned the species name *O. atratus,* since only two different species of *Osilinus* have definitely been recorded on Lanzarote and Tenerife (Poppe & Guido, 1991). The geographical location of specimens of *O. atratus* from the NHM (London) dry and wet Trochidae collections include the Selvagens, Canary Islands, Cape Verde Islands, Senegal, Lower Guinea and Cape of Good Hope, S. Africa. Specimens from Madeira are conspicuous by their absence. Only one species of *Osilinus* was recorded on Madeira by Nobre (1937): *O. colubrinus (Trochocochlea colubrina*), which was considered the same species as that found in Portugal.

Beck (1995) assigns *O. colubrinus* and *O.edulis* as listed synonyms of *O. sauciatus*. These names have become interchangeable, either as synonyms or representing two species (e.g. *O. edulis* and *O. colubrinus,* Nordsieck, 1982; *O. sauciatus* and *O. edulis* Crothers, 2001). *O. edulis/ O. colubrinus/ O. sauciatus* may represent a species complex (Serge Gofas *pers comm.*). *O.edulis* is, however, the more commonly used species name and has been given priority over *O. sauciatus* and *O. colubrinus* (Nordsieck & Garcia-Talavera, 1979; Poppe & Guido, 1991).



The geographic distribution of this species, or complex of species, extends from the Atlantic coast of Northern Spain and Portugal south to Northwest Africa, including the Atlantic Islands of Madeira, the Selvagens and the Canaries. The taxonomic uncertainty regarding *O. edulis* is based on whether the individuals occurring on the Iberian Peninsula are the same species as those occurring on the Atlantic Islands and North Atlantic coast of Africa (Serge Gofas *pers comm.*; Crothers 2001). The distribution of *O. colubrinus* according to Fischer-Piette, (1957) is shown in Figure 1.2. The classification of two separate species, based largely on geographic distribution, is maintained by Crothers (2001), but the Iberian species has been assigned the name *O. sauciatus* with *O. colubrinus* as a listed synonym. The Atlantic Island species is more commonly referred to as *O. edulis* (Nordseick & Garcia-Talavera, 1979; Poppe & Guido, 1991; Crothers, 2001).

Figure 1.2. The distribution of *O. colubrinus* along the French, Iberian and North African coast (after Fischer-Piette 1957).

The taxonomic confusion surrounding *O. edulis* has arisen due to a widespread but disjunct distribution in the Northeast Atlantic (particularly along the Iberian Peninsula Atlantic coast), as a result of high intraspecific morphological variation and inconsistencies within the taxonomic nomenclature. For the purpose of this investigation, *O. edulis* will be used as a working name to

refer to all specimens previously described as *O. colubrinus* or *O. sauciatus* until the evolutionary relationship of the Iberian and Atlantic Island/N. African specimens is clarified.

1.3 The Biology and Ecology of *Osilinus*

In the main, the following text is taken from Fretter and Graham (1994) unless otherwise stated. For the purpose of describing the morphology, life history and ecology of these species, a generalised approach will be taken, most notably referring to *Osilinus lineatus* the common littoral species of the UK. Differences in other species are given where known.

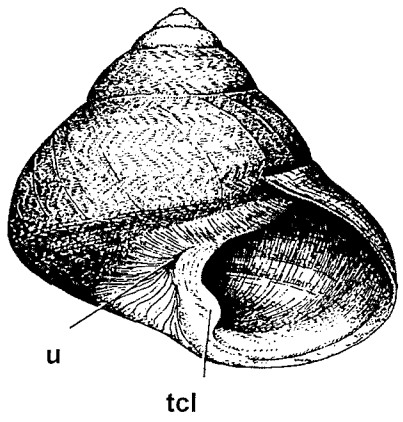


Figure 1.3. *Osilinus lineatus*: shell in apertural view. tcl, tooth on columella; u, umbilicus (after Fretter & Graham, 1994).

The trochoidean shell is typically dextrally coiled (Figure 1.3). The first whorls remain from the larval shell; the protoconch. The remainder represent the adult shell, the teleoconch (Hayward & Ryland, 1990). The shell is secreted by the mantle (also called pallium); the epithelial covering of the visceral hump and mantle skirt. During growth, rings of conchiolin impregnated with calcium carbonate are added to the edge of the existing shell and an inner layer of calcium carbonate is secreted over its internal surface. Externally, a layer of conchiolin forms the periostracum.

The shell shape of the trochids concerned here is cyoconoid, straight sided or slightly convex when viewed in profile of the spire (Hayward & Ryland, 1990). The inner surface of the whorls form a pillar through the centre of the shell which may be solid, or hollow opening to the surface at the umbilicus (as in *Gibbula umbilicalis*). During growth, non-uniform pigment secretion results in the often beautiful shell patterning. Most archaeogastropods pigmentation is attributed to porphyrins, particularly uroporphyrin I, (Creese and Underwood 1976a). Intraspecific variation in banding pattern and colour occurs in many prosobranch species (e.g. *Turbo Cornutus* (Ino, 1949) and *Acmaea digitalis* (Giesel 1970)). In *Austrocochlea constricta* (Underwood & Creese 1976; Creese & Underwood 1976) and other gastropods (see Leighton & Boolootian, 1960; Leighton, 1961) ecophenotypic variation such as intraspecific shell colour variation is thought to be driven by environmental factors, notably food availability and water chemical cues.

The body is normally described as having two major parts, the head-foot and the visceral mass. (see Figure 1.4)

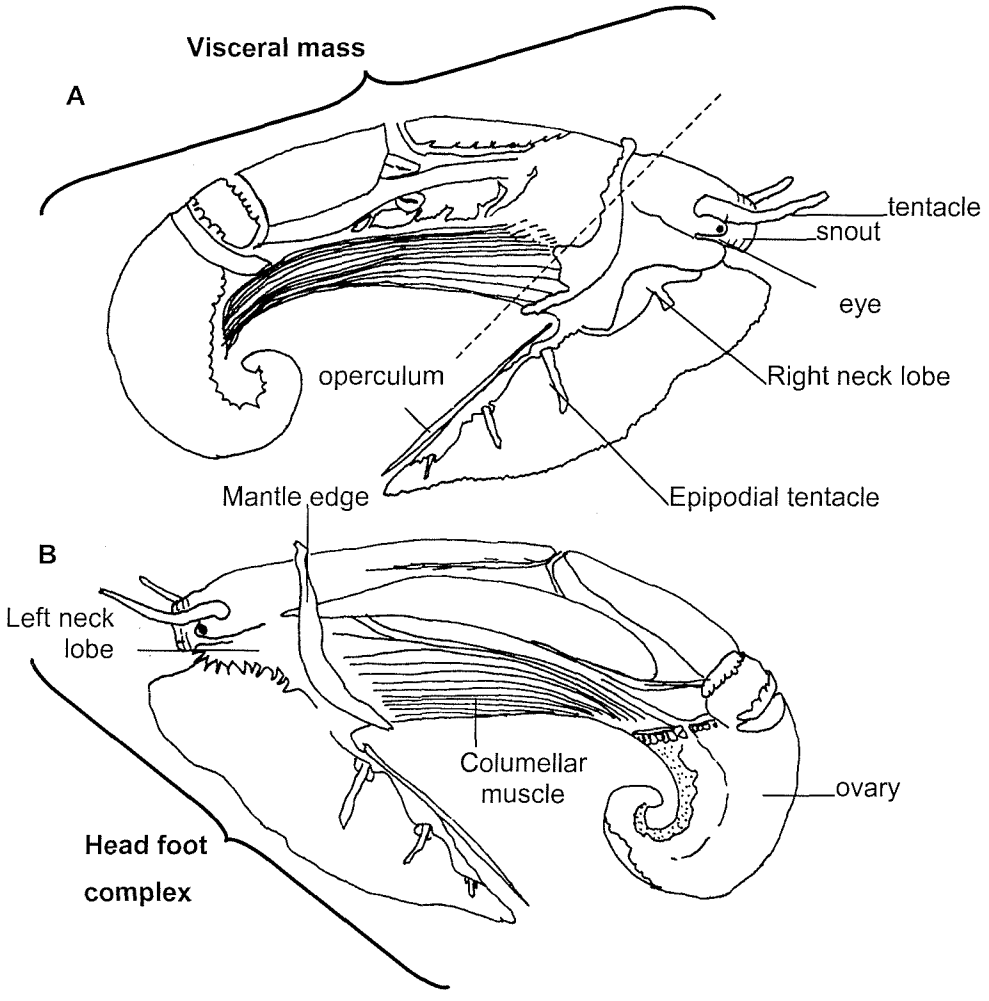


Figure 1.4. *Osilinus* lineatus. Animal removed from shell and seen A, from the right, B, from the left. After Fretter & Graham, (1994).

The head-foot complex itself has many specialised areas. The main function of the foot, described by Kier & Smith (1985) as a muscular hydrostat, is locomotion. Various glands on the foot produce lubricating mucus and other secretory products involved in creeping and adhering to the substratum. The creeping movement exhibited by the trochids is facilitated by direct waves of muscular contraction, an advanced state relatively uncommon amongst prosobranchs (Miller, 1974a). The operculum (a horny plug secreted by the operculigerous disc of the foot), lies posteriorly and confers protection from predation and desiccation when the body is withdrawn into the shell. The operculum consists of two proteinaceous layers, arranged in a polygyrous spiral.

The head area comprises a short snout at the end of which lies the mouth, a slit like opening. A pair of cephalic tentacles and eye stalks extend anterior-dorsally. The head also contains the buccal cavity containing the food-gathering radula. Radulae have provided highly useful characters in gastropod classification (Reid, 1999). *Osilinus* species possess primitive rhipidoglossan radulae. A multi-fine toothed radula that scrapes the substrate with an action

likened to a broom brushing (Steneck & Watling, 1982; Hawkins *et al.,* 1989). Its denticulation is described by the following formula:

$$a)\ \infty +1+4+R+4+1+ \infty \text{ OR } \qquad b)\ \infty +1+D+3+R+3+D+1+ \infty$$

Where R represents the middle tooth or rachidian; the integers the number of other laterals and; ∞ a high or uncountable number of teeth. In instances where the radula possesses a distinct lateral tooth, D represents this. The five major types of radula are depicted, Figure 1.5.

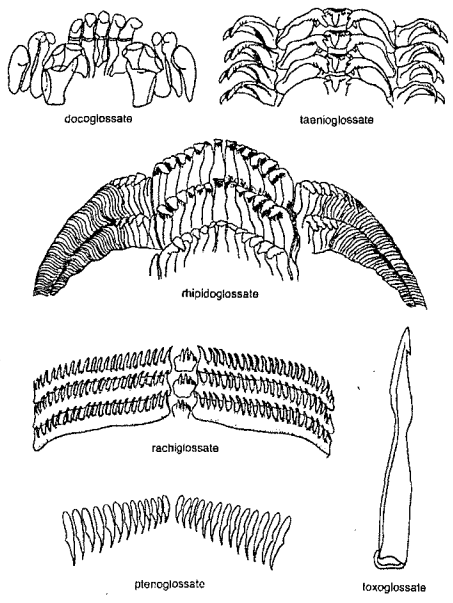


Figure 1.5. The five major radular types of patellogastropods and caenograstropods: docoglossate *(Scutellastra,* Patellidae); taenioglossate *(Vermetus,* Vermetidae); rhipidoglossate *(Margarita,* Trochidae); rachiglossate *(Fasciolaria,* Fasciolariidae); ptenoglossate *(Janthina,* Janthinidae); toxoglossate *(Conus,* Conidae). From Reid (1999a). After Cox (1960).

Trochids, like most prosobranchs are gonochoristic and as in most archaeogastropods fertilization takes place externally. They are perennial and iteroparous; reproducing at least once a year, and often during more than one reproductive season. Variation in reproductive strategies is exceptionally high within gastropods. Archaeogastropods have the shortest embyonic life amongst prosobranchs. *Osilinus lineatus* reaches sexual maturity at approximately two years old; gametogenesis lasts approximately 1 year (Fretter & Graham, 1994). *Osilinus lineatus* lay their eggs singly and unencapsulated, surrounded by a vitelline membrane and sphere of albumen produced by the ovum. Fertilisation substances called gamones secreted by both gametes facilitate attraction of the sperm to the egg prior to fertilisation and penetration. They also prevent post-fertilisation attraction. On spawning, the eggs are 150-200μm wide, increasing to 500μm as the jelly coat swells (Fretter & Graham, 1994).

It is thought that *Osilinus lineatus* has a lecithotrophic larval stage (Fretter & Graham, 1994), and therefore although planktonic, it is not considered a high-dispersal species (see Hawkins & Hiscock, 1983). There seems to be significant variation within the literature regarding the time and length of spawning. It can occur from May to September for a period of two weeks to three months (Gaillard, 1965; Crisp & Southward, 1958; Desai, 1966; Underwood, 1972; Kendall, 1987; Garwood & Kendall, 1985).

The fertilised eggs hatch as trochophore larvae 1-2 days after spawning. The trochophore larvae are lecithotrophic, having enough food reserve to sustain them through most of larval life. Larval life is short lasting up to approximately one week. Thereafter, the trochophore rapidly develops into a veliger larva, developing a shell, mantle and increased foot size. The larvae settle with a shell approximately 1mm in diameter and grow to 7-8mm before temporarily ceasing growth over winter (Kendall *et al.,* 1987). At year one, the shell of O. *lineatus* is approximately 10mm. By two years, the shell is 15mm in diameter. The life span of *Osilinus lineatus,* is a robust 8-10 years (Kendall *et al* 1987), individuals have been aged up to 15 years (Williamson & Kendall, 1981).

Osilinus species are generalist herbivorous grazers (Hawkins *et al.,* 1989) feeding off the microbial film on intertidal rock surfaces. Large chunks of crustose calcareous algae and bivalve fragments have also been observed in the *Osilinus* gut, inferring a wide diet. The preferred habitat is sheltered to moderately exposed rocky shores or boulder fields. Absence of trochids from very exposed shores is probably because of their limited adhesion power (Kendall, 1987). *Osilinus lineatus* is found in the intertidal zone between Mean High Water Spring and Mean Low Water Neap, although specimens have been dredged below MLWS.

O. lineatus exhibits seasonal and circadian migration up and down the shore (Williams, 1965; Desai, 1966; Underwood, 1973). *O. lineatus* exhibits a consistent behaviour pattern correlated to natural tidal position (Underwood 1972b). It is thought the behaviour shown by *O. lineatus* to follow mucus trails may assist in such vertical shore migration and winter aggregation (Wells & Buckley, 1972). A gradual upshore movement of larger animals has been found for several prosobranch species, such as *Olivella biplicata* (Edwards 1969, see also Creese & Underwood 1976). Direct relationships exist between the vertical position of littoral trochids on the shore and the oxygen consumption in air, mirrored by an inverse relationship between height on shore and oxygen consumption in water (Micallef 1966, Bannister *et al.,* 1966, Micallef & Bannister 1967, Houlihan & Innes 1982). The suggested increase in adaptation to activity during periods of emersion may be linked to the ability to retain water in the mantle cavity (Houlihan *et al.,* 1981). Adult *O.lineatus* individuals are found on exposed rock surfaces in the summer but in the winter individuals display aggregation. The spat are found under stones on damp gravel and juveniles inhabit crevices (Kendall *et al.,* 1987).

1.4 *Osilinus* as a model organism

Coastal marine invertebrates are ideal candidates as model systems for biogeographic studies at the regional scale (such as the Mediterranean and Northeast Atlantic). They are accessible, have simple one-dimensional ranges along coastlines, and often have excellent fossil records (Cunningham & Collins, 1994). The species of *Osilinus* are a conspicuous element of the fauna on sheltered to moderately exposed rocky shore habitats throughout the Atlantic and Mediterranean (Fretter & Graham, 1994). An understanding of the evolutionary patterns and

processes determining the distribution of the species of *Osilinus* may therefore be applied to understanding the biogeography of other gastropods occupying similar niches in the Northeast Atlantic (such as *Littorina, Gibbula* and *Patella* species found on more exposed rocky shore coastlines).

Osilinus has a complex biogeography that prompts many questions regarding the role of larval dispersal ability, ecological niche availability, historical interactions and palaeoceanography in speciation within the Northeast Atlantic-Mediterranean. *Osilinus* species are found in sympatric pairs at different wave exposures on the shore throughout its geographic range. *Osilinus atratus* and *O. edulis* occur sympatrically on the Atlantic Islands (Poppe & Guido, 1991). *O. lineatus* and *O. edulis* occur sympatrically along the Portuguese, Spanish and N. African Atlantic coasts (Fischer-Piette, 1955, 1957, 1958, 1959). *O. articulatus* and *O. turbinatus* occur sympatrically throughout the Mediterranean (Giannuzzi-Savelli, 1994; Oliverio, 1996). All the above species appear to be reproductively isolated based on the lack of observed hybrids or intermediate forms (Barton, 2001). Understanding interspecific phylogenetic relationships of this genus will provide a tool to interpret these striking biogeographic patterns, using the approach of Barraclough & Vogler, (2000).

The genus *Osilinus* is thought to represent a complex of sibling species (Hickman & McLean, 1990). Knowlton (1993) presents a case that reproductively isolated sibling species are common in the marine environment, and that understanding these species complexes is the key to understanding evolutionary processes in the sea. Such species provide a good model for examining the number of genes implicated in speciation, since the species are so similar, few genes are likely to be involved. Sibling, or cryptic species lack conspicuous, diagnostic morphological features but are identifiable from genetic, biochemical, behavioural or ecological characters. Classical taxonomy based solely on morphology cannot always recognise sibling species, with the results that reproductively isolated, yet morphologically apparently homogeneous entities are assigned to single species, seriously under-estimating biodiversity (Gomez, *in prep*). In particular, *O. edulis* and *O. atratus* may present examples of sibling speciation in the Northeast Atlantic.

1.5 Molecular tools in systematics

The molecular tools of PCR and DNA sequencing are the main molecular methodologies applied in this work to establish the interspecific evolutionary relationships of *Osilinus*. The genetic variation between individuals can be used as a direct measure of the evolutionary change that has occurred between them (Nei, 1987). By quantifying and describing this genetic difference between individuals from different populations or species, population structure can be analysed, and the ancestral sequences reconstructed to gain information regarding speciation patterns and phylogenetic relationships (Avise, 1994). Population structures and speciation patterns provide the founding blocks for investigating the processes that drive micro- and macroevolution respectively (Avise 2000). DNA sequences provide the ultimate level of evolutionary information.

The genes passed from parent to offspring determine an individual, and are in turn a reflection of an individual's ancestry (Maddison, 1996). This is a non-random process; DNA replication is driven by strictly controlled mechanisms, and DNA behaves in a regulated manner (e.g. substitution rates and complementary base pairing) (Alberts *et al.,* 1994). The evolutionary information inherent in DNA sequences is obtained by transforming observed differences between sequences into models of evolutionary relationships, presented as tree diagrams (Felsenstein, 1981). Since the behaviour of DNA is understood, models of evolution (substitution matrixes) are imposed on sequences to convert the observed distances (variation between sequences) into measures of actual evolutionary change (Avise, 1994). These measures of actual evolutionary change between groups of taxa reflect their ancestral relationships. The ancestral relationships and states provide insight into evolutionary processes such as speciation (e.g. Graybeal, 1994).

In addition to the molecular anlaysis, a morphological approach, based largely on radula characters has been employed to complement the molecular phylogeny. Radulae have been used extensively in systematic studies of gastropods at all levels of taxonomic resolution and provide a valuable source of phylogenetic information (Reid, 2000). Radula characters provide robust and significant sources of morphological characters for phylogenetic inference of higher level taxonomy (e.g. Ponder & Lindberg, 1997) and can be diagnostic at the species level. As an evolutionary informative character, the radula is second only to the shell for use and implementation in taxonomic studies for the discrimination of species (Reid, 2000). The choice and coding of discrete morphological characters is a fundamentally subjective process that aims to determine phylogenetically informative characters from observation. Genuinely homologous characters are required for the cladistic analysis to be phylogenetically useful, since only homologous derived characters can provide synapomorphies to determine clades (Reid, 1999a). When superimposed on molecular phylogenetic trees such characters can be used to study the evolution of adaptation and convergence.

1.6 Rationale of thesis and specific aims

The questions posed by the geographical distribution and taxonomic uncertainty surrounding the species of *Osilinus* is in essence a phylogeographic problem. Phylogeography aims to place the relationships of organisms in a spatial *and* temporal context. It is *'a field of study concerned with the principles and processes governing the geographic distributions of genealogical lineages, especially those within and among closely related species'* (Avise, 2000:3). The field of phylogeography requires an interdisciplinary approach. The interpretation of lineage distributions over space and time requires the integration of biogeography, molecular genetics (in this case phylogenetics), historical geography (in this case palaeoceanography) and palaeontology (where the fossil record is available) (Avise, 2000).

In addition to the multidisciplinary approach discussed above, this work needs also to take into consideration the theories on mode and tempo of speciation, particularly in reference to the marine

environment. Therefore, the first objective of this study (chapter 2) was to give an outline of the current theories of speciation with particular reference to marine invertebrate organisms.

My work aims to investigate the evolutionary processes that have determined the number and distribution of the closely related species within *Osilinus*. Palaeoceanography can be used to contextualise the temporal patterns of evolution (the phylogenetic relationships based on DNA sequences and morphology) and therefore to infer the processes of speciation (in the case of allopatric speciation, by vicariance or dispersal) that has given rise to the current biogeographic distribution. Therefore, in chapter 2 an overview of the palaeoceanography of the Northeast Atlantic and Mediterranean region since the late Cretaceous to the present day is given, within timescales relevant to the speciation of *Osilinus*.

Descriptions of the different species of *Osilinus,* as resolved subsequently in this thesis (chapter 6), are presented in chapter 3. Their resultant biogeography, based on fieldwork observations and a review of previous *Osilinus* distribution descriptions, is also given in light of the taxonomic revision. As suggested by the present distribution of *Osilinus*, hypotheses are presented on the mechanisms (palaeoceanographic events) that may have caused the observed radiation within this genus. These hypotheses are tested by the molecular phylogenies presented in chapter 6.

Chapter four, the general methods, provides a theoretical background to the molecular ecology techniques and phylogenetic analysis used in this work. The protocols and methodologies optimised and applied are detailed in Appendix B.

In order to correctly resolve interspecific phylogenetic relationships within *Osilinus* appropriate outgroup taxa must be selected (Reid, 1999a) (Chapter 5). I have used the 28S nuclear gene and 16S mitochondrial gene to construct a molecular phylogeny for the following taxa from the *Gibbulini* Stoliczka, 1868: *Austrocochlea constricta* (Lamarck, 1822), *Monodonta labio* (Linnaeus, 1758), *Oxystele tigrina* (Anton, 1839), *Oxystele variegata* (Anton, 1839), *Oxystele sinensis* (Gmelin, 1791), *Gibbula umbilicalis* (da Costa, 1778) and the ingroup taxa: *Osilinus kotschyi* (Philippi, 1849), *Osilinus edulis* (Lowe, 1842), *Osilinus articulatus* (Lamarck, 1822), *Osilinus atratus* (Wood, 1828), *Osilinus turbinatus* (Born, 1780), *Osilinus lineatus* (da Costa, 1778). This phylogeny is used to establish if *Osilinus* is a monophyletic group and to identify the sister taxon to *Osilinus* as the most appropriate outgroup for interspecific phylogenetic analysis (Chapter 6).

The taxonomic status, and interspecific evolutionary relationships of species within *Osilinus* is determined (Chapter 6). I used 16S and COI mitochondrial DNA sequence data to construct a phylogeny of *Osilinus edulis, Osilinus articulatus, Osilinus atratus, Osilinus turbinatus, Osilinus lineatus* using the outgroup taxa *Gibbula umbilicalis* and *Osilinus kotschyi*. The evolutionary relationships are discussed in context of the palaeoceanographic events detailed in chapter 3 to test and discuss hypothesis on the speciation of *Osilinus*. The geographic pattern of speciation is estimated from this species-level phylogeny (Barraclough & Vogler, 2000).

A morphological cladistic approach is used to complement the molecular analyses of interspecific relationships of *Osilinus* and thereby provide a more robust phylogeny (chapter 7). A morphological cladistic phylogeny is given for the gibbuline outgroup taxa: *Austrocochlea constricta*, *Austrocochlea concamerata* (Wood, 1828), *Austrocochlea porcata* (Adams, 1853), *Monodonta labio*, *Oxystele variegata*, *Oxystele sinensis*, *Gibbula umbilicalis* *Osilinus kotschyi* and ingroup taxa *Osilinus edulis*, *Osilinus articulatus*, *Osilinus atratus*, *Osilinus turbinatus* and *Osilinus lineatus* based on radula characters. In particular the phylogenetic usefulness of radula characters in resolving evolutionary relationships within *Osilinus* and the selected outgroup taxa is investigated. The molecular and morphological phylogenies are compared and the evolution of radula characters are discussed.

An interdisciplinary approach to understanding the evolution of *Osilinus* has been applied throughout this work. Biogeography, palaeoceanography, molecular and morphological phylogenetic analysis have been incroporated to present a phylogeographic model of allopatric speciation in the marine environment. Chapter eight, the general discussion, thus provides an overview of the phylogeography of *Osilinus.* This summarises the key results and discussion points of the relevant chapters to present a model of speciation in *Osilinus* at the Mediterranean/Atlantic interface. This chapter will also list limitations of the thesis and makes suggestions for future work.

Table 1.3 Glossary of sytematic terms used throughout this thesis, after Kitching *et al.*, (1998).

Term	Meaning
Apomorphy	A derived character or character state
Plesiomorphy	An ancestral or primitive character or character state. An apomorphy of a more inclusive hierarchical level than that being considered.
Monophyletic group	A group that includes a most recent ancestor plus all and only all of its descendants.
Synapomorphy	An apomorphy that unites 2 or more taxa into a monophyletic group
Symplesiomorphic	The occurrence in two or more taxa of a monophyletic group of a plesiomorphic character or character state
Paraphyletic group	A group that includes a most recent common ancestor plus only some of its descendants
Autapomorphy	A derived character or character state (apomorphy) that is restricted to a single terminal taxon in a data set.
Cladogram	Branching diagram specifying hierarchical relationships among taxa based upon synapomorphies. It includes no connotation of ancestry and has no implied time axis
Phylogenetic tree	A hypothesis of genealogical relationships among a group of taxa with specific connotations of ancestry and an implied time axis.

Chapter 2

A review of speciation theories and the palaeoceanography of the Northeast Atlantic and Mediterranean

2.1 Introduction

The study of speciation in the natural environment is essentially the observation of patterns such as geographic distribution, phylogenetic relationships and taxonomic diversity. Hypotheses to explain these patterns of diversity are based largely on the allopatric speciation model, and processes of vicariance and dispersal (Avise, 2000). Sympatric speciation is also known to occur, based on disruptive selection and intrinsic barriers (Bush, 1994; Palumbi, 1994; Turner & Burrows, 1995).

The aim of this chapter is firstly to provide a literature review on species concepts and modes of speciation with special reference to the marine environment. The scientific literature abounds with debate and controversy on both, a reflection of the importance and complexity of the subject (Barraclough & Vogler, 2000). There are still many unanswered questions and the discussion often tends towards the semantic and philosophical. Opposing schools of thought are defended and attacked vigorously (discussed below), often obscuring significant common ground. The literature on speciation is too vast to be reviewed in depth here. Examples of speciation in the marine environment are therefore considered in light of the different concepts, with reference to how processes in the sea differ from the terrestrial environment, upon which much of current theory concerning speciation is based (Palumbi, 1992).

Palaeoceanography, the study of the past oceans, can provide information on events that may have driven speciation in the past. The second aim is thus to present a contextual overview of the palaeoceanography of the Northeast Atlantic and Mediterranean, thereby providing a framework for constructing hypotheses of speciation processes in marine organisms. The north Atlantic and Mediterranean are geographically complex areas that have been subject to several geological and oceanographic processes that could have driven the allopatric speciation of marine organisms. Tectonic processes such as plate movements, crust formation (notably sea-floor spreading), volcanism and island formation have interacted with climate driven glacial movements and consequent sea level changes (Skinner & Porter, 1991). This chapter will therefore provide a brief overview of the formation of the Mediterranean and Northeast Atlantic with particular reference to the late Cenozoic events, most notably the Messinian salinity crisis and the ice ages. It will conclude with a description of the present north Central Atlantic and Mediterranean. Geological timescales are given in Appendix A.

2.2 Definitions of Species and Speciation

2.2.1 Species concepts

Species have long been recognised as units produced by nature, identified by Aristotle as sharing the same *'eidos'* (essence), which are constant and maintain their own integrity. Buffon (1749) first defined species in terms of reproduction. Dobzhansky (1937), Mayr (1940) and Rensch (1959) expanded this concept: they are the fathers of the modern Biological Species Concept (BSC), later renamed the Isolation Species Concept. Because the BSC defines species in terms of a reproductive community, gene pool and genetic system, it has been widely applicable to population genetics (White, 1978a). The BSC has been the most widely accepted species concept over the last 50 years, although is now facing serious challenge in the light of new knowledge acquired through the application of molecular techniques to systematics (Avise, 1994). The BSC is a far from ideal species concept, reflected in the many alternative concepts presented in the literature. This is most notably because the definition of species, in terms of reproductive isolation, is often impractical or even implausible to confirm or observe in nature (Cronquist, 1978; Sokal & Crovello, 1970; Masters & Spencer, 1989). Reproductive isolation is therefore inferred from indirect observations of morphological differences. When structures associated with reproduction differ between species, reproductive isolation may indeed be a by-product of morphological divergence. The assumption that any observed morphological differences are concordant with reproductive isolation is not based in empirical evidence. This has lead to accusations that the BSC is essentially a morphological concept, which, according to Guiry (1992), *'is essentially the default taxonomic base'*.

Aside from the practical limitations of its application, the Biological Species Concept can also be challenged on theoretical grounds. There is no reason to assume that there must be any observed morphological characters associated with a speciation event; speciation mechanisms and selection for morphological differentiation are often assumed independent *a priori* (e.g. Turner & Burrows, 1995). During allopatric speciation, reproductive isolation mechanisms arise as a by-product of divergence and are thus not directly involved in the early stages of the speciation process. Reproductive Isolating Mechanisms are not the barriers responsible for initial isolation, and therefore some authors believe they should not be included in a species concept (Nelson & Platnick, 1981; Cracraft, 1983; McKitrick & Zink, 1988; Masters & Spencer, 1989). Furthermore, because the Biological Species Concept does not necessarily reflect monophyletic groups (and therefore species as single evolutionary units) phylogeneticists do not favour this concept. On these grounds, the Phylogenetic Species Concept of Cracraft (1983) is probably the favoured alternative. The definition of a species as a monophyletic group has a broad application to sexual and asexual, sympatric and allopatric populations alike (Gosling, 1994).

Like the Phylogenetic Species Concept, the Recognition Species Concept (Paterson, 1985) omits Reproductive Isolating Mechanisms from its definition, but replaces them with the converse, specific-mate-recognition systems (SMRS), which are a product of directional

selection. It differs from the Biological Species Concept (BSC) in being non-relational as it is independent of the identification of close relatives. The significance of this concept is that it focuses on those processes that maintain the reproductive behaviour and associated traits of the species, rather than the incidental effects of such behaviour. Thus the recognition concept species has a 'self-defining nature' (Masters & Spencer 1989:273).

The species concept that most lends itself to studying speciation as a process is probably Templeton's (1989) Cohesion Species Concept that includes evolutionary forces within its cohesion mechanisms. This concept can be hard to apply in real biological terms (Bisby, 1994). Many authors have provided synopses of the different species concepts (see Cracraft, 1983; Endler, 1989; Templeton, 1989; Avise, 1994, 2000; Gosling, 1994; Bisby, 1995) with the common conclusion that no single rigid definition of species can be successfully applied in all circumstances (Hartl & Clark, 1997): since each species will possess a different evolutionary history. Clearly, a pluralistic approach is required (Mishler & Donaghue, 1982). Table 2.1 provides a summary critique of the different concepts, illustrating the limitations and applications.

Table 2.1. Summary of species concepts. The definition of each concept is given with characteristics, limitations and applications.

Concept definition	characteristics	Limitations	Applications
Morphological Species concept (Du Rietz, 1930; Cain, 1954; Mayr, 1963; Shaw. 1964) *'the smallest natural populations permanently separated from each other by a distinct discontinuity in the series of biotypes'* Bisby (1995:41)	Relies on heritable characteristics, be it morphological, behavioural, phytochemistry, microanatomy to define continuity of variation within, and distinct discontinuity between species.	Some evidence to contradict theory: morphologically dissimilar forms can interbreed successfully whereas similar forms do not, therefore this approach does not always reflect that which defines a group of organisms in real biological terms (e.g. cryptic species, polytypic species, Mayr 1963, 1982).	Commonly used by morphological systematicists.
Phylogenetic Species Concept (PSC) (Rosen, 1979, Eldredge & Cracraft, 1980; Nelson & Platnick, 1981; Cracraft, 1983; Nixon & Wheeler, 1990, 1992). *'A species is the smallest group of organisms that is diagnosably distinct from other such clusters and within which there is a parental pattern of ancestry and descent.'*	Based on the same premises as the EvSC, species are considered the terminal branches on an evolutionary tree. Therefore shared characters of a group relate to the sharing of a common ancestor-identifying monophyletic groups. In practice this concept relies on shared distinguishing characteristics, and therefore is not so far removed from the morphological species concept	Monophyletic groups can be hard to recognise. Distinguishing between the histories of traits and organisms can be difficult. This approach tends to produce a far greater number of species than that recognised by the biological species concept, especially with use of molecular methodologies.	Probably the closest to an operational definition of species.
Biological Species Concept II (Mayr, 1982) *'A species is a group of interbreeding natural populations that are unable to successfully mate or reproduce with other such groups and which occupies a specific niche in nature'*	Basically the ISC with a niche criterion added on, and thereby including asexual and parthogenetic species within the definition.	Same criticisms as above. Difficult to define a niche in real terms.	Basically the original BSC with an ecological criterion.
Recognition Species Concept (RSC) (Paterson, 1985; Vrba, 1984) *'A species is a group of organisms that recognise each other, for the purpose of mating and fertilisation.'* Bisby 1995:42	The opposite of the ISC. The recognition concept considers the positive functions that assist the reproductive mechanisms, rather than negative isolation mechanisms. Therefore the recognition species concept is defined by populations sharing most inclusive common fertilization system, - achieved by specific-mate recognition systems (SMRS).	Difficult to decide what characteristics are important in mate recognition. Under this definition, 2 species that infrequently hybridize would be classified as one species.	Only applicable to sexually reproducing organisms.

Table 2.1 continued. Summary of species concepts. The definition of each concept is given with characteristics, limitations and applications.

Concept definition	characteristics	Limitations	Applications
Cohesion Species Concept (CSC) (Templeton, 1989) *'The smallest group of cohesive individuals that share intrinsic cohesion mechanisms.'*	Similar to ISC and RSC, in that it recognizes interbreeding as key factor in delimiting a species group, but includes other cohesion mechanisms (e.g. niche requirements).	Cohesion hard to recognize and define in real biological terms, particularly degrees of cohesion between groups.	Unlike the ISC, and RSC, this concept makes no assumptions about the evolutionary processes behind speciation, only the evolutionary pattern.
Ecological Species Concept (EcSC) (Van Valen, 1976) *'A lineage which occupies an adaptive zone different in some way from that of any other lineage in its range and which evolves separately from all lineages outside its range.'*	Considers niches to be distinct and separate zones, and makes the false assumption that two species cannot occupy same niche, even briefly.	Hard to define an adaptive zone in reality, and therefore this concept is difficult to apply.	Not widely applied.
Evolutionary Species Concept (EvSC) (Simpson, 1944,1951; Wiley, 1978). *'A species is a single lineage of ancestor-descendant populations which is distinct from other such lineages and which has its own evolutionary tendencies and historical rate.'*	This concept considers speciation the process by which new lineages arise. Not dependent on interbreeding, or reproductive isolation.	The criterion of 'evolutionary tendency' is hard to observe in real biological terms.	As a broad definition, it includes extinct, extant, sexual, asexual, and parthogenetic organisms.
Concordance Principles (Avise & Ball, 1990) Recognises species *'by the evidence of concordant phylogenetic partitions at multiple independent genetic attributes'*	Based on the ISC, assuming that reproductive barriers are intrinsic factors and not extrinsic, geographical factors. When concordance if afforded by genetic characters due to extrinsic reproductive barriers, authors suggest sub species status	Cannot be applied to asexually or parthogenetically reproducing species, it therefore omits a large number of organisms.	Only applicable to sexually reproducing organisms.
Genotypic Cluster Definition (Mallet, 1995) *'we see two species rather than one if there are two identifiable genotypic clusters. These clusters are recognized by deficit of intermediates'*	Based on Darwin's species concept, 'to define species .... as distinguishable groups of individuals that have few or no intermediates when in contact' (Mallet 1995:296) using additional genetic criteria. Rejects isolating mechanisms as useful in determining species.	Only really applicable to sympatric populations.	Includes polytypic species. Species are defined by the pattern, not processes, of speciation.

The lack of resolution of the speciation debate may seem a hindrance, and for some proves an obstacle to understanding the process of speciation (Templeton, 1980), however the reverse is true for others.

'The process and outcome of speciation (sympatric or otherwise) does not depend on the a priori invocation of any particular species definition.....it is an understanding of the factors that result in the reduction and eventual elimination of gene flow between sister populations - the very process of speciation itself - that is necessary before a clear species definition is possible. (Bush, 1994:286).

Therefore, since arrival at a single, superior, theoretically valid and universally applicable species concept is highly unlikely, a multidisciplinary and pluralist approach is advocated (Knowlton, 1993; Avise, 1994; Gosling 1994).

2.2.2 *Pattern and tempo of speciation*

Darwin (1859) described the origin of species as *'the mysteries of mysteries'* and today it is far from resolved. Species are widely held as distinct evolutionary units, the product of speciation (Cracraft, 1983; Koeslag, 1995 but see Nelson, 1989). There are two distinct patterns of speciation recognised, represented at their extremes by anagenesis and cladogenesis (Figure 2.1). Anagenesis is the gradual accumulation of genetic differences within lineages over long periods, and is driven by natural selection that results in adaptation to different environmental conditions. Cladogenesis is speciation by division of lineages and results in the multiplication of species.

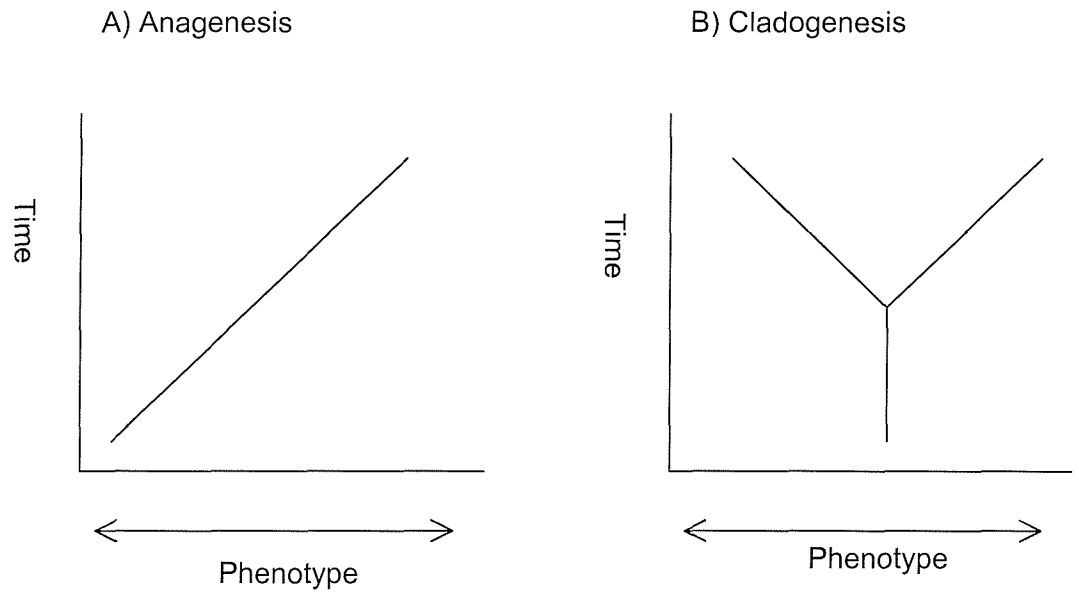


Figure 2.1. Anagenesis compared to cladogenesis. a) Gradual change of an entire lineage. b)The splitting of a lineage into two. After Barbault *et al.*, (1995:226).

These two patterns of speciation are reflected in contrasting theories of the rate and mode of speciation in evolution (see Figure 2.2). Gradual change in response to natural selection was at the heart of Darwin's theory of evolution, for both micro- and macroevolutionary processes. Speciation is therefore considered an extrapolation of the evolutionary processes determining inter-population differentiation, albeit with the evolution of reproductive isolation. Thus, since Darwin, phyletic gradualism has been widely held to account for the process of evolution during speciation events. It is still reflected today in the view of the Neo-Darwinian synthesis (Fisher, 1930, 2000) that most evolutionary change occurs within species: cladogenesis is implicit in the multiplication of species (addition of new lineages), but not accountable for the majority of macroevolutionary change (Stanley, 1979; Eldredge, 1989; Barbault *et al.*, 1995). Observing a common pattern of prolonged periods of stasis followed by geologically rapid speciation events in the fossil record, the Punctuated Equilibrium model challenged phyletic gradualism (Eldredge & Gould, 1972; Gould & Eldredge, 1977). In contrast to the Neo-Darwinian view, their model assumes the most significant evolutionary change occurs with speciation events, and that little or no evolutionary change occurs within species. Thus, the processes working at the level of population genetics do not equate to those driving speciation. In between periods of rapid change, species exhibit approximate equilibrium. Microevolutionary and macroevolutionary processes are thus decoupled to some degree. Other more recent theories relevant to the role of small and large scale processes in evolution include niche saturation (Brown, 1995) and the dynamic equilibrium theory of biodiversity dynamics (Sepkoski, 1992).

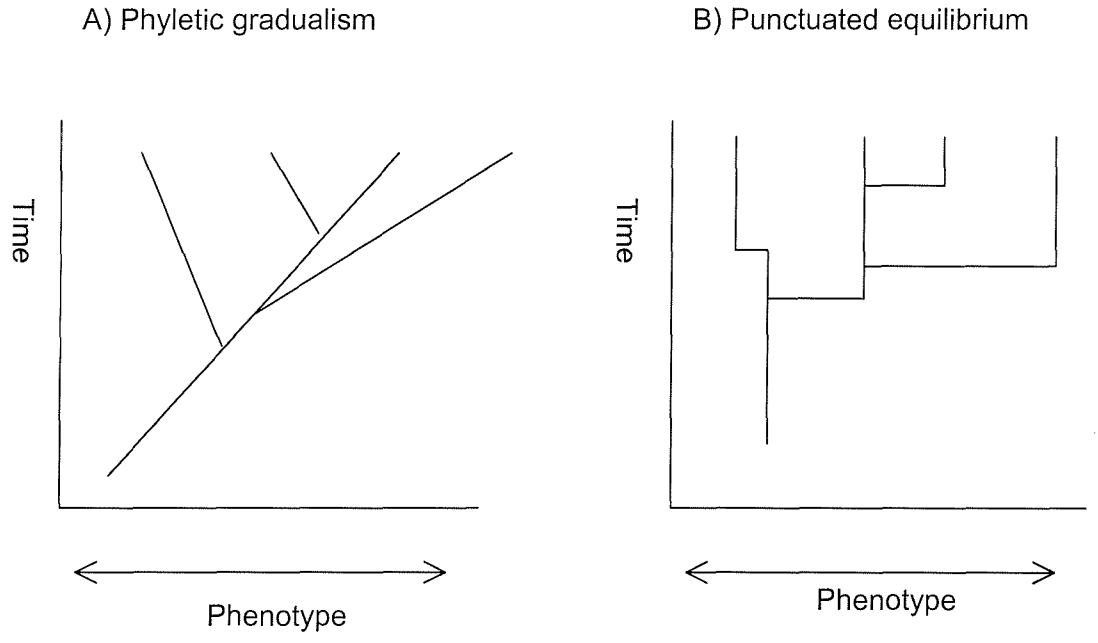


Figure 2.2. Phyletic gradualism versus punctuated equilibrium. Redrawn from Barbault *et al.*, (1995:226).

Templeton (1986) considers micro- and macroevolution to be interdependent hierarchical levels of the evolutionary process. The relationship between micro- and macroevolution is not a simple extrapolation, but that each evolutionary level is a result of the emergent properties from the level below, wherein the whole is greater than the sum of its parts. This argument will never be fully

resolved until the relative importance of the different genetic mechanisms and evolutionary process driving speciation are understood. The main questions, yet unanswered, are as follows (Coyne, 1992; Avise, 1994):

1. How many genes must change to make a new species?
2. Do such genes occur at consistent positions on chromosomes?
3. Are the changes involved conventional changes in allelic frequency or are other evolutionary processes involved?
4. What are the relative roles of genetic drift and natural selection?
5. Are genetic bottlenecks important in facilitating speciation events?
6. Is geographical isolation the main mechanism?
7. How frequently does sympatric speciation occur?

The first three questions are addressed by examination of the different mechanisms of evolutionary change and then reviewing evidence for the genetic basis of speciation. The latter four questions are addressed below in section 2.2.3.

There are seven main processes of genetic evolution: mutation, recombination, genetic drift, natural selection, gene flow, hybridization and lateral genetic transfer. The process of **mutation** is predominantly independent of environmental conditions experienced, although some environmental stresses, such as heavy metal loading and radiation at hydrothermal vents, can affect rate dramatically, (Barbault *et al.*, 1995). Therefore, mutation is generally an unpredictable source of genetic variation that does not usually respond to the adaptation needs of an organism in response to stimuli from environmental conditions. Mutations of individual nucleotides (changes, insertions, and deletions) occur at a low rate (4×10^{-9}) that is about constant throughout the genome and between eukaryote species, (Kimura 1983). Different forces govern the frequencies of new alleles produced by such mutations. Some genetic loci have relatively high mutation rates. Insertion of transposable elements is a further source of mutation. The rate of transposition is relatively low, about 10^{-5} /generation. An exception to this is found in *Drosophila* and other insects when, under certain environmental conditions, the rate dramatically increases (Carson, 1971, 1975 1987). Gene duplication can also play an important adaptive role, especially under strong selection, as proposed in the evolution of anti-freeze proteins in Antarctic notothenioid fish (Chen *et al.*, 1997).

Recombination creates new combinations of alleles. The effect of recombination on the genome varies between species, and is to some extent determined by mode of reproduction (Barbault, *et al.*, 1995). Recombination has greater importance in out-crossing sexually reproducing species than in those self-fertilising, and does not occur in asexual reproduction.

Genetic drift is the mechanism of change in allele frequencies arising from the stochastic events of Mendelian inheritance. The effect of genetic drift is inversely proportional to effective population size, its greatest effect being exerted on small populations (Nei *et al.,* 1975, Nei, 1977). It is of note that the lowest effective population sizes have been estimated within marine organisms (Frankham, 1995). An example of genetic drift that could have an important role in speciation is the *Founder effect* (described below); the reduction of genetic variability that occurs when a population originates from a small number of individuals. The significance of this in speciation events is, however, challenged by several authors (see below).

A mechanism considered to play the dominant role in speciation, especially in vicariant allopatric speciation, is **natural selection**. Natural selection can work at different levels: selection on phenotypic characters, selection on individual loci, spatial and temporal variation in selection, and selection on multiple characters (Barbault *et al.*, 1995). Variation over large distances can result in geographical clines in one or several phenotypic characters (Endler, 1977), for example Ldh expression within Fundulus fish along a thermal cline (Crawford & Powers, 1992).

Dispersal and gene flow are also important processes in speciation, since reduction of gene flow may result in speciation (Wolpoff, 1989). Dispersal and gene flow between populations creates homogeneity, decreasing local differences. But the level of gene flow varies significantly between species (Slatkin 1985, 1987). In allopatric speciation, geographical separation of populations within a species disrupts dispersal and gene flow. This can result in genetic divergence and the consequent reproductive isolation of the separated populations. This is essentially the Neo-Darwinian view of how reproductive isolation arises, as a by-product of divergence of conspecific populations separated by geographic barriers. A commonly held perception is that reproductive isolation is completed upon secondary contact of such populations, when the then sympatric species undergo `reinforcement'. Theoretical research now shows, however, that reinforcement is an unlikely mechanism in speciation (Spencer *et al.*, 1986; Butlin, 1989; Coyne, 1992).

Hybridisation and lateral genetic transfer are also important processes in evolution. **Hybridisation** results in the exchange of genes between two species that are usually reproductively isolated, and is the only mating-dependant method of horizontal transfer. For a comprehensive review of the role of hybridization in evolution see Barton (2001). **Horizontal gene transfer** is distinct from the vertical transfer of genetic information from parent to offspring (which facilitates the above processes of genetic evolution); it defines the lateral transfer of DNA sequence from one genome to another, usually implying transfer between contemporary individuals of different species. Findings from the Human Genome Project have provided evidence that horizontal gene transfer occurs at a greater frequency than previously thought, and is not a process restricted to bacteria. This process seems to have played an important role in the emergence of the earliest kingdoms of life a few billion years ago (Kidwell, 2001).

The neo-Darwinian view predicts that adaptation is polygenic (involves many genes), and therefore that isolating factors are polygenic (Coyne, 1992). This viewpoint leads to the question of how many genes are involved in speciation. This prediction is often true for *Drosophila* (see Avise, 1994) and other taxonomic groups (Coyne, 1992 and references therein). For example, 150 genes were found to be involved in hybrid viability in chromosomal races of the grasshopper *Podisma pedestris,* and 55 in the toad species *Bombina bombina* and *B.variegata* (Barton, 1988). An extreme example to the contrary is found in snails (Orr, 1991), where prezygotic isolation is mediated by a single mutation changing coiling direction, thereby making internal copulation physically impossible. Rapid speciation involving few genes controlling sperm bindin proteins has also been observed in closely related echinoids of the genus *Echinometra* (Palumbi 1994). Changes in gene regulation may also be a mechanism of reproductive isolation, and the driving force behind adaptive radiation (Dickinson, 1989). Chromosomal rearrangements, already known to be an important mechanism of speciation in plants (White, 1978b; Weiss and Maluszynska, 2000), could also be important in animals, conferring sterility on carriers of certain heterozygous rearrangements (White, 1978b). Templeton (1986, 1989) suggests that coadapted gene complexes, involving changes at a few loci of major genes with many pleiotropic effects, can provide the basis for speciation.

Regardless of the pattern or tempo of speciation, the question of how novel species arise and maintain their integrity lies at the centre of the debate surrounding speciation. The most widely held and accepted mode is that championed by Mayr (1963) and Dobzhansky (1970) of allopatric speciation by geographic isolation. There are, however, three main types of speciation: allopatric speciation, parapatric speciation and sympatric speciation (Bush, 1975) summarised and discussed below.

2.2.3 Modes of speciation

Allopatric speciation can occur by geographic separation (also called speciation by subdivision or vicariant allopatry), by dispersal, or by the founder effect (also called the peripheral isolates model, or peripatric speciation) (Bush, 1975). Vicariant allopatry is initiated by the separation of two large populations by a geographic barrier (Rosen, 1978). Gene flow is therefore prevented and the two populations diverge genetically as they adapt to changing environmental conditions. There is no direct selection for reproductive isolating mechanisms (RIMs) which arise as a by product of divergence, or as a consequence of selection for other characteristics. Reproductive Isolating Mechanisms include pre-mating isolation, seasonal or habitat isolation, behavioural isolation, mechanical isolation (male end female sexual organs are incompatible), post-mating isolation, gametic mortality, zygotic mortality, hybrid inviability, hybrid sterility (Hartl & Clark, 1997). Divergence tends to occur slowly under vicariant allopatry, and speciation is often completed upon secondary contact of the sister populations, as hybrids are selected against and the pre-mating Reproductive Isolating Mechanisms are established (see Figure 2.3) (Hartl & Clark, 1997). Allopatry by dispersal occurs when '*propagules manage to overcome an existing barrier and succeed in colonising a new territory.*' Briggs (1999:647). Speciation by the founder effect or

peripatric speciation (Mayr, 1940) occurs when a small population is geographically isolated at the periphery of the range of the large parent population, and it diverges rapidly under the effects of genetic drift, inbreeding and selection pressures of a novel environment (Knight *et al.*, 1987; Carson, 1989; Dickinson, 1989; Nevo, 1989; Provine, 1989; Templeton, 1989).

Parapatric Speciation (White, 1968, 1978a) is defined as the divergence of adjacent but non overlapping populations between which gene flow is possible and there is no physical barrier preventing interbreeding. To facilitate speciation, selection must favour differing alleles in adjacent populations. This occurs when species evolve from contiguous populations along a continuous cline (see Bush, 1975) as recently modeled by Doebeli & Dieckmann, (2003).

Sympatric Speciation (Maynard Smith, 1966) or Competitive Speciation (Rosenzweig 1995) is defined as the divergence of populations not subject to spatial separation. Divergence occurs within the dispersal range of the parent population. This mode of speciation is initiated by disruptive selective forces associated with a shift in habitat or host, or the partitioning of resources (Tauber & Tauber, 1999). Barriers to gene flow are intrinsic as opposed to extrinsic; it implies strong disruptive selection on a polymorphic population since the '*process of divergence is constantly opposed by interbreeding of the divergent polymorphic types*' (Gosling, 1994).

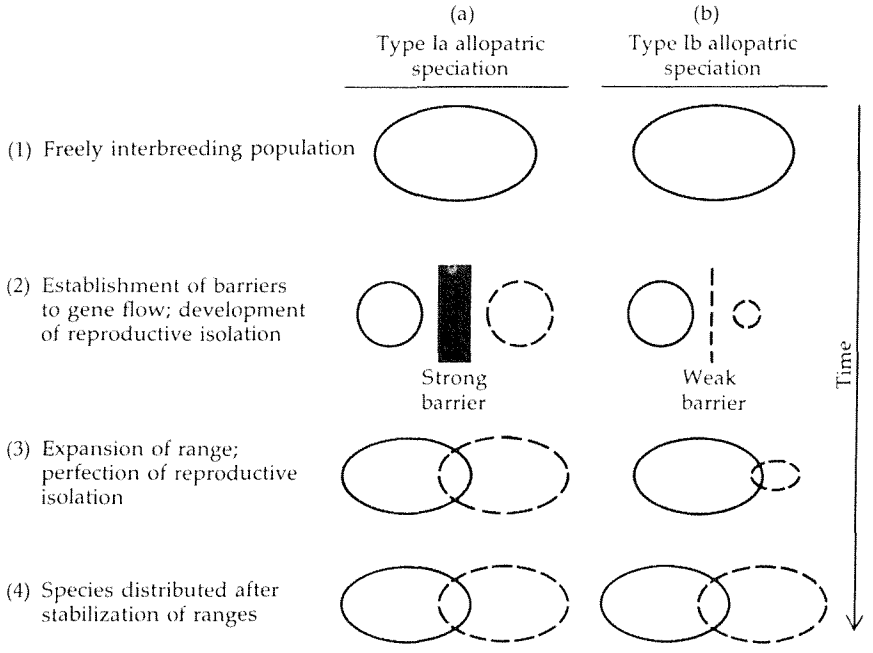


Figure 2.3: (a) Type Ia allopatric speciation, in which a strong geographical barrier to gene flow splits a population (1) into two large subpopulations (2) that evolve independently and develop incipient reproductive isolation. Reproductive isolation is perfected should the barrier then disappear or become surmounted (3), leading to overlapping species ranges. (4). Type Ib allopatric speciation, in which small subpopulation (with consequent large founder effects) splits off from a large population (2) and, by random genetic drift, develops incipient reproductive isolation; the populations thereafter follow patterns (3) and (4) of Type Ia allopatric speciation (after Bush, 1975. modified from Hartl & Clark, 1997)

Lynch (1989) considers the peripheral isolation model of allopatric speciation as the most common mode, followed by vicariant allopatry, with sympatric speciation least common. Gosling (1994:3) states allopatric speciation as the *`almost exclusive'* mode for animals and *`likely the prevailing one in plants'.* Mayr's model of peripatric speciation (1940) is considered by many as a major source of new species (see Templeton, 1980 and references therein; Templeton, 1989). The mechanism by which it arises (originally described by Mayr, 1940 as '*a molecular revolution*', but see Mayr, 1982) is challenged. Genetic drift, genetic bottlenecks and reinforcement are unlikely to be direct mechanisms of pre- or post-reproductive isolation, and appear to be infrequent in nature (Bush, 1994). The founder effect required by Mayr's original peripatric model of speciation is challenged by evidence of the role of adaptation in the speciation of subterranean mole rats (Nevo, 1989). Moreover, mathematical models do not confirm the predictions of the founder effect model, as far as genetic drift is necessary for speciation by peripheral isolation (Coyne, 1992). The observations of isolated species fit the traditional view that islands provide geographical isolation and new environments leading to strong selection. Coyne (1992) therefore supports Nevo (1989), by considering it most likely that observation of extreme morphs on islands that have evolved rapidly can be explained by adaptive radiation.

In a later paper Mayr (1982a) did, however, acknowledge that his proposed `genetic revolution' does not occur very often; also that speciation does not occur only in founder populations and that not all genes were affected every time a small population was founded. Although founder speciation may be rare, some propose it can still have significant effects within a genus, such as the rapid speciation of *Drosophila* on the Hawaiian archipelago (Templeton, 1986). Frey (1993) agrees that dispersal, central to the Peripheral Isolating Model (PIM), is more likely to occur on archipelagos that provide stepping stones away from mainland populations. An alternative model involving peripheral isolates may in fact be a more common mode of speciation. This is the Centrifugal Speciation Model (CSM), proposed by Brown (1957) in light of the centrifugal evolution originally described by Simpson (1944), and apparently ignored in recent literature (Briggs, 1999a). The main mechanism of the Centrifugal Speciation Model is range retraction, and the predictions of this mode of speciation seem to be supported by empirical evidence (Frey, 1993). A case for centrifugal speciation is presented by Briggs (1999a) from the biogeographic diversity pattern of the Indo-West Pacific marine province.

Many agree that sympatric speciation in less obvious, testable or common than allopatric speciation (Bush, 1975; Diehl & Bush, 1994). Alongside the numerous authors advocating its occurrence (White, 1968, 1978a; Bush, 1969, 1975, Endler, 1977; Tauber & Tauber, 1977a, 1977b; Caisse & Antonovics, 1978; Templeton, 1980; Wood & Guttman, 1983; Rice, 1984; Bush & Howard, 1986; Kondrashov & Mina, 1986; Feder *et al.,* 1988; McPheron, *et al.,* 1988; Turner & Burrows, 1995) alternative explanations are also offered (Carson, 1987b; Coyne & Barton, 1988). Others still contest whether sympatric speciation can occur at all (Mayr, 1963; Futuyma & Mayer, 1980; Futuyma, 1986; Paterson, 1981). The main argument against non-allopatric speciation is its requirement for intrinsic barriers to gene flow, which are considered only to arise in allopatry

because of extrinsic barriers. Detractors of sympatric speciation consider that most purported cases of sympatric speciation can be explained by secondary contact between species that have diverged allopatrically. Evidence is mounting, however, to support sympatric speciation in certain taxa under disruptive selection regimes (e.g. Bush, 1994; Palumbi, 1994; Turner & Burrows, 1995; Johannesson, 2000) and that the predictions of sympatric speciation are reflected in nature (for review on challenges to the arguments against non-allopatric speciation, see Bush & Howard, 1986).

Sympatric speciation appears to be rarest in sexually reproducing vertebrates (Lynch, 1989) and invertebrates (Bush, 1975). The literature suggests that sympatric and 'quantum speciation' is found primarily in plants (Gosling, 1994). It may, however, play an important role in shallow-water marine invertebrates (Knowlton, 1993). Sympatric speciation is also thought to be an important evolutionary process in insects, the most widely cited and accepted being that of tephritid fruit flies *Rhagoletis* (Bush, 1975, but see Futuyma & Mayer, 1980). Evidence supports the occurrence of sympatric speciation in nature, most notably in organisms for who mate choice is directly associated with habitat or resource choices (e.g. insects, mites, nematodes, fish and some birds, Bush, 1975, 1994). *'Adaptive traits, such as habitat or host preference, selection and fitness, intimately involved in the shift to a new niche are often the same traits that result in habitat specific assortative mating and the evolution or partial or complete reproductive isolation between sympatric populations'* (Bush, 1994:285). Rolan-Alvarez *et al.,* (1999) provides evidence for incipient sympatric speciation in two *`ecotypes'* of *Littorina saxatilis* exhibiting incomplete prezygotic isolation. The two main components of this isolation are behavioural mate choice resulting in assortative mating and microhabitat selection. Evidence for sympatric speciation is, however, often indirect. For example, sympatric population divergence and speciation is inferred from the observation of resource polymorphisms exhibited in sympatric populations of birds, amphibians and fishes (Smiths & Skulason, 1996).

The biogeography and phylogenetic relationships of young, sibling species, which are yet to be obscured by dispersal events, can also be used to infer speciation mode (Barraclough & Vogler, 2000). An indication of possible sympatric speciation (sibling species with sympatric distribution) has been observed in East Indies fish populations (Briggs, 1999b). Allopatric speciation, however, is strongly supported as the dominant mode of speciation in the East Indian Ocean, driven by sea level changes during the Cenozoic era (Paulay, 1977; Springer & Williams, 1990). The classic model of sympatric speciation is that of cichlid fishes in African lakes: mate choice of females for different male coloration maintains reproductive isolation between sympatric populations (Turner & Burrows, 1995; Galis & Metz, 1998).

Gosling (1994) considers both sympatric and allopatric speciation to be gradual processes in comparison to `Quantum speciation', a rapid process involving major chromosomal changes in one or a few individuals that hence become reproductively isolated from the parent population, and consequently establish a new species. The subterranean mole rats of Israel are an example of this

(Nevo, 1986). The *Spalax ehrenbergi* species complex consists of four chromosomal species (of different diploid numbers) each adapted to a specific climatic regime. This species complex is thought to be in the final stages of speciation via chromosomal evolution under different selection pressures.

The above definitions of speciation modes are based on the observed geographic range of species. An alternative mechanistic classification of speciation, based on genetic transilience, is offered by Templeton (1980). This is recognised by Valentine & Jablonski (1983) and Glazier (1987) who call for a predictive theory of speciation. In considering the peripheral isolate model of allopatric speciation, Glazier (1987) presents a testable, predictive theory of speciation based on the working hypothesis that speciation is ecologically biased. It is based on the assumption that incipient daughter species tend to have smaller population sizes and geographic ranges than parent populations, conferring a greater vulnerability to extinction. Therefore, the theory predicts species arising from such isolates are expected to be biased towards stable population dynamics. An obvious prediction of this would be for island populations: endemic species will have stable populations, and accordingly occupy K-selecting habitats as compared with widespread undifferentiated island populations. Several terrestrial taxonomic groups observe this trend including higher plants (Smith, 1981), birds (Ricklefs & Cox, 1978) and carabid beetles (Darlington, 1971). The general pattern arising from this is an inverse relationship between habitat stability and geographical range, with restricted species occupying stable/persistent habitats, and wide-ranging species more likely to occupy fluctuating/ephemeral habitats. Exceptions are uncommon but include intertidal molluscs on tropical shores (Vermeij, 1972). Ranges are more restricted on the highly variable, harsh upper shore than the more constant and less harsh lower shore environment. This general pattern could also be a reflection of successful species often occurring across multiple habitats, being abundant and less vulnerable to extinction where as rare species often exhibit the converse ecological attributes (McKinney & Drake, 1998).

The vast majority of work on speciation to date has been focused on the terrestrial environment, and applying theories of speciation created for the terrestrial environment to the sea is not without its problems. Only recently has the literature started to reflect a renewed interest in speciation in the marine environment, but already significant progress has been made (e.g. Palumbi, 1992, 1994; Knowlton, 1993; Gosling, 1994; Oliverio, 1996; Hellberg, 1998; Jackson & Cheetham, 1999; Briggs, 1999).

2.2.4 Speciation in the Marine Environment

Early theories of speciation were based on terrestrial observations, classically Darwin's finches on the Galapagos Islands (Darwin, 1909). The terrestrial environment has been far more accessible to the scientist, or natural historian, and is far more easily replicated in the laboratory than the marine environment, most notably using the model organisms *Drosophila* (Lawrence, 1992) and *Neurospora* (Nelson *et al.,* 1997). The terrestrial environment also provides obvious boundaries,

greater heterogeneity, sharper gradients between habitats, greater temperature and other climatic variations and, perhaps most importantly, has resulted in different life history strategies upon its occupants (Barton & Charlsworth, 1984; Kay & Palumbi, 1987; Palumbi, 1992). In contrast the world's oceans offer a vast, three-dimensional, continuous, relatively stable and less explored environment that is difficult to sample (Hellberg, 1998).

These characteristics are reflected in the apparent homogenous and broadscale geographical distributions exhibited by many marine taxa (e.g. Porifera; Hooper & Van Soest, 2002). Obvious geographical barriers are lacking in the marine environment, and those present are often hard to define (e.g. warm water bodies dividing temperate species with antitropical distributions, for review see Hellberg, 1998). Most marine organisms, especially invertebrates, exhibit planktonic larval stages, conferring a greater dispersal potential and therefore greater gene flow, hence seemingly inhibiting allopatric speciation events. Furthermore, *in situ* research in the marine environment has only recently been feasible with the advance of diving and other sampling technology (e.g. deep-sea submersibles). The marine environment still presents a difficult environment to study, especially at great depth. As a reflection of this logistical barrier, our understanding of the life histories and dispersal abilities of many marine organisms is limited and creates a major hindrance to understanding the mechanisms of speciation.

In the marine environment, communication modes are different, primarily facilitated by chemical and physical cues (in marine invertebrates) that are undetectable by humans (Masters & Spencer, 1989). Speciation rate is also thought to be slower in the sea, reflected in the life spans of genera (Rensch, 1959): the average age of a mammalian genus is 15 million years, a insect genus 12 million years, a pulmonate gastropod 40 million yrs, and a prosobranch gastropod 75 million years. Long-lived genera may possess greater evolutionary potential, or their longevity may be a reflection of the environmental stability of marine ecosystems (Fretter & Graham, 1963). The questions raised by these contrasts between the terrestrial and marine environment are discussed below.

Firstly, with the lack of visible geographic barriers in the sea, wide geographic ranges, high dispersal potential and consequent gene flow of many organisms, it seems unlikely that the classic slow, vicariant allopatric models can account for the high species diversity and richness exhibited in the marine environment (Palumbi, 1992). Therefore, how does reproductive isolation (RI) arise between two large, high-dispersal populations, and if allopatric speciation does not seem likely, what are the major speciation mechanisms working in the marine environment? Secondly, with many organisms exhibiting planktonic larval stages, what is the role of life history strategies in marine speciation mechanisms. Thirdly, defining allopatry, sympatry and parapatry is difficult when the reproductive biologies of organisms are largely unknown, and the species boundaries are sometimes unclear (Knowlton, 1993). Species boundaries are most clearly documented in sympatry, which leads to the question, why are there so many sibling species in the sea?

What speciation processes are taking place?

In general, marine species with low dispersal abilities tend to exhibit strong genetic structure over small geographic areas (e.g. some littorinids, Johannesen, 2000; non-planktonic prosobranchs, Oliverio, 1996; the marine snail *Tegula,* Hellberg, 1998; the wrasse tribe Labrini, Hanel *et al.,* 2002), and are better understood than high dispersal species (Jablonski, 1986). Population structure and genetic divergence appear correlated: many high dispersal marine species exhibit low genetic differentiation among populations (Avise, 1994, Palumbi, 1992, 1994). Conversely, organisms with lower dispersal abilities have higher rates of speciation (Palumbi, 1992; Rolan-Alvarez *et al.*, 1995).

There is a widely held intuition that effective population size and speciation rate are inversely related (Wright, 1940a, b; Nei *et al.,* 1975; Nei, 1977), which is supported by the divergence rate predictions for vicariant and founder event allopatric speciation (e.g. Mayr, 1940, 1982). This has been more recently challenged by evidence to the contrary for terrestrial speciation (Orr & Orr, 1996). The time to speciation was found to be essentially equal between small and large population divisions when divergence was driven by genetic drift. When divergence was driven by natural selection, however, time to speciation increased with population subdivision; that is speciation was most rapid when a species was split into two large populations (Orr & Orr, 1996). Evidence supporting the apparent contradiction of speciation in high-dispersal populations is prevalent in the marine environment (e.g. Schroth *et al.,* 2002; Howell *et al.,* in press).

The combination of high dispersal with diverse sympatric species prompted Palumbi (1992) to question how reproductive isolation could arise among large populations, and if allopatric speciation could account for such observations. In the fossil record of gastropods, high-dispersal species appear geographically at random with respect to their closest relatives, suggesting that gradual allopatric speciation has not occurred (Jablonski, 1986). Sea urchins have also speciated during the last 1-3 Ma in the largest continuous marine habitat, the Pacific Ocean. Strong genetic divergence among populations is present despite high dispersal potential. A strong pre-fertilization reproductive isolating mechanism is thought to be responsible; eggs and sperm of different *Echinometra* species are unable to bind and fuse (Palumbi, 1997).

The interaction of gametes appears to be a key mechanism of reproductive isolation. This is consistent with the Recognition Species Concept (RSC) (Patterson, 1985). Gametic interactions are a barrier to cross fertilization in abalones (Palumbi, 1992). Fine scale genetic differentiation has also been observed in other high-dispersal species (Hare & Avise, 1996). Further mechanisms by which population structure can arise in high-dispersal species include natal homing, stable migration routes and, larval dispersal and behaviour patterns (Palumbi, 1997). Marine larvae may be able actively to influence their dispersal by behavioural responses to currents (Doherty *et al.,* 1995). Isolation by distance can affect population structure over large scales. Speciation associated with isolation by distances greater than 5000km has been observed in planktotrophic invertebrates (Palumbi, 1997). In the Macronesian gastropod *Littorina striata,*

however, isolation by distance does not affect the genetic structure of the population over distances of 500km; planktonic development appears to facilitate gene flow throughout the geographic range of *L. striata* (De Wolf *et al.,* 2000). Peripatric origins are suggested for the evolution of the species-rich cosmopolitan marine moon jelly *Aurelia* driven by climatic adaptation forced by divergent selection (Schroth *et al.,* 2002). Subsequent loss of intraspecific gamete recognition is also thought to have contributed to the formation of new species within this genus. Only one *Aurelia* lineage was thought to have arisen via allopatric speciation

Fossil records of the upper Ordovician marine invertebrate fauna of the geosyncline of northeast North America, however, provide evidence, for competitive (sympatric) speciation as the main mode of speciation. Furthermore, speciation rate was shown to be inversely proportional to biodiversity - the reverse of that expected for allopatric speciation. Species richness increased rapidly by a broad invasion followed by a steady state phase wherein sympatric speciation occurred. It has thus been suggested that although most species were produced by sympatric speciation, the common ancestors may well be traced back to a rare successful allopatric speciation event allowing exploitation of a new ecological niche (Rosenzweig & Taylor, 1980). Speciation is often thought to increase following extinction and then decrease, reaching an equilibrium wherein origination approximates extinction; the niche saturation point (McKinney & Drake, 1998).

Allopatric speciation is not excluded, however, in the marine environment by the above factors. Except for a few echinoderms (for review see Palumbi, 1994; 1997) almost all molecular phylogenetic studies of marine species have suggested allopatric speciation (e.g. Hellberg 1998, 2001; Barraclough & Vogler, 2000, see chapter 6). Allopatric speciation is strongly supported by biogeographic distributions in the East Indies, thought to be facilitated by Cenozoic changes in sea level (Springer & Williams, 1990; Paulay, 1997, Briggs, 1999a). Evidence for punctuated equilibrium in marine organisms led Jackson & Cheetham (1999) to conclude that '*most but not all cases of speciation in the sea are punctuated*' and hence associated with the splitting of lineages by allopatric speciation. This evidence prompts further questions about the importance of natural selection and genetic drift in speciation. Jackson & Cheetham (1999) argue, however, that punctuated evolution does not contradict such conventional neo-Darwinian mechanisms but '*constrains the range of probable evolutionary scenarios for speciation*' (p72).

The fossil record has been used to look at the macroevolution of shallow sea communities (Jablonski & Bottjer, 1983; Valentine & Jablonski, 1983). It was found that the near-shore environments seem to provide regular opportunities for founder events. Most near-shore species are planktotrophic. In contrast, offshore species tend to be non-planktotrophic and therefore more likely to initiate colonisation and founder events than planktotrophic species (Johannesson 1988), similarly, Knowlton (1993) predicted that larvae with limited dispersal and sedentary or sessile adults are characters that are expected to facilitate founder-event speciation. Evidence for

founder events facilitating reproductive isolation has been demonstrated in the laboratory with polychaetes, and inferred for littorinids (Knight *et al.,* 1987).

A further important consideration regarding variation in high-dispersal populations is historical interactions between populations (Hilbish, 1996). Traditionally, it is considered that panmictic populations with high dispersal abilities rarely exhibit geographical variation. In cases where divergence is observed, they can be attributed to selection pressures since panmixis virtually excludes the possibility of divergence from random processes. Hilbish (1996) and others (Gosling, 1993) however, explained complex distributions of genetic variation to mixed ancestry (*Mytilus* in the British Isles, Gosling, 1990, 1993), secondary contact between historically differentiated populations, hybridisation and probable natural selection (e.g. a relic of hybridization between the stone crabs *Menippe mercenaria* and *M.adina* in Florida, Hilbish, 1996). Conversely, historical allopatry is offered as an explanation for the modern sympatry of *Nucella* species occupying adjacent and overlapping zoogeographic provinces in Central California (Marko, 1998), as opposed to sympatric speciation. The potential for allopatric speciation at zoogeographic province boundaries is well established (e.g. Jablonski and Valentine 1983, Avise, 1994, Maggioni *et al.,* 2003), and Marko (1998) suggests that this is especially true along linear coastlines.

'Evidence that instances of sympatric overlap between sibling species in adjacent provinces is actually secondary contact would support a scenario of transient allopatric isolation on continuous linear coastlines.' (Marko, 1998)

Clinal speciation may be occurring in many sibling species, especially with different depth distributions (Knowlton, 1993). This could even occur vertically along the shore because of the steep environmental gradient facilitating habitat selection pressures that drown out gene flow from dispersal (Valentine & Jablonski, 1983). Small-scale genetic variation has been described for the western Australian littorinid *Bembicium vittatum* (Johnson & Black, 1991) associated with local habitat changes (non-plankotrophic larvae), the intertidal limpet *Siphonaria jeanae* (Johnson & Black, 1984) and the mussels *Mytilus californianus* and *M. edulis* (Levinton & Suchanek, 1978; see also Gosling & McGrath, 1990). Distinct distributions of New Zealand intertidal species pairs from North to South are attributed to hydrological conditions of the coastal waters (temperature, salinity and current systems), most notably its transversion across the Subtropical Convergence (Pantin, 1963). North-South within-species morphological clines are also apparent (e.g. the chiton *Sypharaochiton pelliserpentis* and the hydroid *Obelia geniculata,* Knox, 1963). Furthermore, the sympatric ranges of five Trochidae species were ecologically separated by habitat preferences, attributed to range expansion after evolution via allopatric speciation (Knox, 1963). Knox concluded that most examples of species distributions could be attributed to allopatric speciation, the main mechanisms being changes in coastlines, seawater temperature and current fluctuations.

The above examples accentuate the difficulties in deducing the mechanisms and geographic context of speciation from modern species distributions alone. Genetic divergence estimates, and

temporal changes in spatial distribution from palaeographic records are required to fully understand speciation events (Marko, 1998). Examples of sympatric speciation are often associated with habitat choice and selective mating (as discussed above). Many of the debates regarding speciation arise, however, because of the difficulties of observing speciation in nature, especially sympatric speciation (Maynard Smith, 1996).

Barriers acting in the sea

Technically, the world oceans present a continuous environment, lacking obvious barriers. Ultimately, temperature, salinity, desiccation, oxygen availability, and other environmental factors, are known to delimit the distribution of marine organisms (Day, 1963); however, species distributions show that other barriers are working within this potential range. It is clear that speciation does take place, and more significantly, in allopatry by vicariance and by founder events. This section therefore presents a brief overview of barriers that have been identified in the marine environment.

Connections between the tropical Atlantic and Pacific were cut off with the rise of the Isthmus of Panama, approximately 3-3.5 Ma. This led to the reproductive isolation of the taxa consequently separated (see Knowlton, 1993 and references therein). Anti-tropical distributions are well known for several organisms (Lindberg, 1991; Vermeij, 1992), the equator presenting a barrier to gene flow by distance or the need to migrate across warm shallow, or cold, deep water. Most anti-tropical marine invertebrate populations in the Atlantic were last connected more than 3Ma, the majority of which appear to have achieved reproductive isolation (Vermeij, 1992). Connections are thought to be more recent for most algae, and some taxa in the East Pacific (Rosensblatt & Waples, 1986; Richmond, 1990; Knowlton, 1993, for review). Genetic divergence is often greater between than within oceans indicating that long distance filters to dispersal must be occurring. The Eastern Pacific Barrier (EPB) presents the biggest marine obstacle to shallow-water organisms (Lessios *et al.*, 1998). There is little doubt that the establishment of the EPB during the Cenozoic has been a significant vicariant event (see Briggs, 1995), however despite the 5400km of uninterrupted deep water, transpacific species do exist and gene flow has been shown to occur across this massive barrier. Significant gene flow between eastern and western Pacific sea urchin *(Echinothrix diadema)* populations has been mediated by larval transport during El Nino events (Lessios *et al.*, 1998). Conversely, when the Bering Strait opened approximately 3.5 Ma, removal of the northern land barrier between Atlantic and Pacific at the North Pole enabled the expansion of cold-water species and the consequent transarctic distributions presently observed. This is migration is called the transartic interchange (Vermeij, 1991).

It has been suggested that as a result of the relatively constant conditions of the marine environment, many marine organisms do not exhibit the evolutionary flexibility of their terrestrial counterparts. Smaller ranges of environmental conditions could accordingly present significant barriers, especially for planktonic species (David, 1963). The different dominant chaetognath species present in oceanic plankton is thought to be attributed to few ecological niches limiting

species distribution by competition (David, 1963). It is evident then, that isolation by distance, behavioural limits to dispersal, selection, and the recent history of a species can create opportunities for genetic differentiation in high-dispersal species. Currents and changes in currents also create barriers in high-dispersal species (De Wolf *et al*, 2000)

Role of life history in speciation mechanisms

Life history strategy is a major determinant of dispersal abilities and evidently plays a significant role in the rate of speciation. Genetic population structure is largely determined by the presence or absence of planktotrophic larvae (Templeton, 1986). As discussed above in relation to speciation processes, non-planktotrophic groups exhibit higher speciation rates and species turnover. Conversely, planktotrophic near-shore species exhibit less speciation, a lower speciation turnover but longer species persistence. This is evident in prosobranchs: there are 75,000 species of the almost universally internally fertilizing monotocardian prosobranch, compared to the 1500 species of externally fertilising diotocardian prosobranchs (Fretter & Graham, 1994). The occurrence of high dispersal planktonic life stages in marine organisms may also account for the many hypotheses of non-allopatric speciation compared to terrestrial environments. Whether this is due to a lack of knowledge regarding species reproductive biology and larval dispersal behaviour and ranges remains to be seen.

Why so many sibling species in the sea?

Sibling species are described by Knowlton (1993 p.190) as *'any species whose distinctiveness has been the source of substantial taxonomic debate or whose discovery was based on non-morphological characters is included under the rubric of sibling species.'* Sibling species are very common in marine invertebrates, with 21 sibling pairs from 16 genera identified from the literature for Mollusca alone (Knowlton, 1993). Knowlton provides five main reasons for the abundance of sibling species: a lack of knowledge about the biology of many species, the loss of many useful characters during fixation and preservation of specimens (such as soft tissue pigmentation and patterns), the difficulties of direct observation *in situ* in the marine environment, the difficulty in measuring the importance of sources of morphological variability has led to high intraspecific variation being accepted as the norm, and wide geographic ranges of species are readily accepted as a reflection of large dispersal potential within the oceans. These reasons have been discussed above and summarise the wider problems faced in research on speciation in the marine environment.

2.3 Palaeoceanography of the North Atlantic and Mediterranean

The modern gibbuline taxa are thought to have been extant since the Jurassic (Hickman & McLean, 1990). The first appearance of *Osilinus* in the fossil record occurred during the upper Cretaceous 142 – 65 Ma (Keen in Moore, 1960), or possibly earlier. Hickman ascribes a Palaeozoic or deep-Mesozoic origin to the genus (Hickman, 1996). The age of origin of this trochid implies many potential vicariant events that could have driven allopatric speciation within *Osilinus*.

The major palaeoceanographic events affecting the Mediterranean and North East Atlantic since the Cretaceous period are therefore detailed below (see Appendix A for a standard geological timescale).

2.3.1 Formation of the central North Atlantic and Mediterranean

During the early Jurassic period, 200 million years ago (Ma), continental convergence and sea floor spreading led to the rearrangement of the northern and southern land masses of the super continent Pangea, dividing them into the modern continents (Maldonado, 1985). Continental drift is mediated by plate tectonics of the mantle, the movement of rigid lithosphere plates over a hotter and more plastic asthenosphere (Pinet, 1992). The six major and several minor plates of lithosphere making up the surface of the earth (Skinner & Porter, 1995) are produced and destroyed in equilibrium, maintaining a constant diameter of the earth (Pinet, 1992).

The movement of these plates determined the oceanography of the pre-Jurassic period. Reconstruction of a world before the present oceans existed is an imprecise science, based on the shape of continents and indirect data. Models of the oceans during the Triassic suggest a wedge-shaped Palaeotethys Ocean existed between the northern and southern continental masses of Pangea, which disappeared during the early Mesozoic (Hsu and Bernouilli 1978; Carey 1958; Bullard *et al.*, 1965; Smith 1971). The distribution of continents and oceans since the Jurassic is more certain, based directly upon magnetic lineations (Piccoli *et al.,*1986).

Two main processes acted in the evolution of the Atlantic and Tethys that served to establish the Central North Atlantic, and account for the complexity of the Mediterranean. Firstly, the opening of the equatorial Tethys during the Triassic to late Cretaceous periods. The Tethys proper was created during the early Jurassic, an Equatorial Ocean extending east to west comprising several basins such as the Proto-Caribbean and Palaeo-Mediterranean (for a detailed reconstruction of Tethys evolution, see Piccoli *et al.*, 1986; Carey 1986; McKenzie 1986). Second was the longitudinal Atlantic opening during the mid Cretaceous. Approximately 165 Ma, during the middle Jurassic, the Central North Atlantic began to open, as oceanic crust formed between North America and Africa. The sinistral movement of Africa relative to Europe during the late Jurassic and early Cretaceous resulted in the Palaeo-Mediterranean undergoing a prevailing left-lateral movement, and alternating periods of compression and distension (Maldonado, 1985). This is reflected in the ancient nature of the eastern Mediterranean basin (Por, 1989). The Central North Atlantic basin was closed to the north but connected to the Mediterranean and Caribbean Tethys via a narrow passage. By 120 Ma, the early Cretaceous, a 4000m deep connection had formed between the mid-ocean of the North Atlantic and the Tethys, remaining as an equatorial ocean until the late Cretaceous. The Tethys was comprised of deep basins and shallow sills, connected to the world ocean in the east. The South Atlantic also opened during the early Cretaceous (110-125 Ma), but was separated from the North Atlantic by the bulge of Africa. During the mid to late Cretaceous period (95-65 Ma) Europe separated from North America, opening up deep

connections between the North Atlantic, South Atlantic and Tethys (Maldonado, 1985). A reconstruction of the oceans of the world during the late cretaceous is shown in Figure 2.5.

A biotic crisis in the Palaeo-Mediterranean occurred at the end of the Cretaceous period caused by intensified current systems that led to the replacement of organically rich sediment with red clays, resulting in decreased biodiversity and impoverished pelagic communities. Concurrently, during the Early Tertiary (65 Ma), the rate of North Atlantic sea-floor spreading increased north of the Azores-Gibraltar fracture zone, closing the gap between Africa and Europe as Africa moved dextrally relative to Europe. As a result most of the original Tethys was lost and the Mediterranean basins were formed (Maldonado, 1985). By the Eocene (40 Ma) most of the topographic features of the North Atlantic were established, although a deep channel still separated Spain and Africa facilitating the connection of the remnant Tethys and Atlantic. The connection between the Indian Ocean and Tethys was severed during the middle Miocene (16-14 Ma) ending connection with the eastern oceans (Benson *et al.*, 1991) (see Figure 2.5). Prior to this there is evidence for traffic of benthic Mollusca between the Indo-Pacific and Mediterranean during the Miocene (Robba, 1986)

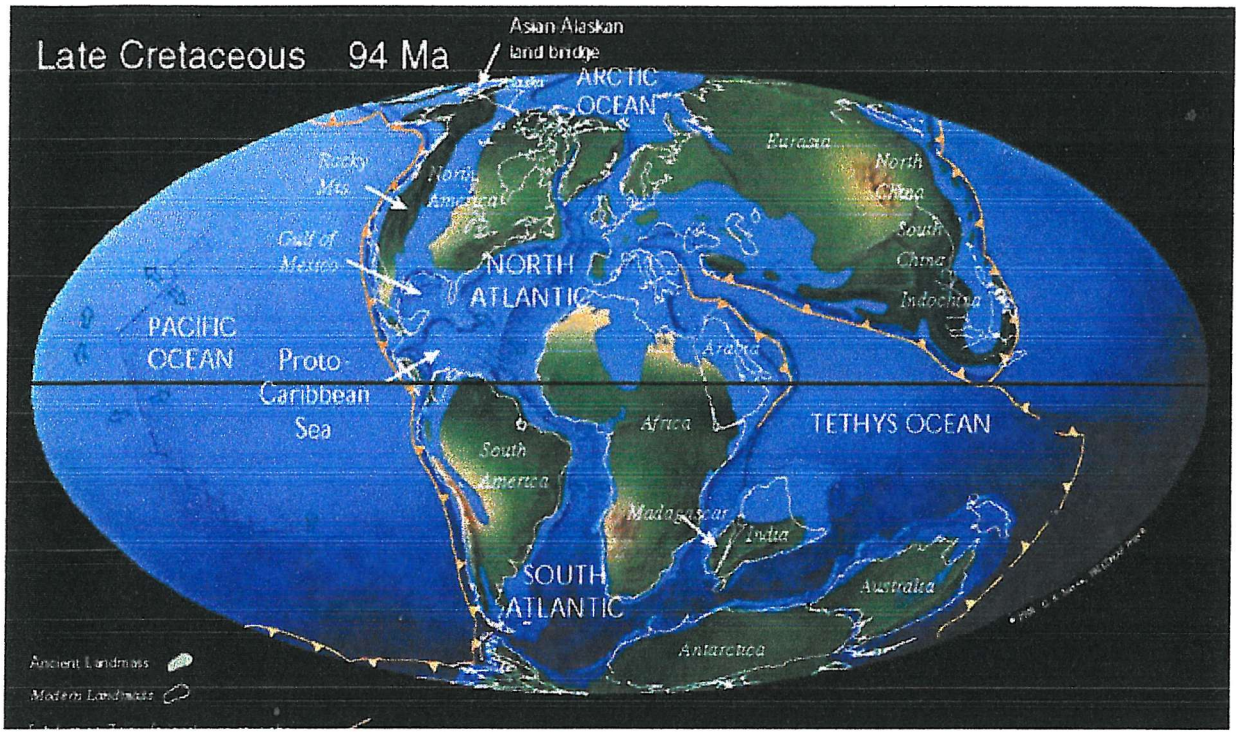


Figure 2.4. The reconstruction of the worlds land masses and oceans during the late Cretaceous. http://www.scotese.com/cretaceo.htm

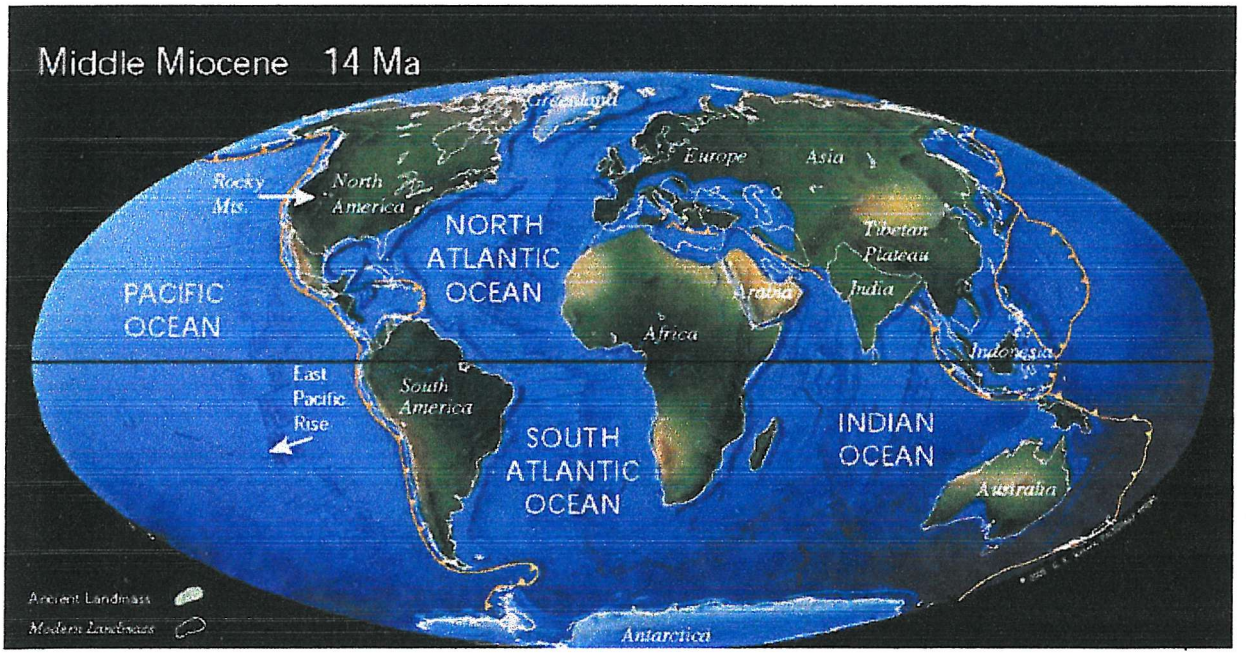


Figure 2.5. A reconstruction of the worlds land masses and oceans 14 Ma during the middle Miocene. http://www.scotese.com/miocene.htm

2.3.2 *Formation of the Atlantic Islands*

The Azores Archipelago, Madeira archipelago, Canary Islands and Cape Verde Islands represent long-lived hot spots under the earth's crust, centres of volcanism. The islands are the result of volcanic eruptions as the lithosphere moves over a fixed source of magma deep in the asthenosphere (Skinner & Porter, 1995).

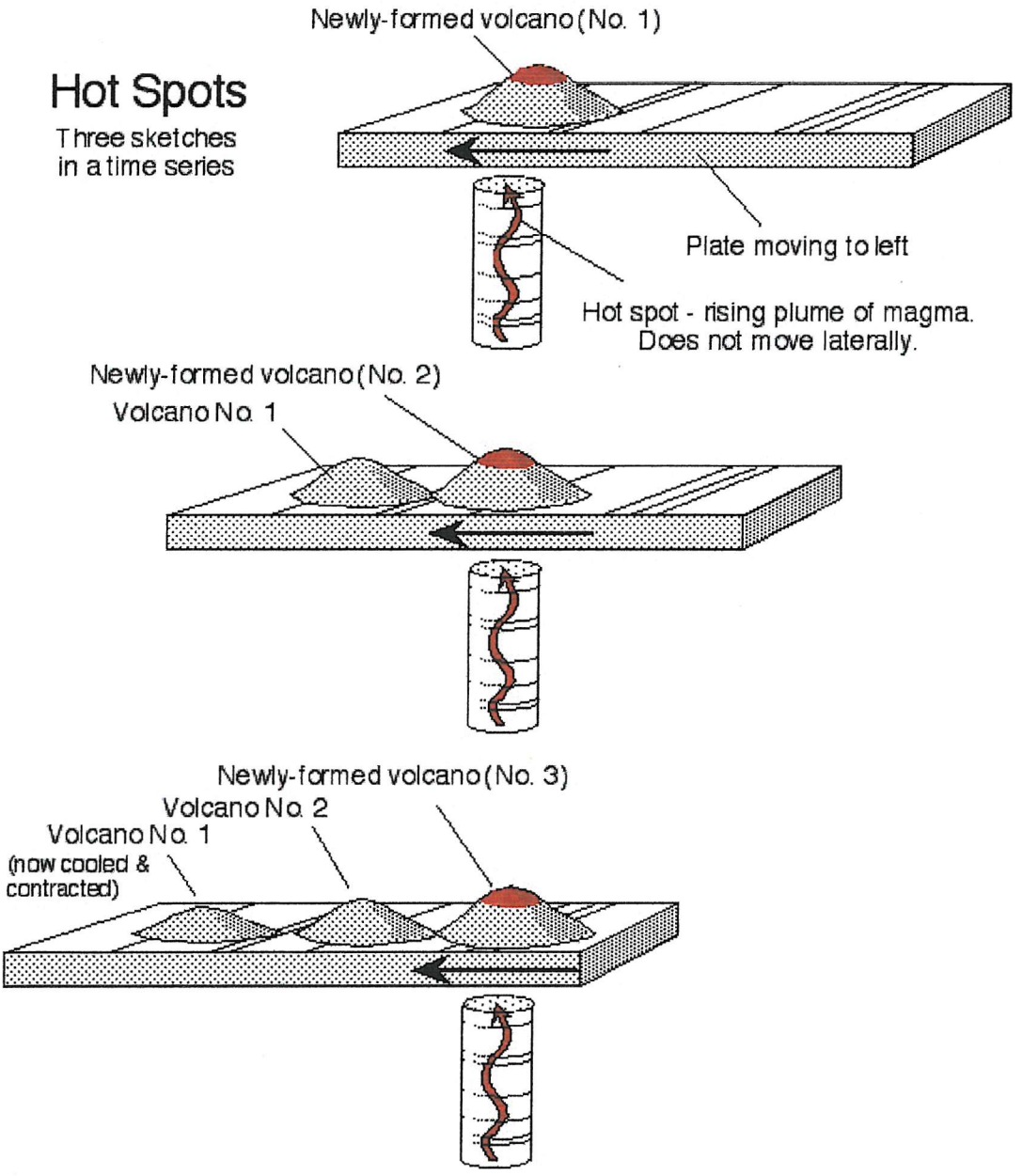


Figure 2.6. The process of island formation by hotspots, as thought to be responsible for the Northeast Atlantic Islands.

A 72Ma hotspot (see Figure 2.6) is responsible for the Madeira Archipelago, Desertas Islands and several seamounts between Madeira and the Serra do Monchique complex of SW mainland Portugal (Geldmacher & Hoernle, 2000). The Selvagen Islands are aged 20-24 Ma. Porto Santo is aged between 11-14 Ma, Madeira around 5 Ma with the Desertas Island between 3.2-3.6 Ma

(Figure 2.8). A similar northeast to south west age gradient is exhibited by the Canary Islands, also attributed to hotspot volcanisms, a propagating fracture from the Atlas mountains.

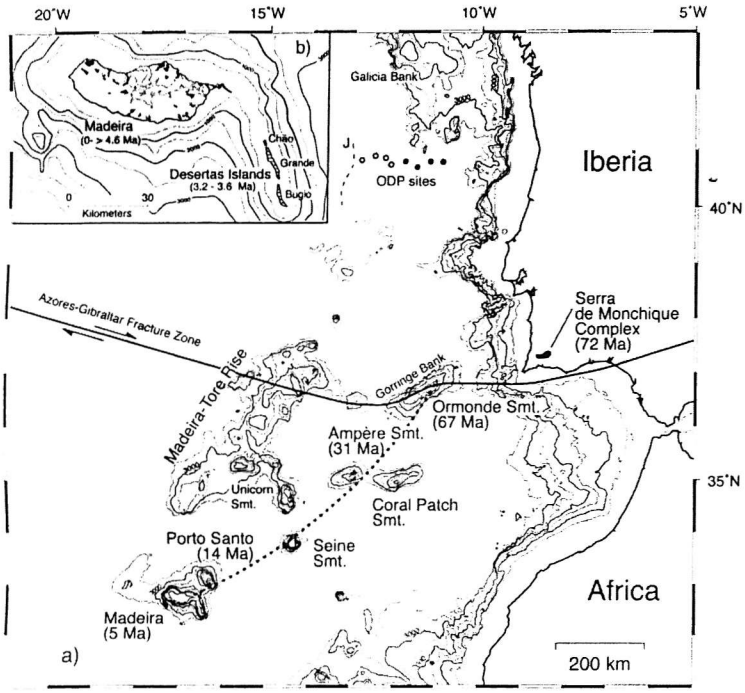


Figure 2.7. (A) Bathymetric map of seamounts and islands in the eastern North Atlantic. Source: TOPEX (Smith & Sandwell, 1997). Proposed Madeira hotspot track (shown as dotted line) and oldest available age data for each island or seamount. (B) Geological map of the Madeira Archipelago. After Geldmacher & Hoernle (2000).

The seven islands comprising the Canary Archipelago were formed within the last 20 Ma by volcanic eruptions. The ages decrease from east to west: Fuerteventura is the oldest at 20 Ma, then Lanzarote 15.5 Ma, Gran Canaria 14-16 Ma, Tenerife 11.6 Ma, La Gomera 10 Ma, La Palma 2 Ma and El Hierro 1 Ma. It is debated whether the islands arose from a hot spot origin or the result of a propagating fracture from the Atlas Mountains. Fuerteventura and Lanzarote have experienced continuous sub aerial volcanic activity during the last 20-22Ma which has resulted in an age gradient over the surface terrains of both the islands from the old SSW to the young NNE. Tenerife has had a disjunct volcanic evolution, originally composed of two islands - the separated massifs of Anaga (north-east) and Teno (north-west). These were joined recently by a massive lava flow from the volcanic eruptions that resulted in the central portion of Tenerife. On Gran Canaria the last volcanic cycle occurred 2.8 Ma, earlier eruptions (3.4-4.5 Ma) are thought to have caused a local extinction from the violent emission of volcanic agglomerates over most of the island (Juan *et al.*, 2000).

Although it is tempting to compare the Canary Islands with the stepping stone model of the Hawaiian Archipelago, the Canary Islands are different in their closer proximity to the mainland, their longer life spans and more complex volcanic evolution (Juan *et al.*, 2000). The Canary Islands represent a cluster, not a clear linear stepping-stone model. The terrestrial species composition of the islands is determined primarily by stochastic factors and there is evidence of repeated colonisation, which conflicts with a stepping stone model. This pattern is related to the volcanic events that have played a key role on the islands, including vicariance events, creation of diverse habitats and mass extinctions. The presence of terrestrial species seems to depend

mainly on availability of suitable niches for colonisers, and competition in these niches (Juan *et al.*, 2000). This may, or may not, be reflected in the marine fauna.

The Azores archipelago is the youngest of the archipelagos. It straddles the Mid-Atlantic ridge and represents the junction of three major lithosphere plates: the North American plate, the African plate and the Eurasian plate. Although the islands arose from volcanic eruptions, attributed to a hotspot near a spreading area, the movement of these major oceanic plates has influenced the area. At the centre of this junction lies the Azores microplate, from where most of the islands arose. The nine islands comprising the Azores vary in age form Santa Maria (8Ma) to Pico and Faial (0.5Ma). *Osilinus* do not occur on the Azores.

2.3.3 Late Cenozoic and Quaternary Events

Terminal Miocene Events

There were several `Terminal Miocene Events' during the Messinian Stage (7.12-5.32 Ma) including the Messinian Salinity Crisis, global cooling and a carbon-13 shift (Kennett, 1982). The global cooling likely caused increased polar ice sheets and a 40-50m decrease in sea level. The shift in carbon-13 is related to increased aridity during the late Miocene (6-7 Ma) that caused a shift from C3-dominated to C4-dominated ecosystems. This climatic change may also be responsible for the negative water budget of the Palaeo-Mediterranean that eventually resulted in the Mediterranean Salinity Crisis. Foraminifera cores place the Messinian/Pliocene boundary at between 4.91 - 5.6 Ma, and less precisely the Messinian/Tortonian between 6.36-7.18 Ma. The latter is indicated in the coiling direction change of *Neogloboquadrina acostaeusis* (Zhang & Scott, 1996). There were several glacioeustatic sea level changes in the North Atlantic and Mediterranean during the Messinian and early Pliocene. Intertidal turbidite deposits indicate sea-level low stands of 80-100m below present sea level at 6.59, 6.22, 6.01, 5.89, 5.75, 5.7, 5.65, 5.60, 5.55 Ma. The formation of Lower and Upper Evaporites in the Mediterranean is associated with the greatest climatic changes during the Messinian and periods of low stands (5.75 & 5.55 Ma), which are linked to the Messinian Salinity Crisis (Zhang & Scott, 1996).

Messinian Salinity Crisis

During the late Miocene (7-5 Ma) there were two passages connecting the Palaeo-Mediterranean (part of the remnant Tethys) and Atlantic. The Rifian Corridor through Northern Morocco and the Iberian Portal (Betic passage) through southern Spain (Figure 2.8). This resulted in the influx of cold waters from the Atlantic through the corridor into the Palaeo-Mediterranean, and the outflow of Palaeo-Mediterranean Overflow Water (hereafter PMOW) via the Iberian Portal (Benson *et al.*, 1991). This siphon phase began at the Tortonian/Messinian Boudary (6.4 Ma) and ended 5.3 Ma. Climatic conditions causing a deficit in the Palaeo-Mediterranean water budget facilitated the MSC. During the siphon phase, surface temperatures were 20°C higher than today, reflected by the coral reefs that flourished. Decreases in sea level by 50-70m are associated with the strong phase of this event (Figure 2.9).

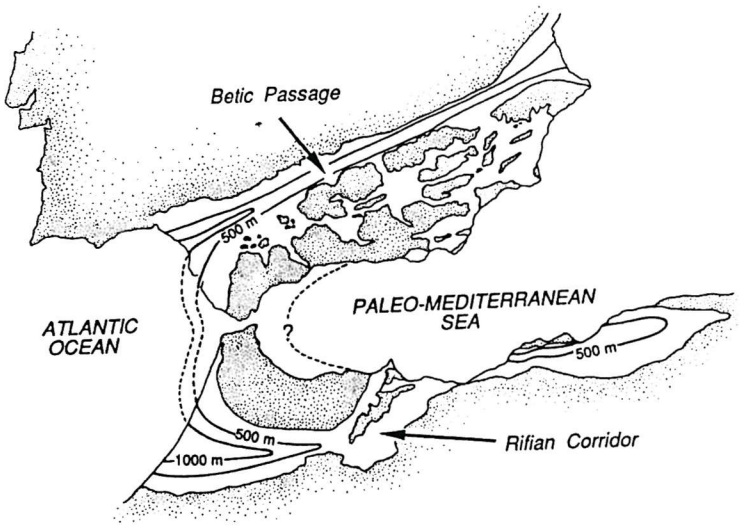


Figure 2.8. Palaeographic reconstruction of the Betic and Rifian passages near the Tortonian-Messinian boundary (Benson *et al.*, 1991)

The closing of the Iberian Portal began around 6.5 Ma, and by 5.6 Ma blocked the channel. The Rifian corridor remained open until approximately 5.3 Ma. Both straits remained closed for the last 400 kyr of the Messinian due to tectonic activity in the Alboran Microplate causing the African and European Plates to collide. As lateral tectonic movements occurred along the Guadalquivir fault in Spain and the South Atlas fault in Morocco, the microplate was compressed isolating the Palaeo-Mediterranean and the connection between the Atlantic and Tethys was finally lost (Benson *et al.*, 1991). The Mediterranean basin suffered significant tectonic subsidence after the Messinian, which is still active in the area of the Hellenic ridge (Por, 1989).

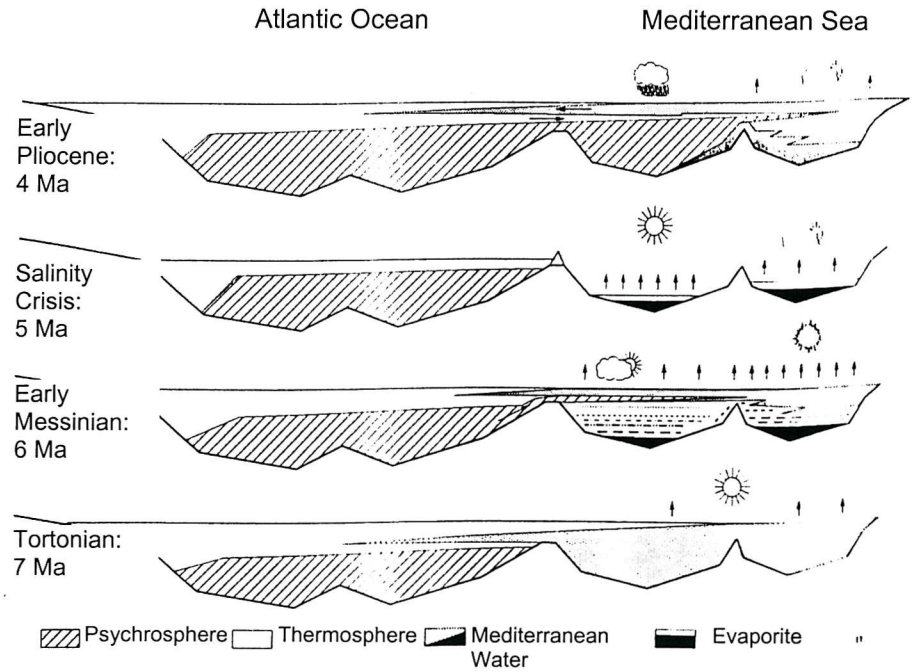


Figure 2.9. Hypothetical reconstruction of late Neogene water masses in the Atlantic and Mediterranean. After Benson *et al.* (1991).

The water budget deficiency responsible for driving the siphon, subsequently caused the desiccation of the Palaeo-Mediterranean basins, and resulted in the salinity crisis. Differences in evaporation and concentration of the deep water brines occurred between east and west basins during the Messinian Salinity Crisis, the reason for which is not clear (Benson *et al.*, 1991). The Mediterranean basins desiccated several times, with recurrent episodes of invading Atlantic water. There is no unanimous consensus that the Mediterranean dried out during the Messinian Salinity Crisis. Sterilisation was highly probable due to combinations of high salinity and at least partial desiccation. Sabelli & Taviani (1984) challenge this view, suggesting some Miocene benthic molluscs survived the Messinian Salinity Crisis in an area of the Atlantic near Gibraltar, and recolonised the Mediterranean once conditions became suitable. The deposition of salts from the desiccations could have removed 6% or more of all dissolved salts in the world oceans (Zhang & Scott, 1996). The Messinian sea-level fluctuations had a direct impact on the Mediterranean Salinity Crisis, and in fact could have been controlled by the repeated Mediterranean desiccation cycles. Atlantic water refilling the Mediterranean, or conversely water transfer from the Mediterranean to the global oceans could cause a fall or rise, respectively, of global sea level by 10m (Zhang & Scott, 1996).

The loss of Palaeo-Mediterranean Overflow Water into the Atlantic with the closure of the Palaeo-Mediterranean also had consequences for the Atlantic water mass structure and the European climate. The North Atlantic Gyre system had reached near maturation by the onset of the siphon event. With the supply of PMOW removed, or significantly reduced, the accompanying heat transfer from the Palaeo-Mediterranean to the eastern North Atlantic was removed, and consequently the climate of Europe was affected. The Messinian crisis was brought to an end as a result of further faulting during the taphrogenic stage that reopened the Palaeo-Mediterranean to the Atlantic, via the Gibraltar Strait. Rapid flooding by Atlantic waters gave birth to the Neo-Mediterranean, marking the beginning of the Pliocene Epoch. Table 2.1 summarises the current theories of the Messinian Salinity Crisis and corresponding events. The reopening of the Mediterranean and the concurrent Atlantic transgression into the Mediterranean (Trubi transgression) marked the boundary of the Pliocene Epoch (Por, 1989).

Approximately 3.5 Ma, the Bering Strait between Alaska and Siberia opened, connecting the North Pacific and Arctic-Atlantic basins. This led to the 'trans-Arctic interchange', a massive predominantly westward migration of fauna between the oceans. *Littorina* and *Nucella* species are two of the 295 Molluscan species took part in, or descended from taxa that took part in, this radiation (Vermeij, 1991).

Table 2.2. An outline of the history of the Messinian Salinity Crisis in the Medtierranean region. After Hsu *et al.*, (1997) and Benson *et al.* (1991).

Age	After Hsu *et al.*, (1977)	Benson *et al.* (1991)
Burdigalian Serravallian Tortonian	Loss of Palaeo-Mediterranean passage to the Indian Ocean: increase in aridity	
	Loss of connection to Paratethys, water budget deficit begins	
Tortonian/Messinian	No event recognised	Siphon formed in Rifian Corridor PMOW outflow through the Iberian Portal
After beginning of Messinian	Closure of Betic and Rif passages Drawdown of Palaeo-Med. Sea level	Straits in Rifian Corridor and Iberian Portal open; diatomites and coral reefs form.
Messinian Lower evaporite	Continous influx of Atlantic; no reflux Salt precipitation	Continued inflow through the Rifian Corridor Portal closes, siphon stops, inflow slows Eva orites in Spain; draw-down begins
Intra-Messinian	Cut off of Atlantic influx; erosion	Olistostrome blocks Rifian Corridor Influx from Atlantic reduced to trickle
Intra Messinian	Second influx of Atlantic water Marine deposition	General unconformity formed
Messinian Upper evaporite	Alternating closure and influx	Cyclic evaporites of continental origin formed Caspi-brackish lakes and lagoons
	Influx from Paratethys; Lago Mare formed	Deep basins/shallow water Strong climatic oscillations
Messinian/Zanciean	Influx of Atlantic water Opening of the Strait of Gibraltar	Massive influx from the Atlantic Portal location a mystery.

2.3.4 Quaternary Ice ages

The earth is presently near a time of maximum warmth in one of the many glacial-interglacial cycles that have occurred since the beginning of the late Cenozoic glacial era. This is reflected in Mediterranean sea levels that have risen by 5-6m in the last 2-3000 years (Margalef, 1995). There have been more than 20 glacial ages throughout the Pleistocene Epoch. Earlier glaciations lasted approximately 40,000yrs. During the last 800,000yrs, however, each glacial-interglacial cycle has lasted approximately 100,000yrs (Skinner & Porter, 1995). Global sea level has concurrently fluctuated for the last 125,000 years (see Figure 2.11) with the glacial and interglacial cycles, with dramatic repercussions for marine life, especially intertidal and shallow-water organisms. The most dramatic sea level change occurred 18, 000 years before present (Figure 2.12). A 120m fall in sea level corresponds to separation of the eastern and western Mediterranean basins during the last glacial period.

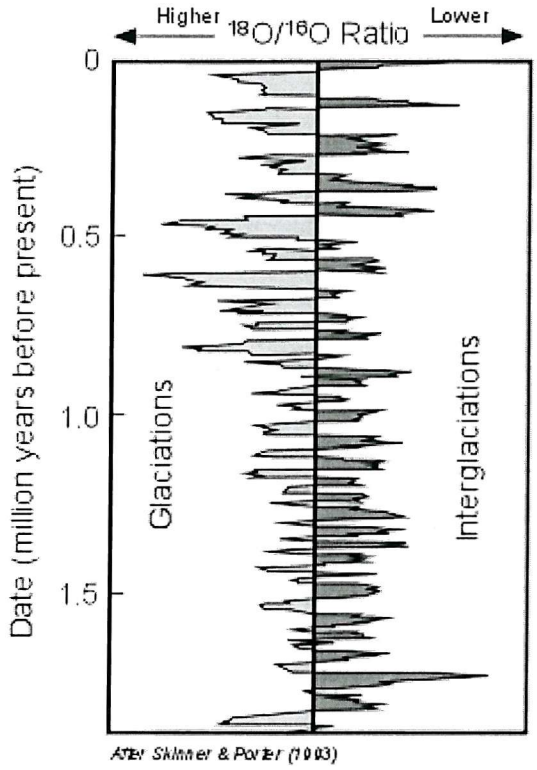


Figure 2.10. Average Curve of oxygen isotopes in deep sea cores representing global changes in ice volume during the last 2 million years (fromSkinner & Porter, 1991)

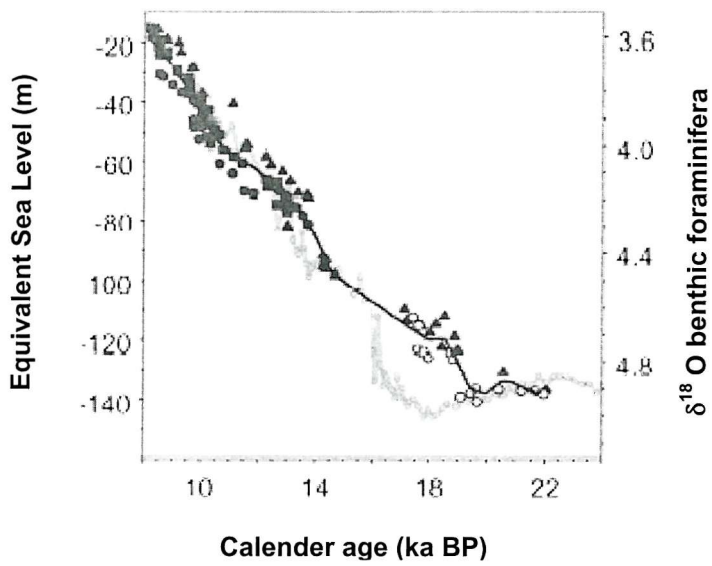


Figure 2.11. Global sea level changes over the past 25 000 years. The last Equivalent sea level (ESL) estimates based on coral data corrected for global isostatic effects. (After Mix *et al.,* 2001).

The water depth in the Straits of Gibraltar dropped to 230m, thus reducing influx of water from the Atlantic to the Mediterranean. Planktonic foraminiferal analysis reveals that sea surface temperatures in both basins dropped at this time, the sea surface temperature between Spain and Morocco was approximately 7°C, and the eastern Mediterranean 18°C, as compared with present temperatures of 22°C and 26°C respectively. Concurrently there was an increase of cold surface water entering the Mediterranean from the Aegean Sea as a result of meltwater from the Eurasian ice sheet draining southward through the Black Sea and Caspian Sea Basins into the Aegean. Drainage into the eastern basin from the Nile was decreased as a result of the increasingly arid climate in North Africa. During the late Pliocene and early Pleistocene increased precipitation and fresh water run off from the Nile may have also altered surface water conditions in the eastern basin (Raffi & Thunell 1997).

Many of the 44 seamounts (Figure 2.8) north and east of the Madeira Archipelago and the nine seamounts north and northeast of the Canary Islands (Epp & Smoot, 1989) were exposed by the falling sea level (Talavera, *in press*). Candidate seamounts include: Ashton Seamount 38°00'00"N, 11°25'00W; The Horseshoe Seamounts of Gettysberg Smt. 36°30'00"N, 13°00'00"W (presently rising to 20-28m depth); Ormonde Smt. 36°35'00"N, 11°25'00"W (rising to 33-46m depth); Ampere Smt. 35°05'00"N, 12°55'00"W (rising to 60m depth); Coral Patch Smt. 34°56'00"N. 11°57'00"W ; The Unicorn Seamount 34°45'00"N, 14°30'00"W; The Seine Smt. 33°55'00"N, 14°20'00"W; The Dacia Bank 31° 10'00"N, 13°35'00"W and Conception Bank 29°55'00"N, 12°45'00"W (Rogers, 1999). The exposed seamounts may have provided stepping stones or staging points for relatively short-lived pelagic larvae.

2.3.5 The present picture

The modern Atlantic current and circulatory systems were established 20-15 Ma during the Middle Miocene by large masses of water flowing from the Norwegian Sea and Artic Ocean into the Atlantic (Maldonado, 1985). The mid-Atlantic ridge is spreading slowly at 1-5cm/yr (Skinner & Porter, 1995). Today the North Atlantic Gyre is a wide eastern boundary current traveling clockwise past Morocco, and comprises the cool Canaries Current and the North Atlantic Water (>10°C) which is affected by the warmer and more saline Mediterranean Sea Overflow Water invading the Atlantic from the Mediterranean (Benson *et al.*, 1991). The surface water of the Atlantic, extending approximately 35° latitude N and S of the equator is called the central water mass, in which temperature and salinity ranges from 6-19°C and 34-36.5 PSu respectively. This overlies an intermediate water mass that extends to a depth of approx 1500m (Skinner & Porter, 1995). The nutrient rich surface Atlantic waters enter the Mediterranean and travel along the African northern coast. Eventually near the eastern basin they increase in density and become intermediate waters, which consequently form most of the overflow through the Straits of Gibraltar (Benson *et al.*, 1991). The water entering the Atlantic from the Mediterranean is of a higher salinity (37-38 PSu); it flows over the shallow Gibraltar sill and sinks to below the intermediate water mass, spreading over the ocean basin (Skinner & Porter, 1995).

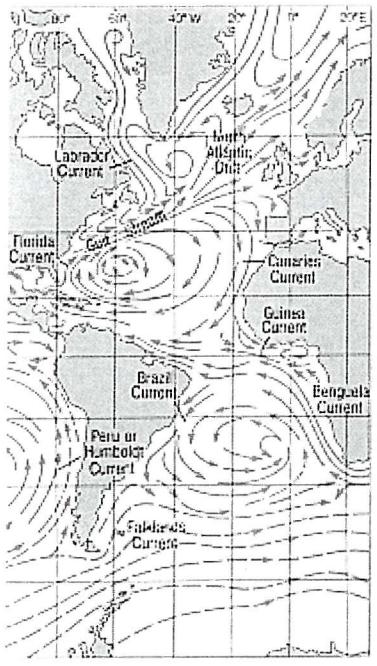


Figure 2.12. Simplified diagrammatic representation circulation in the North Atlantic Ocean (Morton *et al.*, 1998:15)

The Mediterranean represents several basins opening into Atlantic from the Black Sea. Its volume is 2.542 million km^2 and has an average depth of 1500m. Reconstruction models show the Mediterranean to be a dynamic sea, comprised of a mosaic of small and large plates constantly moving, forming ridges, trenches, back-arc basins and island arcs. There are two major and distinct basins, the western and eastern. The eastern Mediterranean is old and influenced by compressional forces, within which lies the Hellenic Trench system that is undergoing active subduction. This is thought to represent the last remnants of the Tethys Ocean. The newer western basins were created during the Cenozoic. The oldest basin in the Mediterranean is, however, the Balearic Sea in the western basin, dating to between the Oligocene and Miocene, whereas the youngest basin is the Aegean sea in the eastern Mediterranean, formed during the Pliocene and Quaternary (Margalef, 1985). Evaporation exceeds precipitation and freshwater run off in the Mediterranean, however, a mass-balance is still maintained. The high salinity and therefore more dense surface water sinks to the bottom. As it flows across the Straits of Gibraltar into the Atlantic, it sinks to reach an equilibrium with water of equal density (this water is then carried north along the Portuguese coast, interacting with an upwelling of deep water and topography along the coast of the Iberian Peninsula). The loss of deep water from the Mediterranean is overcompensated by an influx of nutrient-poor Atlantic water, via the superficial current across the Gibraltar Straits, and eutrophication does not take place (Figure 2.13).

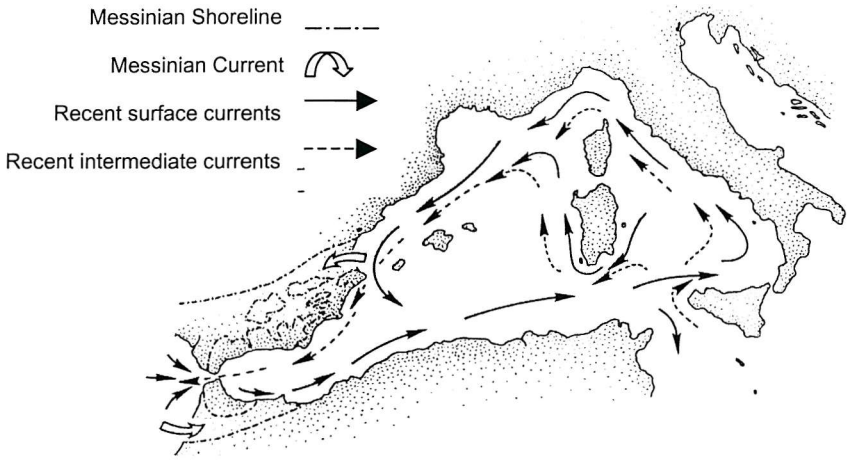


Figure 2.13. Recent and past currents of the Mediterranean (Benson *et al.*, 1991)

The Mediterranean is not a particularly fertile sea, it receives an average solar radiation of 1.5 million kilocalories/m^2/yr, and in the western basin, this is transformed into 60g C/m^2/yr, which is not an effective utilization of solar energy. It has low nutrients due to low freshwater input. Saline lagoons and marshes occupy 6,500km^2 of Mediterranean shores, about half of which occur in western Mediterranean. Brackish environments are generally unstable, however the Atlantic coast is receding, and in the process accumulating large amounts of sediment, creating important deltas, such as the Rhone and Ebro. These brackish environments can act as negative estuaries with higher salinity than the open sea (Benson *et al.*, 1991). The Mediterranean possesses a complex coastline complex because of the recent changes in sea level, pattern of coastal currents and topography of the coast. Tides are small, therefore reducing the intertidal area and enhancing segregation.

2.4 Synthesis

This chapter provides an overview of the general theories on rate, mode and mechanism of speciation. It also gives a summary of examples and evidence of speciation in the marine environment, clearly showing the complexity, multidisciplinary nature and immaturity of the subject. A common theme from the literature is the importance of life history strategies in marine speciation and ecological divergence facilitating sympatry (e.g. interactions with competitors and predators, dispersal abilities, larval settlement preferences, habitat preferences). Neither of these processes are well understood for many marine organisms, including *Osilinus*. Until better understanding of the biology and ecology of organisms, speciation in the marine environment will remain elusive.

Identifying species boundaries is therefore crucial to understanding speciation. Although biogeographic patterns are not sufficient to infer the mechanisms driving speciation, when combined with known historical events (as described in the palaeoceanographic overview of this chapter), they can be used to construct hypothesis on the modes and mechanisms of speciation. This chapter therefore provides a background for hypotheses of the speciation processes that

have determined the present biogeography of *Osilinus,* as given in Chapter 3. These hypotheses, based on the palaeoceanography of the Northeast Atlantic and Mediterranean, and biogeography of *Osilinus* are consequently tested in light of the phylogenetic relationships of *Osilinus* resolved in chapters 5 & 6.

Chapter 3

Patterns of distribution: description and biogeography of *Osilinus* in the Northeast Atlantic and Mediterranean

3.1 Introduction

'Biogeography is not yet a formalized science. The biogeographer must concern himself with a full range of phenomena concerning chorology (area analysis), commonality (both continuous and disjunct), uniqueness (endemism), species mobility (active and passive) and speciation (both course and cause), and these facets are as important in themselves as any rigid rules of procedure for the erection of biogeographical theories.' (Ball, 1983:409).

In the late 19th century, Wallace's (1880) theory of biogeography was published and the subject was essentially born (Patterson, 1983). Wallace's theory is based on the assumption that any distribution can be explained by four basic causes: dispersal, evolution, geographic change and climatic change. This rational remained the prominent and widely accepted theory until the 1960s when the new subject areas of population ecology and genetics became widely studied. Consequently, there was a demand for more analytical, testable and predictive approaches that discriminated between pattern and process. This required a method of capturing pattern that was not dependent on the theories of process being tested. Probably the most famous response is that of Mac Arthur & Wilson (1967) and their "Theory of Island Biogeography". This theory did not require, however, any '*commitment to evolution*'. It is in essence an equilibrium approach to biogeography (Patterson, 1983). Two other schools of thought were concurrently established within the field of biogeography: phenetic (Sokal & Sneath, 1963) and cladistic (Hennig, 1966; Eldredge & Cracraft, 1980) approaches, which led to a dichotomy between advocates of dispersal and vicariance to explain biogeographic distributions.

Those that adhere to one school of thought (i.e. vicariance cf. dispersal), tend to underestimate or over estimate the role of dispersal respectively (Patterson, 1983). '*The dispersal model proposes that present-day distributions were caused by long distance dispersal among disjunct localities. The vicariance model proposes that the distributions resulted from the fragmentation* of *formerly continuous distributions with accompanying speciation'* (Endler, 1982). Since Hennig (1966), cladistic analysis has proved a powerful tool, not requiring the effort and specialist knowledge of phenetic approaches (Paterson, 1981). It has extended to include both vicariance and dispersal approaches (see Nelson & Platnick, 1981) reflecting a trend to integrate the opposing schools of thought (Endler, 1982). Classically, biogeography does rely on the assumption that all speciation is allopatric, and therefore sympatry indicates dispersal. This can create problems where sympatric speciation has been demonstrated (e.g. due to sexual selection in cichlid fishes, Seehausen, 1999)

Biogeography is essentially a pattern-gathering science with the aim to test and predict theories of evolution. It has become clear that species composition around the globe fall into distinguishable groups, called zoogeographic provinces. The primary molluscan provinces of the continental shelves have been described as depicted below (Figure 3.1).

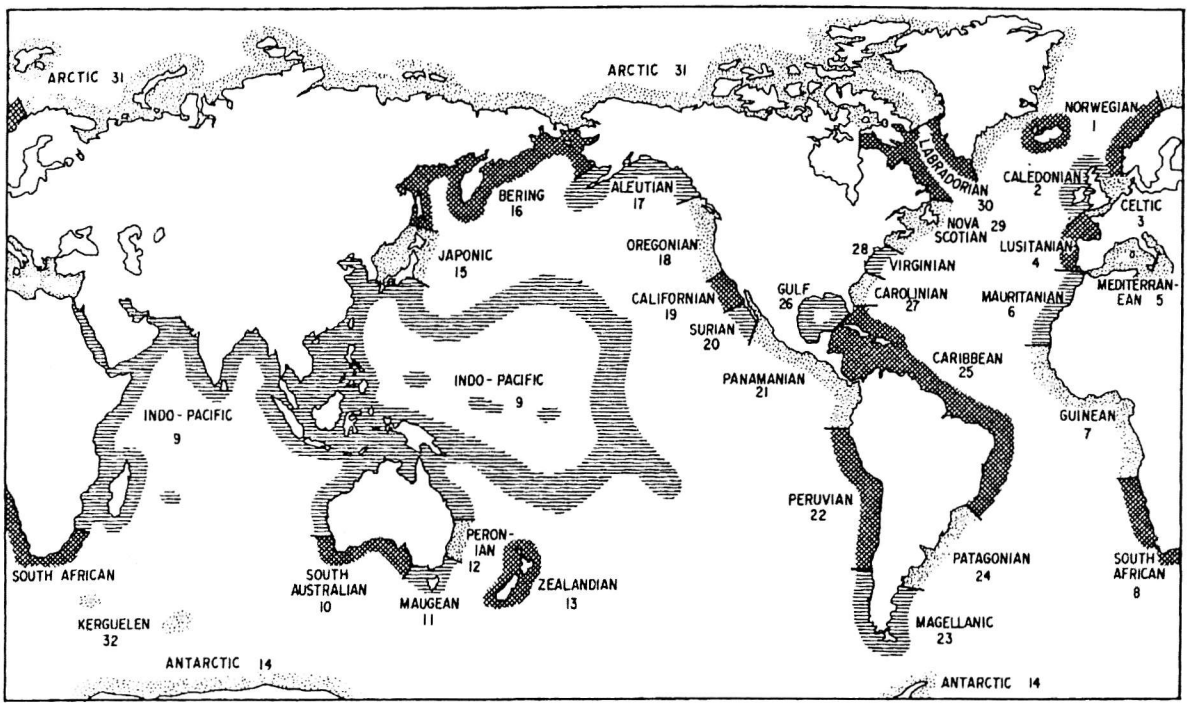


Figure 3.1. The 32 described contemporary, primarily molluscan provinces (numbered on map) of the continental shelves (from Talent *et al.,* 1986).

Marine faunal and floral provinces are known to be determined by the following factors (Talent *et al.,* 1986):

- Degree of separation (especially latitudinal), the effects amplified or diminished by patterns of oceanic circulation.
- Length of pelagic larval life of the benthic organisms.
- Presence or absence of intervening staging points. The enormous extent of the Indo-Pacific tropical province is a result of galaxies of coral reefs providing staging posts for the dispersal of shallow-marine biota.
- Presence of land, deep-water or oceanic circulation barriers.
- Duration of isolation from other provinces.

These reflect the processes discussed in chapter 2. Biogeography is highly dependent on the species concerned being correctly described and their distributional limits known. At the outset of this work, the taxonomic status and resultant distribution of species of *Osilinus* was unresolved and ambiguous (Poppe & Guido, 1991). This chapter provides a revised description and distribution of the genus *Osilinus*. The biogeographic descriptions provide a summary of the fieldwork results, synthesis of the Trochidae collections of the British Museum, Natural History

(BMNH) and cited distributions (Nobre, 1937; Fischer, 1943; Duffas & Johnston, 1969; Burnay & Monteiro, 1977; Nordsieck & Garcia-Talavera, 1979; Nordsieck, 1982; von Cosel, 1982; Poppe & Guido, 1991; Giannuzzi-Savilli, *et al.*, 1994;).

Descriptions of the five Atlantic and Mediterranean *Osilinus* species, determined in part from the molecular and morphological phylogenetic analyses (chapters six and seven), are given below to end the previous taxonomic confusion. The morphological descriptions are based on shell and radula morphology. Shell morphology and variation are described and illustrated in Figures 3.5 to 3.10. The radula descriptions relate to SEM photographs (Figures 7.2 to 7.13 in chapter seven). The recently designated Indian Ocean species *Osilinus kotschyi* is also described. Finally, in light of the biogeography, the relevance of the speciation and palaeoceanogeographic literature presented in chapter 2 is discussed to provide working hypotheses on the processes and mode of speciation that have occurred in *Osilinus*.

3.2 Fieldwork methodology and collection sites

Extensive fieldwork was required to collect samples for molecular and morphological analysis and establish the presence and range of *Osilinus* species throughout the Mediterranean and Atlantic Islands. The fieldwork site locations are shown in Figure 3.2 and 3.3. The name and GPS reference of each site is listed in Table 3.1. Ecological surveys were conducted at most locations. Random clusters of five $1m^2$ quadrats over a 30m transect were placed at three different vertical heights on the shore to survey low, mid and upper shore habitats. The number of individuals of each *Osilinus* species present and percentage cover of other occupying species was recorded for each quadrat. The ecological survey data provides information on tidal range, habitat and species composition, but it is not included in the thesis. It is partly summarised in the species descriptions (section 3.4). At each site between 20-50 individuals of each species were collected for preservation in absolute ethanol for molecular analysis.

3.3 The biogeography of *Osilinus*

The species of *Osilinus* Philippi, 1847 occurring in the Mediterranean and Northeast Atlantic, determined by the phylogenetic analyses conducted for this thesis (chapter 6), are considered as follows: *O. lineatus* (da Costa, 1778), *O. atratus* (Wood, 1828), *O. articulatus* (Lamarck, 1822), *O. edulis* (Lowe, 1842) and *O. turbinatus* (Born, 1780). The distribution of *Osilinus* ranges from the Cape Verde Islands and coast of North Africa (Morocco) to the British Isles (Kendall 1987). McMillan (1944) describes it as a characteristic southern genus. Figures 3.2 and 3.3 illustrate the biogeography of the Atlantic/Mediterranean species. The distribution of the supposed Indian Ocean Tethyan relict *Osilinus kotschyi* is given in Figure 3.4. Biogeographic notes for each species are given below, preceding the species descriptions.

Table 3.1: Locations for the Gibbulini species *Gibbula umbilicalis* (G), *Osilinus lineatus* (L), *Osilinus edulis* (E), *Osilinus atratus* (A), and *Osilinus turbinatus* (T) and *Osilinus articulatus* (Ar).

Map Ref.	Location	Latitude	Longitude	G	L	E	A	T	Ar
1	UK - Lyme Regis	N50°43.267'	W002°56.113'	•	•				
2	UK - Porthguerra	N50°19.223'	W005°13.503'	•	•				
3	Basque Coastline	N43°17.28'	W002°58.08'	•	•	•			
4	Spain - Torre Guadiaro, Cadiz	N36°14.40'	W005°17.28'					•	•
5	Spain - Calahonda, Malaga	N36°29.06'	W004°44.08'					•	•
6	Spain - Ponta Sol, Malaga	N36°23.13'	W005°12.14'						
7	Spain - Puerto duguesa, Malaga	N36°21'	W005°14'			•		•	
8	Cyprus - Coral Bay	N34°46.57'	W032°24.20'					•	
9	Greece - Corinth	N37°56.33'	W022°57.8'					•	
10	Morocco - Anza	N30°26.767'	W009°39.663'		•	•			
11	Morocco - Tifnit	N30°11.619'	W009°38.357'		•	•			
12	Morocco - Imourane	N30°30.374	W009°41.238'		•	•			
13	Morocco - Cap Ghir	N30°38.676'	W009°53.357'		•	•			
14	Tenerife - Las Galletas	N28°06.366'	W016°37.302'			•	•		
15	Tenerife - Los Gigantes	N28°13.444'	W016°50.135'			•			
16	Tenerife - La Rambla	N28°25.589'	W016°30.071'			•			
17	Tenerife - El Medano	N28°01.512'	W016'32.126'			•			
18	Tenerife - Santa Cruz	N28°26.239'	W016°09.102'			•			
19	Fuerteventura - Coleta del Cotillo	N28°40.264'	W014°10.462'			•	•		
20	Fuerteventura - Punta de la Caletones	N28°14.628'	W014°12.735'			•			
21	Fuerteventura - nr Punta Paloma	N28°10.471'	W014°11.536'				•		
22	Fuerteventura - nr Roja Mountain	N28°39.441'	W013°49.888'			•	•		
23	Fuerteventura - Castillo Beach	N28°13.256'	W013°46.724'			•	•		
24	Fuerteventura - Playa de Ugan	N28°16.243'	W014°13.333'			•			
25	Lanzarote - La Santa	N29°06.977'	W013°39.573'			•	•		
26	Lanzarote - Costa Teguise	N28°58.927'	W013°30.879'			•	•		
27	Lanzarote - Jameous de Agua	N29°09.485'	W013°25.876'			•	•		
28	Lanzarote - La Golfa	N28°58.950'	W013°49.901'			•	•		
29	Gran Canaria - Cuintanella, San Andres	N28°08.372'	W015°34.077'			•	•		
30	Gran Canaria - Playa de Balito	N27°44.245'	W015°40.426'			•			
31	Gran Canaria - Playa del Burrero	N27°57.028'	W015°19.372'			•	•		
32	Gran Canaria - Playa de la Aldea	N27°58.311'	W015°50.053'			•			
33	Gran Canaria - San Cristobal	N28°10.213'	W015°46.298'			•	•		
34	Gran Canaria - Playa de Sardina	N28°04.587'	W015°25.264'			•			
35	Madeira - Prainha Beach	N32°44.28'	W016°37.18'			•			
36	Madeira - Ponta do Sol	N32°40.07'	W017°05.06'			•			
37	Madeira - Sao Vincente	N32°48.45'	W017°00.44'			•			
38	Madeira - Seixal	N32°49.35'	W017°07.33'		•	•			
39	Portugal - Viana do Castelo	N41°41.25'	W008°50.14		•				
40	Portugal - Povoa de Varzim	N41°22.41'	W008°45.31'		•				
41	Portugal - Figueira da Foz	N40°08.55'	W008°50.54'		•	•			
42	Portugal - Ericeira	N38°57.59'	W009°25.07'		•				
43	Portugal - Sesimbra	N38°26.48'	W009°04.16'		•				
44	Portugal - Porto Cova	N37°50.08'	W008°48.53'		•	•			
45	Portugal - Sagres	N37°01.14'	W008°56.58'		•	•			
46	Portugal - Burgua	N37°04.08'	W008°46.12'		•				
47	UAE - Khor Al Hamra	N25°40.52'	E055°47.03'						

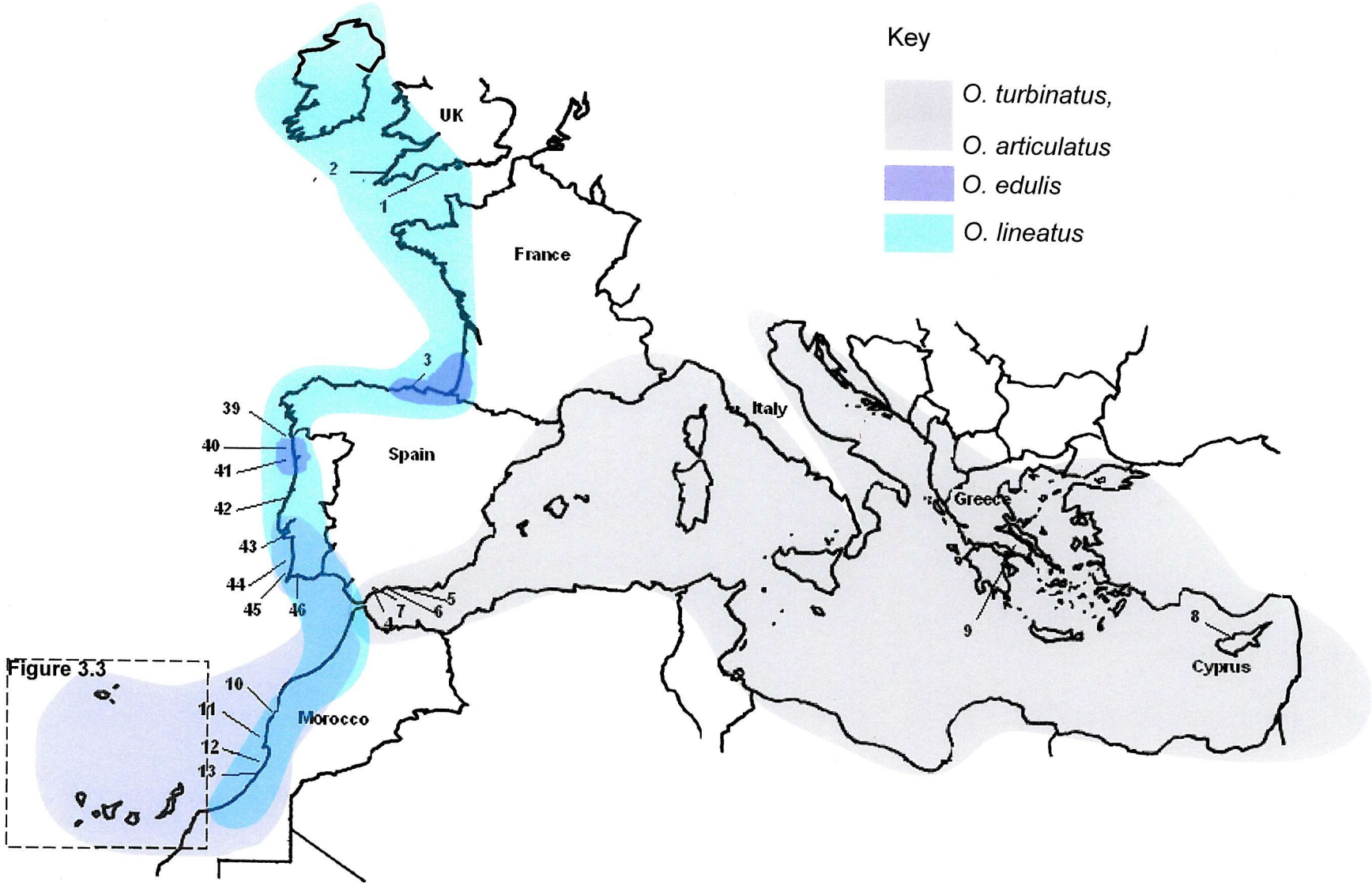


Figure 3.2. The distribution of *O.articulatus, O. turbinatus, O. lineatus and O. edulis* in the Northeast Atlantic and Mediterranean. Fieldwork collection sites are numbered. The name and GPS reference of each site is listed in Table 3.1.

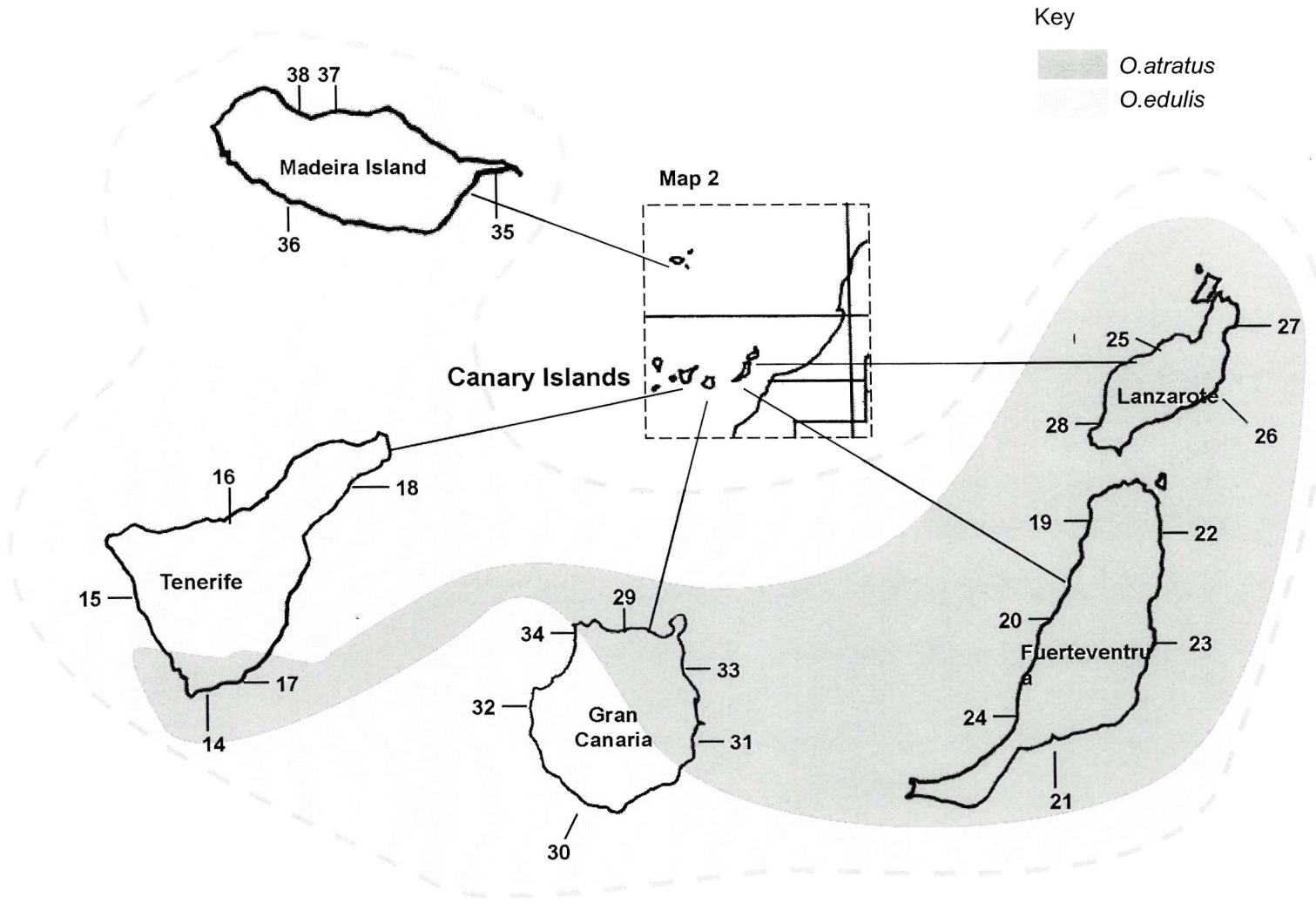


Figure 3.3. Insert from Figure 3.2: Distribution of *O.edulis* and *O.atratus* on Madeira and the Canary Islands. Fieldwork collection sites are numbered. The name and GPS reference of each site is listed in Table 3.1. The solid-outlined, shaded areas represent the distribution of each species based on my fieldwork results. The dark grey dashed line represents the distribution of *O. atratus* based on the literature. *O. atratus* probably occurs in low numbers within this area. Availablility of suitable habitat (*O. atratus* is found on less exposed rocky shores than *O. edulis*) on the western Canary Isles and Madeira is probably the main factor limiting the distribution of *O. atratus*.

Osilinus kotschyi

Until this species was transferred to *Osilinus* by Herbert (1994), this genus had only been recorded in the Mediterranean and Northeast Atlantic. *O. kotschyi* is the only Indo-West Pacific species of *Osilinus,* recorded by Herbert (1994). It is endemic to the Persian Gulf, Gulf of Oman, Arabian Sea and Red Sea (Figure 3.4). Based on this recent taxonomic designation, *O. kotschyi* has been proposed as the sister species to the Atlantic/ Mediterranean *Osilinus*, and is thought to be an ancient relic of the genus (Herbert, 1994).

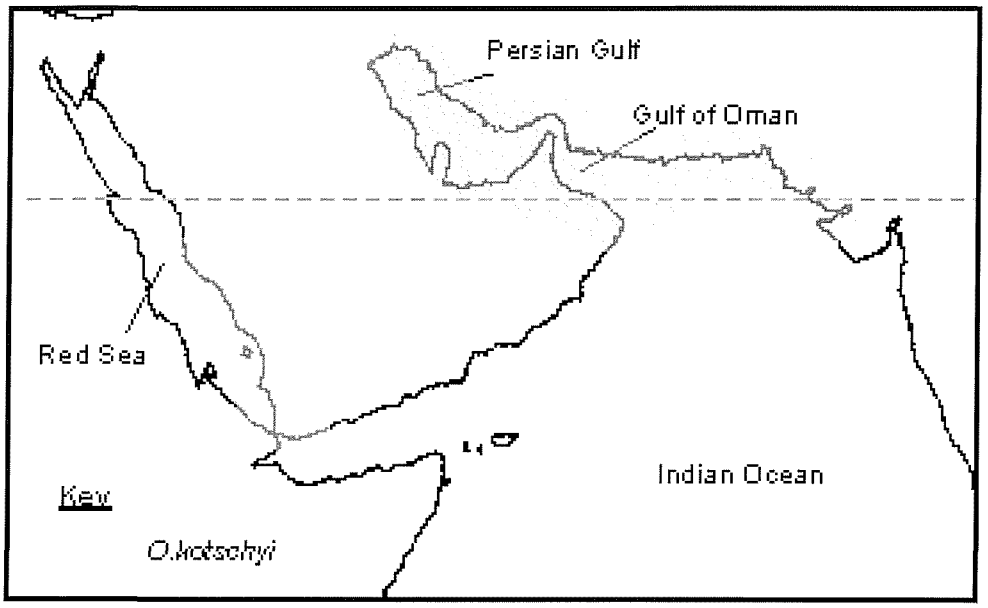


Figure 3.4. Distribution of *O. kotschyi* based on material examined by Herbert (1994).

Osilinus turbinatus and Osilinus articulatus

O. turbinatus and *O. articulatus* are sympatric species found throughout the Mediterranean. *O. turbinatus* is found in higher, mid-energy sites, *O. articulatus* occurs in more protected, lower-energy sites. These Mediterranean species reach their western limit at Sotogrande on the southern Spanish coast. From Sotogrande, west to Fuengirola, occasional incursions of these species have been observed in sympatry with the Atlantic species O. *lineatus* and *O. edulis* (Gofas, *pers comms). O. articulatus* has been described to occur on the Northern coast of Portugal (Poppe & Guido, 1991), however none of my fieldwork observations or specimens in the British Museum, Natural History support this. This observation was most likely due to taxonomic confusion or misidentification of *O. lineatus*.

Osilinus atratus

The taxonomic designation of this species is historically confused (see chapter 1), and its range was consequently unclear at the beginning of my study. *O. atratus* has been often cited as occurring on Madeira (Nordsieck & Garcia-Talavera, 1979; Poppe & Guido, 1991). It was not, however, found at any location on the islands during fieldwork. The Selvagen Island population

(with a paler shell colouration) has been given subspecies status *O. atratus selvagenesis* (Talavera, 1978). *O. atratus* occurs on the Canary Islands, in less abundance than *O. edulis*. It shows a trend of increasing abundance to the east in the Canaries. *O. atratus* was observed at all sites sampled around Lanzarote, the most eastward Isle. On Fuerteventura its distribution was limited to the eastern coast and northwest in Coleta del Cotillo. On Gran Canaria *O. atratus* exhibited a northeastern distribution from Cuintanella, San Andres south to San Cristobal. On Tenerife, it was only found at Las Galletas, the very southern point of the Island. This distribution is most likely a reflection of its preference for less exposed habitats than *O. edulis*. According to specimens from the British Museum of Natural History, *O. atratus* also occurs on the Cape Verde Islands. *O. atratus* was known to share a similar distribution as *O. edulis* with regard to the Atlantic Islands (Nordsieck & Garcia-Talavera, 1979), but thought also to occur along the western coast of Africa and possibly Portugal (Poppe & Guido, 1991). *O. atratus* was not observed at any location along the Portuguese or Moroccan coastline, nor is this supported by the collections of the British Museum, Natural History. It therefore appears to be endemic to the Atlantic islands. It may, however, occur in pockets on the coast of West Africa south of Agidir, Morocco.

Osilinus edulis

Previously, this species was known to occur on the Atlantic Islands of the Canaries, Madeira and the Salvegens (Poppe & Guido, 1991). It does not occur on the Cape Verde Islands (Burnay & Monteiro, 1997). The range was thought to extend to the Atlantic coast of North Africa and along the coast of Portugal in areas of colder upwelling (Fischer-Piette, 1957; Hawkins, *per comms;* Poppe & Guido, 1991). This was, however, unclear due to taxonomic confusion (reviewed in chapter one). The distribution of *O. edulis*, based on fieldwork collection and the molecular analysis (chapter 6) has been clarified. This species occurs on the northern shores of Spain in the Bay of Biscay. It has a disjointed distribution along the Atlantic coast of Portugal appearing in the north around Figueira da Foz, reappearing in the south from Porto Covo to Sagres. It does not occur in abundance along the Algarve coast of Portugal due to the lack of suitable habitat. *O. edulis* reappears along the southern Atlantic coasts of Spain on suitably exposed shores such as at Zahara south of Cadiz. Occasional occurrences of both *O. edulis* and *O. lineatus* have been observed in the Mediterranean east to Fuengirola (Serge Gofas, *pers comm). O. edulis* continues southward along the Atlantic coast of Morocco down to Agidir. The actual southern limit of *O. edulis* most probably extends further south along the western Atlantic coast of Africa.

Osilinus lineatus

As an Atlantic species, the distribution of *O. lineatus* was previously described as extending from the British Isles south to Portugal (Poppe & Guido, 1991). On the African coast it was known to occur south to Asilah, (150km south of Tangiers) (Fischer-Piette, 1959). *O. lineatus* was collected along the entire length of the western and southern Portuguese coast, incurring along the southern Atlantic coast of Spain, east to the Gibraltar straits. The southern distribution of *O. lineatus* is, however, more extensive than previously described (Fischer-Piette, 1959). The range of *O.*

lineatus continues south to Agidir, Morocco, where it I observed it in abundance. As for *O. edulis,* the actual southern limit of *O. lineatus* most probably extends further south along the western Atlantic coast of N. Africa.

The most northern populations of *O. lineatus* are found along the Irish coast. It does not occur ubiquitously around the coast but has a limited range. *O. lineatus* has a predominantly western and southern distribution around Ireland. It is present at all suitable shores (flat rocky beaches, sheltered boulder strewn beaches with minimal sand and shingle) between Dublin on the east coast, clockwise round the coast up to Donegal Bay in the Northwest. Availability of suitable habitat substrate is possibly the limiting factor to the eastern distribution of *O. lineatus* in Ireland.

On mainland Britain, the northern distributional limit occurs on Anglesey (Crisp & Knight-Jones, 1964) with substantial populations reaching the Lleyn Peninsula, North Wales (Kendall, 1987). Recent observations (Nova Mieszkowska, *pers. comm.,* 2003) have shown that it has regained ground lost after the cold winter of 1962/1963 (Crisp, 1964). South of this point, *O. lineatus is* common throughout Cornwall, occurring also on the Isles of Scilly (Kendall *et al.,* 1996) and the Channel Isles (McMillan, 1944). It has been observed in the Bristol Channel to Minehead (Gardiner, 1935). Abrupt eastern limits have been observed on the English and French coasts of the English Channel. On the south coast of England, *O. lineatus* is only present in the Western channel; Lyme Regis supported the last major eastern population. (Crisp & Southward, 1958), although it has been found further east in small numbers (Nova Mieszkowska, *pers. comm.,* 2003). On the French coasts, the distribution of *O. lineatus* reaches significantly further east, ending east of the Cotentin peninsula. The sharp distributional limits reflect suitable habitat availability: there is a predominance of exposed rocky shore along the western Channel, as opposed to the uniform, fine sediment shores of the eastern Channel. The east-west divide of the channel biota may also be correlated to the topographical limits of the eastern and western basins. The western biota of the latter is far richer, containing Atlantic and Lusitanian-Mediterranean forms, which the eastern basin lacks (Crisp & Southward, 1958).

The populations of *O. lineatus* at their distributional limits do not display characteristics typical of near limit populations, but rather regular population structures (see Hutchins, 1947; Lewis *et al.*, 1982; Kendall, 1987; Kendall *et al.*, 1987). Transplantation experiments of *O. lineatus* to the North Yorkshire coast by Kendall and Williamson proved highly successful despite a cold winter (Kendall 1987). An explanation suggested for both of these factors is that the northerly distribution limit of *O. lineatus* in mainland Britain is reached because of physical barriers preventing colonisation of otherwise suitable sites, before it is ultimately limited by climatic factors.

3.4 Descriptions of the species of *Osilinus* Phillipi, 1847

The history of the genus is reviewed above (chapter 1). Crothers (2001) gives a taxonomic description of all the species below but considers *O. edulis* and *O.sauciatus* separate species status. (see also Poppe & Guido, 1991; Nordsieck, 1982). For systematic descriptions of the Mediterranean species, *O. articulatus* and *O. turbinatus* see Cesari & Pranovi (1989), Giannuzzi-Savilli, (1994) and Oliverio (1996). For taxonomic descriptions of the Atlantic Island species *O. atratus* and *O. edulis* see Watson (1897), Nobre (1937), Dufas &Johnston (1969) and Nordsieck (1979). Synonyms, morphological descriptions (shells and radulae), discussion of taxonomic confusion and shell variation of all *Osilinus* species are given below with emphasis on diagnostic features. Shell morphology is shown in Figures 3.5 – 3.10. Radula morphology is shown in figures 7.6 – 7.13 (chapter 7).

3.4.1 Osilinus kotschyi (Philippi) 1849 : Figures 3.5 and 7.6.

Synonymy: This species was previously placed in the genus *Trochus,* see Melvill & Standon, (1901) and Moolenbeek (1995), but is presently considered to belong to the genus *Osilinus.* For a complete systematic description, see Herbert (1994).

Habitat: Low energy intertidal flats on fine soft sediment and hard substrates, rocky beaches and silty creeks. It also occurs amongst *Zostera.*

Morphological description: Based on shell, radula, operculum and external anatomy *O. kotschyi* is consistent with that of members of the Gibbulini (Herbert, 1994). Variation in shell morphology is illustrated in Figure 3.5. Shell shape and size is analogous to *O. atratus* (Figure 3.7).

The radula formula is ∞+6+1+6+∞, (see Figure 7.6). In light of Hickman & McLean's (1990) systematic revision and other published radula figures the *O. kotschyi* radula has been described as an intermediate form between the outgroup species *M. labio* and *G. umbilicalis*. (Herbert, 1994). The rachidian and lateral teeth possess large pointed cusps 50-70μm in length with 2-3 denticles at the base (Figure 7.6B-D). The rachidian has a thin neck shaft and small neck shaft wings (Figure 7.6C). The outer lateral tooth is differentiated from lateral teeth 1-4 by possession of a bifid basal lobe projection, subject to intraspecific variation (Figure 7.6E&F). The marginal teeth are smooth in appearance with no rachis and a rounded cusp tip (Figure 7.6G). 2-3 small feathery denticles are present at the cusp base (Figure 7.11 H).

3.4.2 Osilinus articulatus (Lamarck, 1822): Figures 3.6, 7.7 and 7.8.

Synonymy: *Trochus turbiformis* von Salis, 1793; *Monodonta draparnaudi* Payraudeau, 1826; *Trochus tesselatus* A. Adams (non Gmelin, 1791).

Habitat: Sublittoral to Intertidal, mid to upper shore on moderately exposed rocky shores. Occurs on boulders and in crevices. Not associated with macroalgae.

Morphological description: This species exhibits high intraspecific variation in shell morphology, particularly colouration and banding pattern (see Figure 3.6). The shell is similar in shape and

size to *O. lineatus*. The top whorls are less commonly eroded due occurrence in lower energy habitats, consequently shell height often exceeds diameter. *O. articulatus* is characterised by a broad spiral of red and white bands below the suture of each whorl. This pattern is repeated to varying degrees, within each whorl, interspersed by *O. lineatus* type zig zag patterning (Figure 3.6).

The radula formula is ∞+5or6+1+5or6+∞, (see Figures 7.7 and 7.8). There was considerable intraspecific variation in *O. articulatus* radula morphology. In general appearance, the radula was analogous to that of the other *Osilinus* species (Figure 7.7A). The marginal teeth are typical of this genus with a pronounced dorsal rachis and 2-5 denticles at the cusp base. The first inner marginal tooth possesses a broader and shorter tipped cusp than the adjacent outer teeth (Figure 7.7C&D). The lateral teeth are similar to the rachidian in basal plate morphology with large broad tipped cusps 50-70μm long with 3-5 denticles at the cusp base (Figure 7.7E). The outer lateral tooth is differentiated from lateral teeth 1-4 by a well-defined basal lobe (Figure 7.8E&F) comparable to that of *O. kotschyi* (Figure 7.6E). The rachidian cusp, however, displays intraspecific variation in both cusp morphology and size (Figure 7.7B, 7.8C&D). The rachidian possesses a thin neck shaft with small neck shaft wings in all states. The cusp is either short (15-25μm) lacking distinctive shape and denticulation (7.7B), or exhibits small serrated lobes at the cusp side (Figure 7.8D) comparable to *O. edulis* (Figure 7.10B), *O. lineatus* (Figure 7.11B) and *O. turbinatus* (Figure 7.13A). It can also have a larger broad tipped rachidian cusp 25-40μm long comparable in morphology to the lateral cusps (Figure 7.8C). Of most interest is the variation in lateral tooth number. Although five pairs of lateral teeth were most common, six pairs were also observed (Figure 7.8B&F) therefore six pairs of lateral teeth is not a taxonomic feature exclusive to *Osilinus kotschyi*.

3.4.3 ***Osilinus atratus*** **(Wood, 1828):** Figures 3.7 and 7.9

Synonymy: *Trochus punctulatus* Lamarck, 1882; *Monodonta. trappei* F. Norsieck, 1974; *Monodonta selvagensis Talavera, 1978; Monodonta tamsi Dunker, 1858; Monodonta atratus turbinoides* F. Nordsieck, 1982.

Habitat: Intertidal, mid to upper shore on moderately exposed rocky shores. Occurs at the base of rocks near sand and on small rocks and pebbles, on medium boulders and rocks and in crevices. It is often found in sympatry with *O. edulis* on moderately exposed shores.

Morphological description: The intraspecific variation in shell colouration and banding pattern is illustrated in Figure 3.7A-G. The most common colouration, typical of Madeira, the Canary Islands and Cape Verde Islands is a black background with white zig-zag stripes (Figure 3.7A,B,C,F,G). The Salvagens Island subspecies *O. atratus salvagensis* (Nordesieck & Garcia-Talavera, 1979) displays the reverse colouration (Figure 3.7D & E). The shell is elevated and trochoid with stepped whorls. Shell height nearly always exceeds diameter (see Burnay, 1977; Cosel, 1982).

The radula formula is ∞+5+1+5+∞, (see Figure 7.9). The rachidian has a broad neck shaft with no neck shaft wings and a broad triangular expanding basal plate. The rachidian is cusp small (10-25μm long) in comparison to the lateral teeth cusps (50-75μm long) as throughout the *Osilnus* spp. (excluding intraspecific variations in *O. articulatus*). The cusp shape displays intraspecific variation (Figure 7.9D&E), either with a strong concave cusp tip similar to *O. turbinatus* (Figure 7.13A) but without serrations, or an M-shaped cusp similar to *O. lineatus* (Figure 7.11D). Lateral teeth 1-4 are similar to the rachidian in basal plate morphology with 4-6 denticles at the lateral tooth cusp base (Figure 7.9C). The outer 5th tooth has a differentiated basal lobe that interacts with the lateral tooth denticles (Figure 7.9F). The marginal teeth have a very strongly defined dorsal rachis. The inner most marginal displays a typically shorter cusp with a broad square tip (Figure 7.9G). The marginal teeth have 3-4 feather denticles at the cusp base (Figure 7.9H&I).

3.4.4 ***Osilinus edulis*** **(Lowe, 1842):** Figures 3.8 and 7.10.

Synonymy: *Trochus sauciatus* (Koch, 1845); *Monodonta colubrinus* (Gould, 1849)

Habitat: Intertidal, mid to upper shore on moderate to very exposed rocky shores. Occurs on medium to large boulders and rock and in crevices.

Morphological descriptions:

Crothers (2001) describes *O.sauciatus* (Koch, 1845) as a separate species to *O. edulis* with a geographical distribution along the Portuguese Atlantic coastline, extending north to Catalonia. Conversely, Fischer-Piette (1959) describes *O. colubrinus* as occurring in Portugal. In other taxonomic revisions, *O.sauciatus* (Koch, 1845) and *O.colubrinus* (Gould, 1849) are listed as synonyms of *O. edulis* reflecting the historic ambiguity in the number of species (Nordsieck & Garcia-Talavera, 1979) (see chapter 1). High intraspecific shell variation has led to this confusion (Figure 3.8). Shells A and B display the less distinctly patterned morphotype. This has a flattened globose shell that has historically been diagnostic of the Portuguese variety or subspecies *O.sauciatus/ O.colubrinus*. Shells A and B, however, actually originate from Seixal, Madeira. Shells G and H illustrate the typical shell morphology of *O. edulis* thought to have a distribution limited to the Canaries, Madeira and possibly the Atlantic coast of North Africa. These shells are more elevated and possess a paler background with dark stepped or wavy lines. Shell G was collected from El Medano in Tenerife. Shell H was collected, however, from the Basque coastline in Northern Spain. Shells C to F are examples of intermediate shell morphology, all of which are collected from the Canary Islands. Both morphotypes and their intermediates appear along the Atlantic coastline from the Basque region, along the Portuguese coastline, down to southern Morocco and on the Atlantic Islands of the Canaries and Madeira. From the extensive sampling conducted for this work, there is no evidence for the *O.sauciatus / O.colubrinus* taxonomic division based on geographical distribution and morphology.

Spiral bands of red and white spots occur below the suture of juveniles and some adults (Figures 3.8E & F). This has led to taxonomic confusion with the species *O. articulatus*. Populations of *O. edulis* occurring on the Salvagens Islands have a distinct colour pattern, possessing a white

background with bands of red and dark spots near the top of the spire, rather than the otherwise darker specimen with similar banding patterns.

The radula formula is ∞+5+1+5+∞, (see Figure 7.10). The rachidian (Figure 7.10B) has a small cusp 15-20μm long with an M shaped cusp top (described as typical of the Gibbulini by Hickman & McLean, 1990) and small serrations as in *O. lineatus* (Figure 7.11B) and *O. turbinatus* (Figure 7.13A). The neck shaft is broad with no neck shaft wings. The basal plate is triangular, reflected in the morphology of the four inner lateral teeth. The five pairs of lateral teeth possess large cusps, 50-75μm long, with 3-5 denticles at the base of the cusp (Figure 7.10C). The outer lateral tooth is differentiated by the basal lobe, which interacts with the cusp denticles (Figure 7.10D) as in *O. lineatus* (Figure 7.11C). The innermost marginal tooth has a shorter broad tipped cusp compared to adjacent teeth (Figure 7.10E). The marginal teeth possess well defined dorsal rachis (Figure 7.10E) and 2-4 denticles at the cusp base (Figure 7.10F).

3.4.5 Osilinus lineatus (da Costa, 1778): Figures 3.9, 7.11 and 7.12.

Synonymy: *Trochus crassus* Pulteney, 1799

Habitat: Intertidal, mid to upper shore on moderate to exposed rocky shores and limestone flats. Occurs on boulders and in crevices and at the edge of rock pools. Not associated with macroalgae.

Morphological description: Variation in shell morphology is limited in comparison with other *Osilinus* species (see Figure 3.9). The shell has a distinct alternation of fine dark and light zig-zag patterning. The dark colouration ranges from black-brown to pink. The same zig-zag patterning is observed in *O. articulatus,* between spiral bands of red and white pigmentation (Figure 3.6). The *O. lineatus* shell is globose but elevated. The top whorls are often eroded, however, and width commonly exceeds shell height (L/D = 0.9-1.00).

The radula formula is ∞+5+1+5+∞, (see Figure 7.11 and 7.12). The top of the rachidian cusp is a distinct M shape. It is short (10-25μm long) and differentiated from that of the lateral teeth cusps. The cusp serration shows intraspecific variation (Figure 7.11). Most commonly, it has 3-4 small rudimentary denticles or serrations and a square projection at the cusp tip (Figure 7.11B). There are no neck shaft wings, and the basal plate is of similar shape to *O. kotschyi.* The five lateral teeth either side of the rachidian are similar in size and basal plate morphology but possess large broad ended cusps 85-120μm in length (Figure 7.11C). Two to five denticles are present at the cusp base (Figure 7.11D). The outer lateral tooth possesses a basal lobe that interacts with the cusp denticles (Figure 7.11C). The first, inner marginal tooth has a shorter and wider cusp than adjacent teeth. A dorsal rachis is present on all but the small, outer marginal teeth (Figure 7.11E). All but the outer marginal teeth possess 2-3 feathery denticles at the cusp base. As for all the gibbuline taxa described in here, the extent of the denticulation along the cusp edge increases relative to cusp length as the tooth size decreases from the inner to outer teeth (Figure 7.12A-C).

3.4.6 ***Osilinus turbinatus*** **(Born, 1780):** Figure 3.10 and 7.13.

Synonymy: *Trochus tesselatus* Born, 1778; *O. fragaroides* (Lamarck, 1822); *O. olivieri* (Payraudeau, 1826).

Habitat: Intertidal, mid to upper shore on moderately exposed rocky shores. Occurs on boulders and rocks and in crevices.

Morphological description: The variation in shell morphology is illustrated in Figure 3.10. There are obvious differences in shell patterning between specimens from eastern and western Mediterranean. All shells have a pale background with a dark, spiral-banding pattern. Shells on the left (A, C & E) from the eastern Mediterranean have large dark blocks of pigmentation spaced at regular intervals approximately equal to the length of the pigmentation. The shells on the right (B, D & F) from the western Mediterranean have smaller, closely spaced red or dark green spiral blocks of pigment. No intermediates were observed either in specimens collected for the analyses or the dry collection of the BMNH.

The radula formula is ∞+5+1+5+∞, (see Figure 7.13A). The rachidian of *O. turbinatus* is similar to that of *O. edulis*. The cusp is short in comparison to the lateral teeth cusp, approximately 20μm long. There are three to four small serrations on the cusp as in *O. lineatus* (Figure 7.11B), *O. edulis* (Figure 7.10B) and in some specimens of *O. articulatus* (Figure 7.8D). The broad neck shaft exhibits small neck shaft wings. The lateral teeth are similar to the rachidian in basal plate morphology but have long cusps 50-60μm and 4-5 denticles at the cusp base (Figure 7.13B). The marginal teeth have the same morphology as *O. lineatus,* described above.

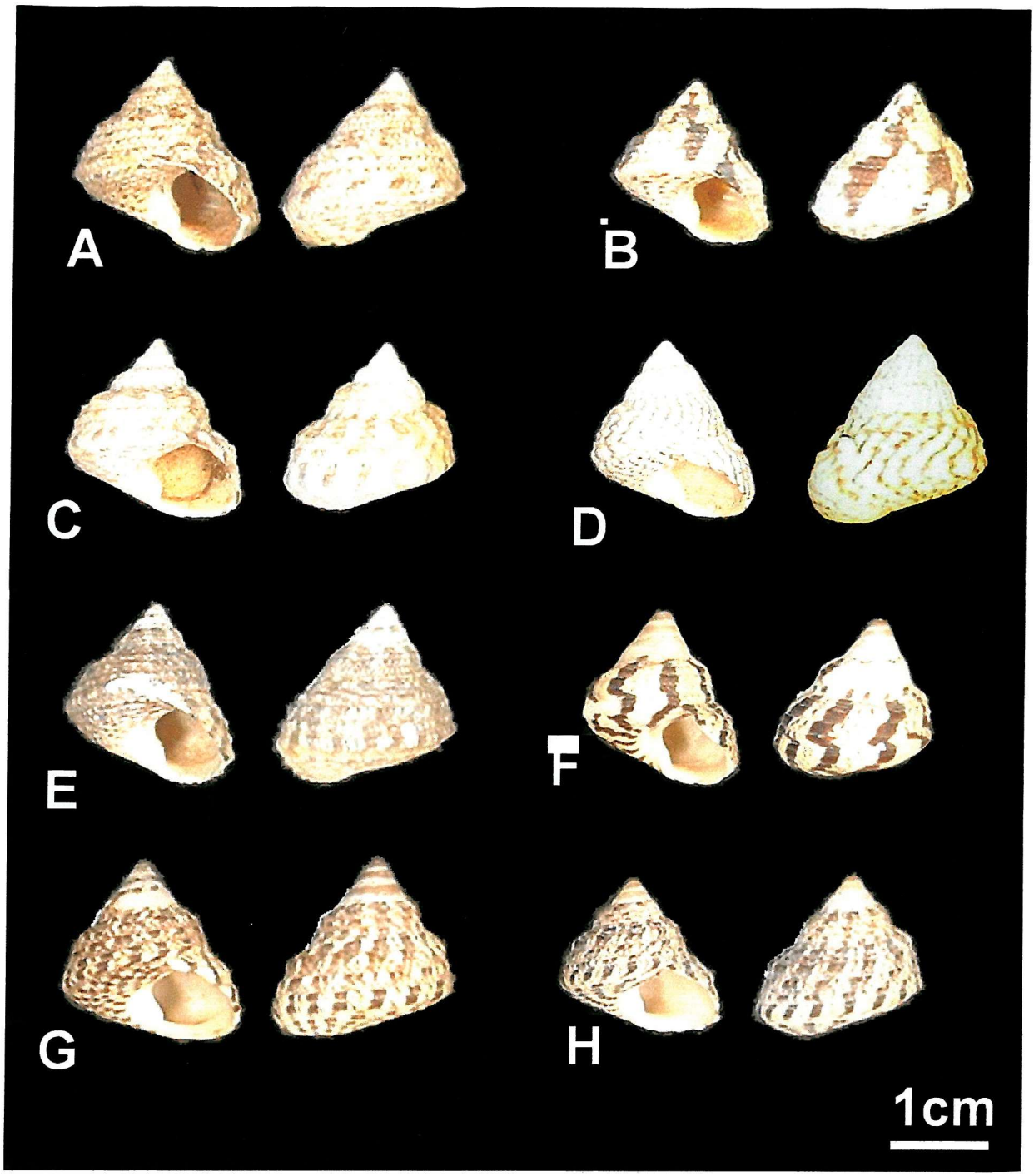


Figure 3.5. Plate of *Osilinus kotschyi* shells.
Specimen locations A - E: Hormuz Island, Persian Gulf. F-H: coast of Iran, Persian Gulf (BMNH acc. no. 2258).

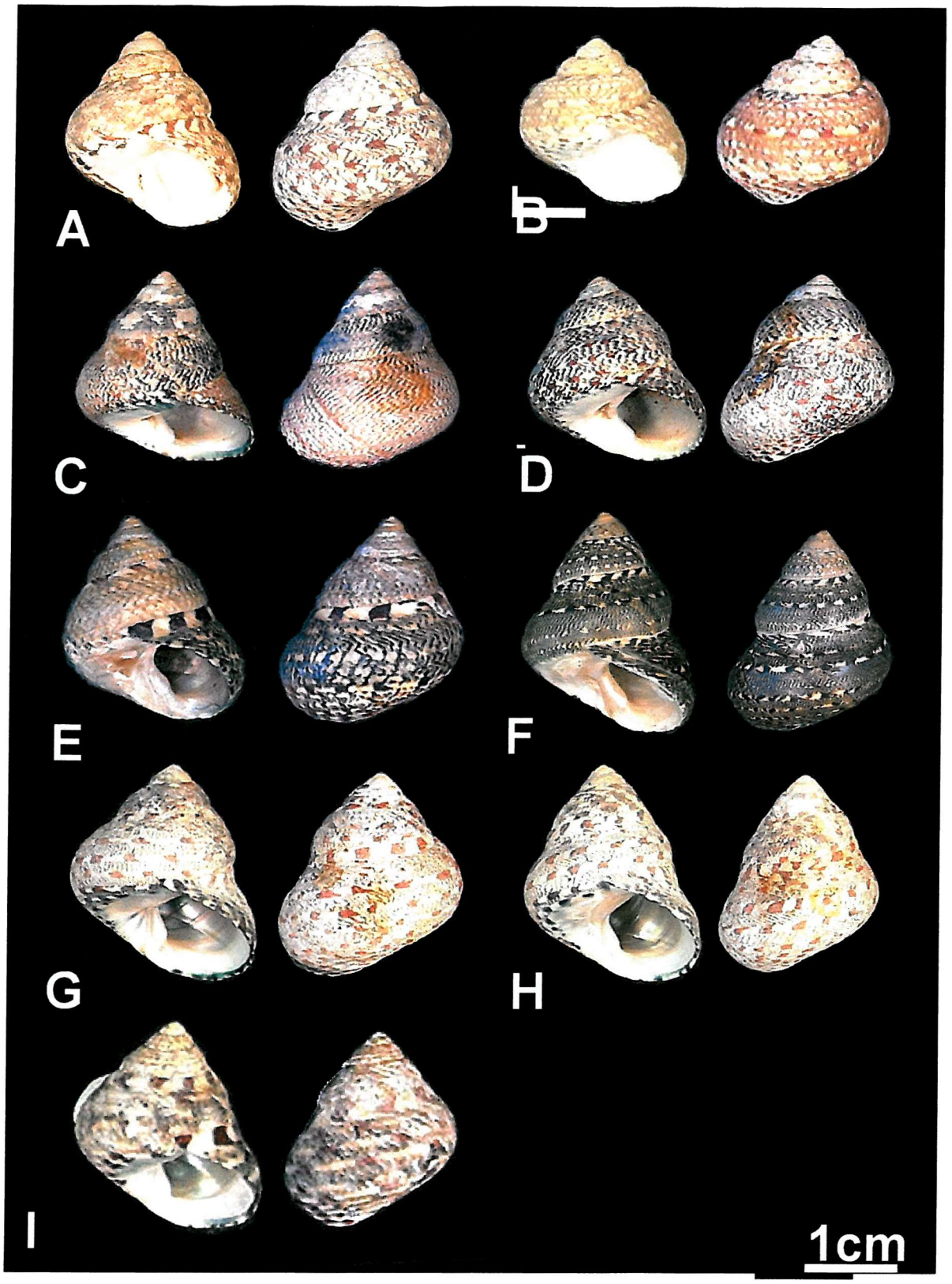


Figure 3.6. Plate of *Osilinus artictulatus* shells.
Specimen locations: A & B: Tobruk, Libya, (BMNH Acc.No.: 2258). C & D: Oran. E - F: Malaga, Spain. G - I: Porto Pollo, West Corsica.

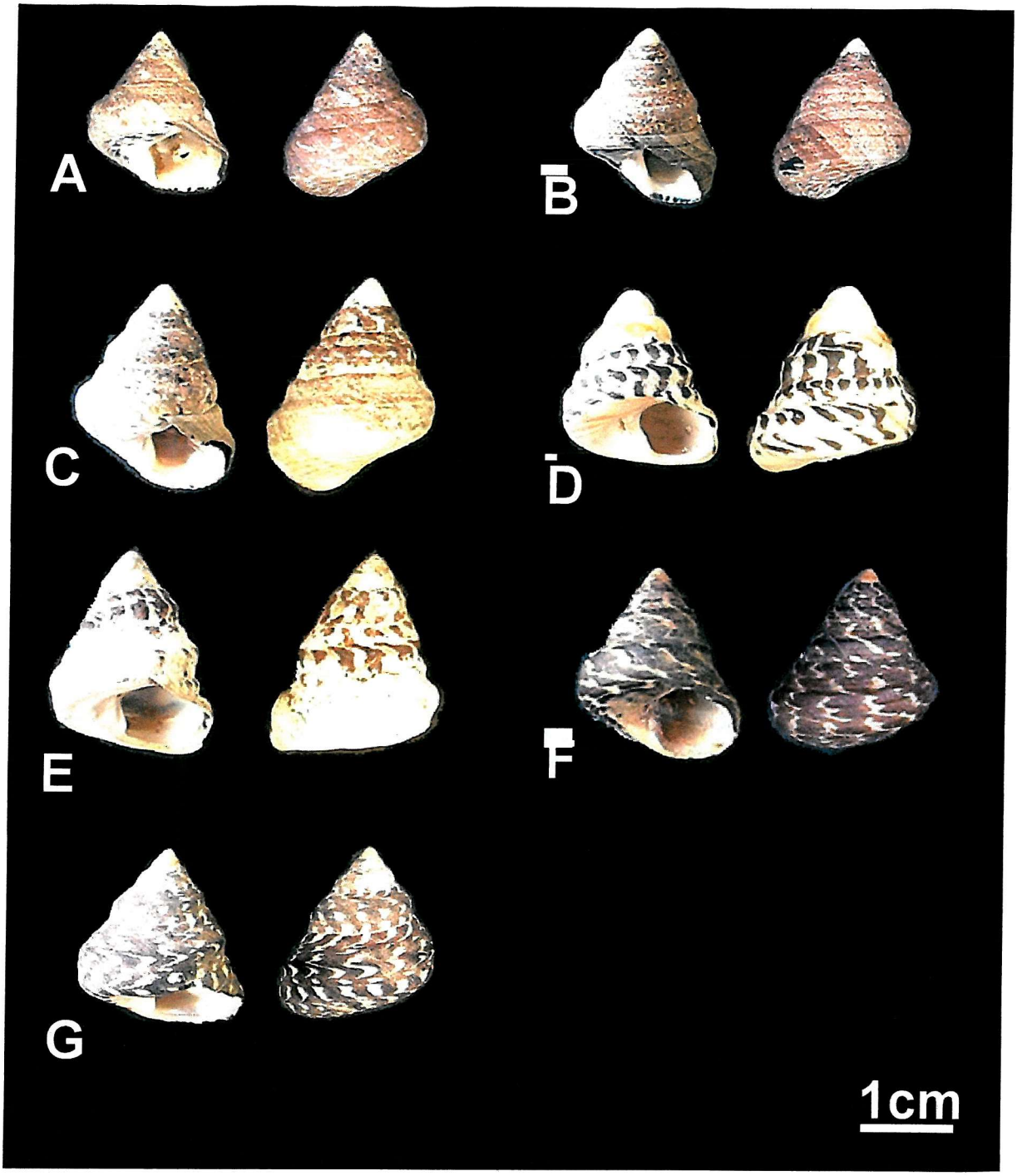


Figure 3.7. Plate of *Osilinus atratus* shells.
Specimen locations: A - C: Las Salinas, Costa Teguise, Lanzarote. D & E: Selvegen Is. F: Canary Islands. G: Cape Verde Islands.

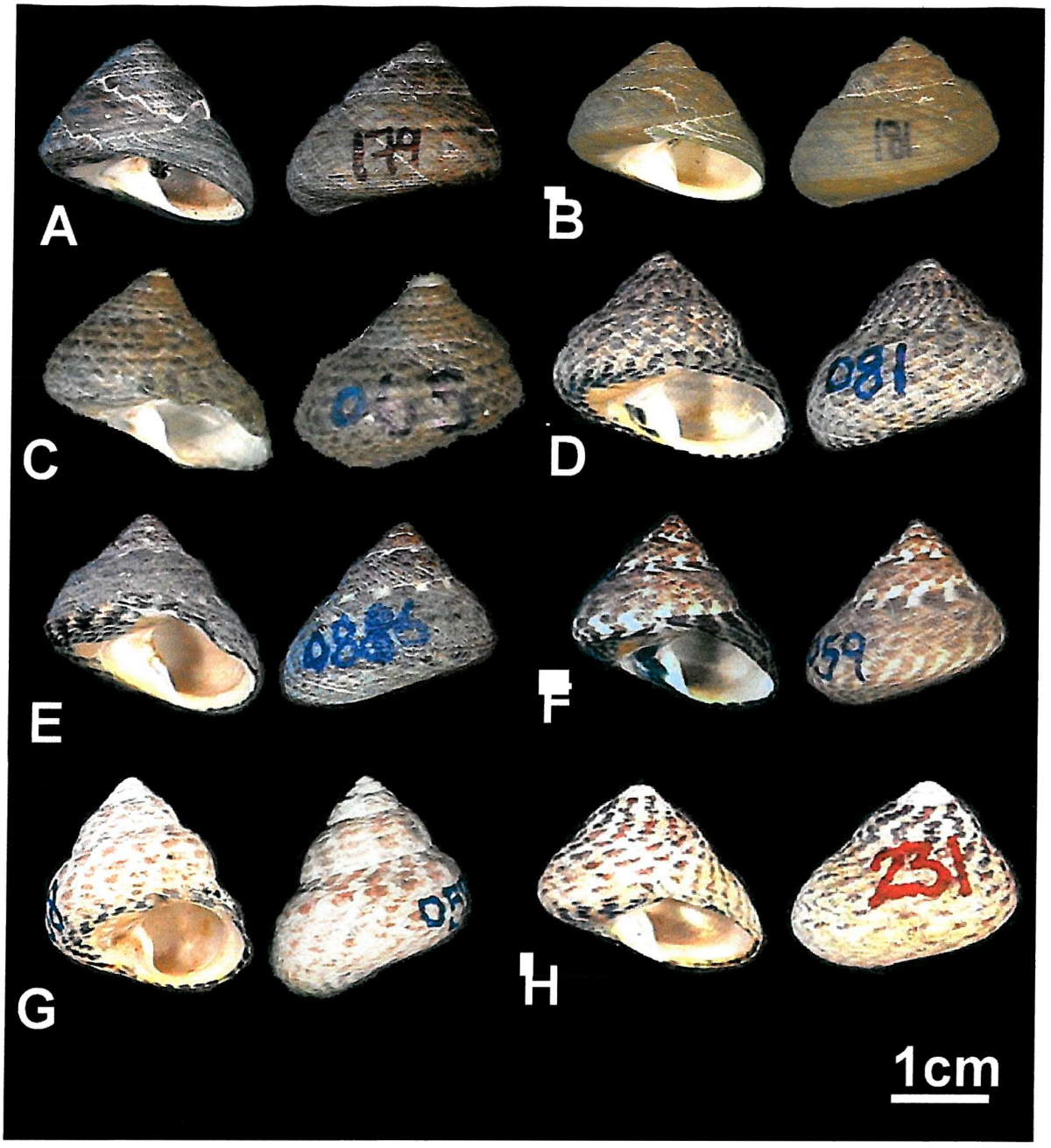


Figure 3.8. Plate of *Osilinus edulis* shells.
The large degree of shell morphological plasticity within this species has led to taxomomic confusion and previous classification of the separate sub-species *O.sauciatus* (see text) typified by shells A& B. Specimen locations: A&B: Seixal, Madeira. C: La Rambla, Tenerife. D&E: Santa Cruz, Tenerife. F&G: El Medano, Tenerife. H: Basque coastline, northern Spain.

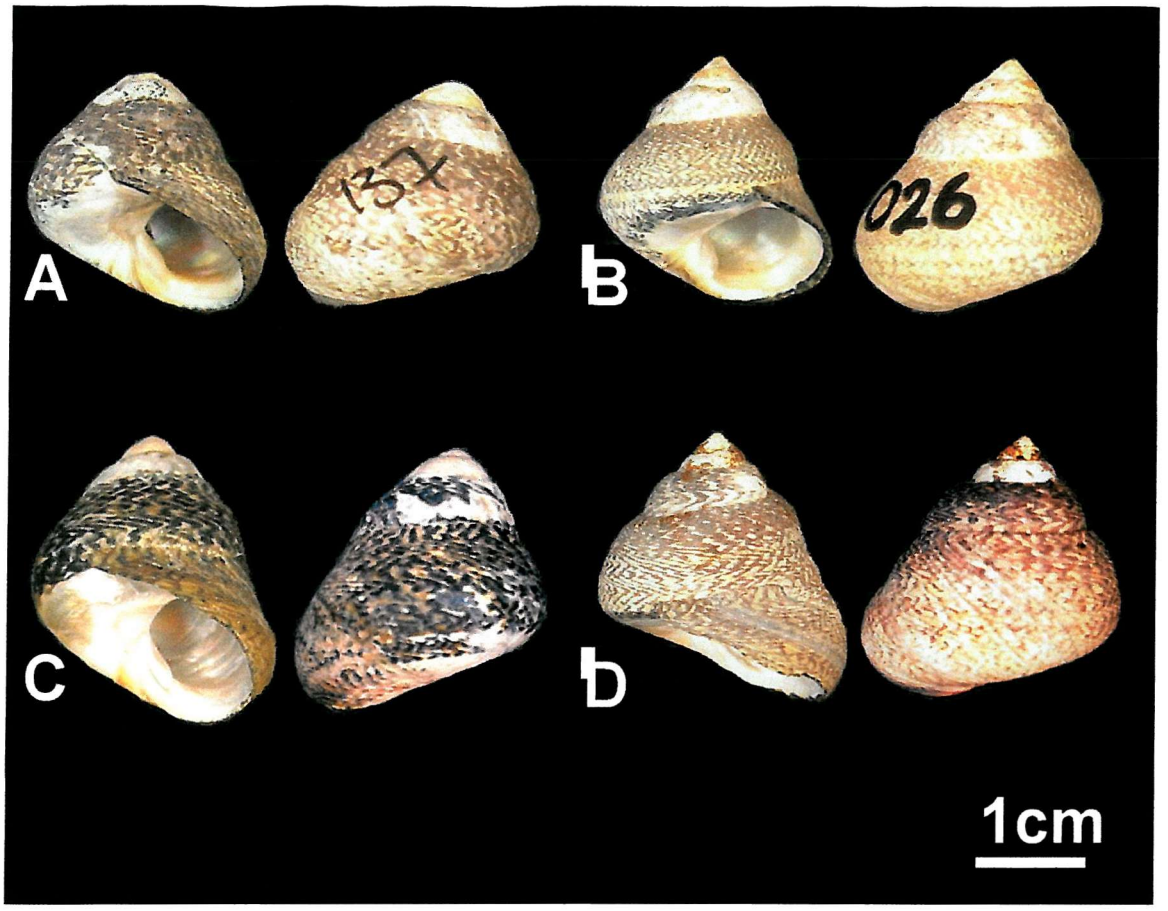


Figure 3.9. Plate of *Osilinus lineatus* shells.
This species exhibits very little morphological variablitity in shell shape, colour or pattern. *O.lineatus* can be readily distinguished by its distinct fine zig-zag patterning. Specimen locations: A: Anza, Agidir, Morocco. B: Lyme Regis, UK. C & D: Scilly Island

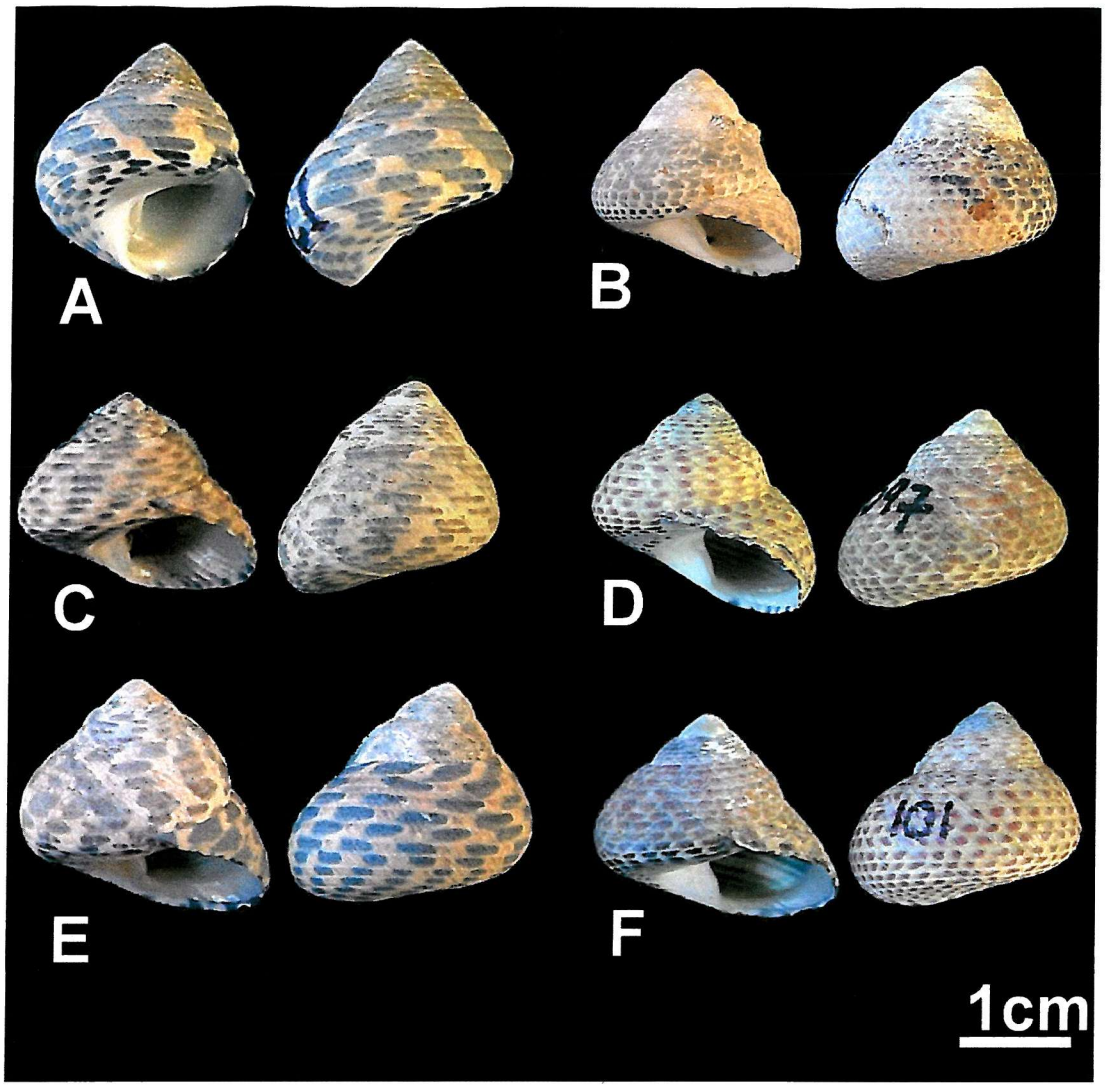


Figure 3.10. Plate of *Osilinus turbinatus* shells.
This species exhibits strong morpholgical variation in shell pattern between populations from the eastern and western Mediterranean Basins. Shells pictured on the left (A, C & E) are from the Eastern Mediterranean Basin, shells on the right (B, D & F) are collected from the Western Mediterranean Basin.
Specimen locations: A: Cyprus. B: Calahonda, Spain. C: Corinth, Greece. D: Puerto Duguesa, Spain. E: Corinth, Greece. F: Puerto Duguesa, Spain.

3.5 Speciation and *Osilinus*

The modern gibbuline taxa are thought to have been extant since the Jurassic 206 –142 Ma (Hickman & McLean 1990). The first appearance of *Osilinus* occurred during the Upper Cretaceous 142-65 Ma (Keen in Moore 1960). It is hypothesised that *Osilinus* became distinct during the Cretaceous, radiating into the central portion of the Tethys Sea 80-66 Ma, as it opened up to the eastern oceans of the world (Maldonado, 1995). Subsequently, the biotic crisis at the Cretaceous/Tertiary boundary (66 Ma) is thought to have annihilated all life within the proto Mediterranean (Maldonado, 1985), and thus *Osilinus* may have contracted its range in either the North Atlantic or eastern oceans (Robba, 1986). Herbert (1994) considers *O. kotschyi* to be a pre-Miocene relict of the genus that reflects its mid-Tethyan origin. During the lower-mid Miocene, 16-14 Ma, the Hathay-Euphrates straits of Syria-Mesopotamia connecting the proto-Mediterranean and Indian Ocean closed (Robba, 1987). The isolation of the Tethys and Indian Ocean is the vicariant event thought to have isolated *O. kotschyi* on the east of the consequent Middle Eastern land bridge from the proto-Mediterranean/ Atlantic species of the genus.

The presence of O. *edulis* on the continental mainland of Portugal and Morocco, and the Atlantic Islands suggests a colonisation event has occurred (Nunn, 1994). Colonisation of the Atlantic Islands by *Osilinus* may have occurred 24 Ma when only the Selvagens and Fuerteventura existed (Geldmacher & Hoernle, 2000). The *O. edulis* and *O. atratus* morphotypes endemic to the Selvagens may therefore be ancestral to the more common morphotypes radiated throughout the younger Atlantic Islands. It is also possible that the current distribution of these morphotypes is the result of the diminishing of a previously widespread species.

Differentiation on the Atlantic Islands followed by subsequent colonisation of the continental coast has been proposed as the most parsimonious explanation of diverse sympatric sibling species within the Northeast Atlantic Islands and mainland (O' Foighil *et al.,* 2001). This may be true of *O. edulis* and *O. atratus*. The absence of *O. atratus* on the continental coastline, however, suggests that its occurrence on the Atlantic Islands may be a result of peripatric speciation (Mayr, 1940, 1982), during which the Atlantic Islands were colonized by peripheral populations of a sister species occurring on the mainland, possibly *O. lineatus*. *O. lineatus* has a geographical range on the continental coastline extending from Africa north to the UK and Ireland. The absence of more northerly extending populations of *O. atratus* may be explained by a relatively short planktonic larval stage, and therefore the absence of suitable habitat within the dispersal range of this species. The distance between Madeira and the Azorean Islands seems to present an effective dispersal filter for *Osilinus*, since no species of this genus occur there (Morton *et al.,* 1998).

The late Cenozoic Terminal Miocene Events that occurred during the Messinian stage, 7.7-5.32 Ma (Zhang & Scott, 1996) are likely to have major impacts on the distribution of *Osilinus* in the Mediterranean and North-east Atlantic. The Messinian hypersalinity crisis in the Mediterranean

probably caused extinction of the occupying molluscan fauna (Sabelli & Taviani, 1984), including *Osilinus.* This vicariant event was possibly fundamental in the evolution of *O. turbinatus* and *O. articulatus.* The closing of the Betic and Rifian corridors created a geographic barrier between the Atlantic and Mediterranean (Benson *et al.*, 1991), fragmenting previously continuous populations. Miocene fauna were thought to have survived this isolation and desiccation of the Mediterranean by taking refuge in the Atlantic near Gibraltar (Sabelli & Taviani, 1984).

The loss of Palaeo-Mediterranean Overflow Water into the Atlantic reduced the heat transfer to the north Atlantic influencing European climate (Benson *et al.*, 1991). Consequently, the geographic range of *Osilinus* is likely to have retracted southward, occupying warmer areas along the African coast and possibly the Cape Verde Islands. The north to south cline of cold to warm climatic conditions may have driven the divergence of *Osilinus* by parapatric speciation. The unique tolerance of *O. lineatus* to more northern conditions may be a reflection of its refuge, adaptation and divergence north along the Iberian coast during the Messinian Salinity Crisis. When the Strait of Gibraltar reopened 5 Ma, the Mediterranean was re-colonised by Atlantic taxa (Por, 1978; Sabelli & Taviani, 1984). The sudden availability of ecological niches may have been key in promoting speciation of the Mediterranean fauna by adaptive radiation.

The glacial-interglacial cycles, and consequent climatic oscillations that occurred since the beginning of the late Cenozoic glacial era were strongest in the North Atlantic and had enormous bearing on North African and European climate (Adams *et al.,* 1999). Dramatic changes in sea levels occurred (Zhang & Scott, 1996). The Quaternary period (1.6 Ma – present) may therefore have been highly significant in the evolution of *Osilinus.* Of the many fluctuations in sea level, the 120m drop 18, 000 years BP (Benson *et al.*, 1991; Mix *et al.*, 2001) may have been most significant for the speciation of the Mediterranean species *O. turbinatus* and *O. articulatus.* During this glacial cycle the eastern and western Mediterranean basins were separated and temperatures decreased by 8°C from present day averages. The eastern and western morphotypes observed in *O. turbinatus* may be a result of this vicariant event causing divergence. This extreme event and previous decreases in sea level over the 125,000 BP of the glacial era (Mix, 2001) may have facilitated potentially repeated colonization of the Atlantic Islands by exposing the chain of seamounts between the Canary Islands and Madeira and the continental mainland (Epp & Smoot, 1989). Conversely, the seamounts may have facilitated re-colonisation of the mainland by *Osilinus* species located in refugia on the Atlantic Islands during glacial periods.

Chapter 4
General Methods

4.1 Molecular techniques

4.1.1 Introduction

Genetic variation among individuals can be used as a direct measure of the evolutionary change that has occurred. By quantifying and describing genetic differences among individuals from different populations or species, we can analyse population structure or reconstruct the ancestral sequences and test hypotheses regarding speciation processes and phylogenetic relationships. Thus the processes that drive micro and macroevolution can be investigated (Avise 2000).

The technologies facilitating the molecular biological methods used to determine genetic variability have progressed at a rapid rate. During the 1950-1980's allozyme work was the predominant tool used in assessing genetic differentiation. A fast procession of technological breakthroughs since the late 1970's has shifted the emphasis to DNA sequencing (reviewed in Ausubel, 1999). These include the discovery of *Taq* polymerase and the consequential development of the Polymerase Chain Reaction (PCR) (Mullis & Faloona, 1987), DNA sequencing (Sanger *et al.,* 1977; Maxam & Gilbert, 1977) and its automation (Smith, 1986). This access to the extensive nucleic acid sequence data combined with the development of electronic databases and novel software has become the basis for many new fields of biology (Blake & Butt, 1996), including bioinformatics (e.g. Woodwark, 2002), genomics (e.g. Lee & Lee, 2000) and bioprospecting (e.g. Abe & Horikoshi, 2001), and has greatly expedited pharmaceutical and biotechnological discoveries (e.g. Chopra *et al.,* 1997). It has also become a fundamental tool in evolutionary biology, enabling phylogenetic and phylogeographic studies (Avise, 1994, 2000; Page & Holmes, 1998).

There are three main molecular techniques that have been used throughout my work: DNA extraction, PCR and DNA sequencing. This chapter will provide a theoretical background for each methodology. The exact protocols implemented in the molecular laboratory work conducted for this thesis are detailed in Appendix B. Three molecular markers have been chosen for use in the molecular work: the partial mitochondrial genes 16S and COI and the partial nuclear gene 28S. These are detailed in section 4.1.2 below, with justification for their selection.

4.1.2 Molecular markers

There are several advantages to using mitochondrial genes for phylogenetic analysis (Ohno, 1997; Boore, 1999; Avise, 2000, see also Chapter 6). Usually animal mitochondrial DNA is a small (15-20kb), circular extrachromosomal genome that facilitates the mitochondrion organelle with autonomous DNA replication, transcription, mRNA processing and protein translation systems. With few exceptions, all animal mitochondrial genomes contain the same 37 genes encoding 13 proteins, two ribosomal RNAs (12S and 16S) plus 22 transfer RNAs (tRNA) and possess no introns (see Table 4.1 from Boore, 1999). Exceptions to this rule are found in some species and

groups, particularly nematode worms. In nematodes the mitochondrial genes show a different arrangement to those of other metazoans, and they are reduced in size or even missing. Some cases have even been found where mitochondrial genomes are multipartite (i.e. split into several small molecules, Armstrong *et al.,* 1998).

Table 4.1 The 13 protein, two ribosomal RNAs and 22 tRNA genes typically found in animal mitochondrial genomes (Boore, 1999)

Protein encoded	Designation for animal mDNA
Cytochrome oxidase subunit I, II, III	*COI, COII, COIII*
Cytochrome *b* apoenzyme	*Cytb*
NADH dehydrogenase subunits 1-6, 4L	*NDI 1-6, 4L*
ATP synthase subunits 6, 8	*A6, A8 or ATP6, ATP8*
Large ribosomal subunit RNA	*LrRNA*
Small ribosomal subunit RNA	*SrRNA*
18 Transfer RNA's each specifying a single amino acid	Corresponding one-letter amino acid code: *trnX*
2 transfer RNA's specifying leucine	Differentiated by codon recognised, *L(CUN)* and *L(UUR)*
2 transfer RNA's specifying serine	Differentiated by codon recognized, *S(AGN)* and *S(UCN)*

The mitochondrial genes 16S and COI were chosen as suitable markers since both provide sufficient resolution at the interspecific level of variation (Whitfield & Cameron, 1998). The DNA sequences for COI and 16S markers could also be combined in analyses, considering the mitochondrial genome a single locus and thereby providing a greater likelihood of reflecting a whole-genome (i.e. correct) tree topology (Cummings *et al.*, 1995). For a robust phylogeny (Zhang & Sang, 1999) and inclusion of paternal inheritance, the nuclear gene 28S was chosen as a suitable marker. The 28S nuclear gene is more conserved and therefore was considered a more suitable marker for intergeneric studies and for identifying the optimal outgroup (Littlewood *et al.,* 2001). Nuclear genes present a risk of paralogy arising from high gene copy numbers (Page, 1998a). They can also, however, offer more phylogenetic information than mitochondrial genes alone (Cronn *et al.*, 2002).

COI has been a widely used marker at species-level phylogenetic studies, and is suitable for use with universal primers. It is accessible and convenient (Palumbi, 1997; Moritz *et al.,* 1987; Folmer *et al.,* 1994; Wares & Cunningham, 2001). COI confers the greatest genetic variability of the chosen markers, particularly within the Mollusca (Hoeh *et al.,* 1997). It can therefore be used in analyses of recent speciation and radiation events (e.g. Caterino & Sperling, 1998; Smith & Bush, 1997; Schubert *et al.,* 2000) that are likely to be reflected in small geographic scale patterns such as between island interspecific variation. COI has also been shown to be an appropriate marker for analysis of population structure (e.g. Beckenbach *et al.*, 1993; Benson *et al.,* 2001).

COI is one of three mitochondrial genes (along with COII and COIII) that encode for subunits of the cytochrome c oxidase complex. Nuclear genes code for the remaining 8 subunits (Ostermeier *et al.,* 1996). The cytochrome c oxidase complex is situated in the inner mitochondrial membrane. It is the terminal enzyme (see Figure 4.1) of the respiratory chain that pumps protons (H+) out of

the mitochondrial matrix into the intermembrane space, creating a transmembrane electrochemical proton gradient used in ATP synthesis and the transport of metabolites across the inner membrane (Alberts *et al.,* 1996).

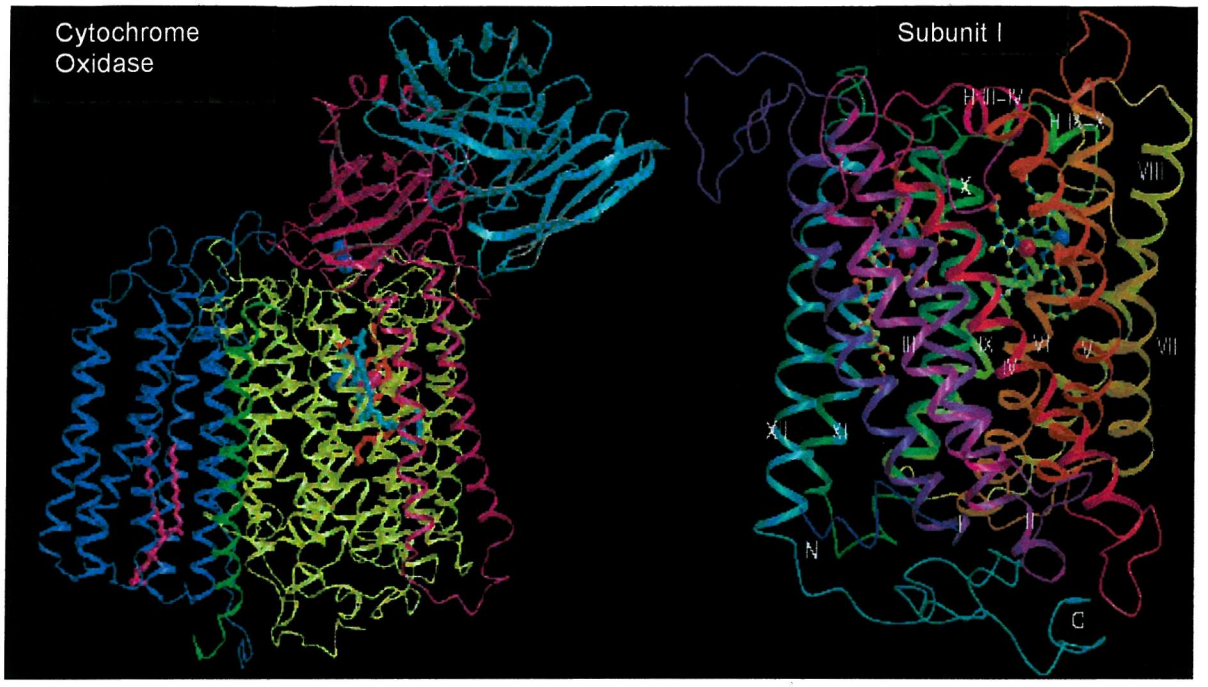


Figure 4.1. Left: Complete Cytochrome oxidase enzyme viewed from the side. Subunit I is yellow-green; Subunit II is purple-red; Subunit III is deep blue. The blue-green subunit is from the antibody used to aid crystallization of the complex. Right: Subunit I viewed from the side. The main features are 11 transmembrane helices, 1 interrupted helix (VII) and two helical connections (III-IV) and (IX-X) lying on the membrane surface. Haem a is red, Haem a_3 is cyan and Cu_B is blue. Pictures from Iwata *et al.*, (1995).

The 16S rRNA gene has been used widely used in species-level phylogenetic investigations. Studies include the molecular ecology of various Mollusca such as genetic variation in land snails (Van Reil, 2001), the evolution of reproductive traits in mussels (Lydeard *et al.,* 1996), the systematics of freshwater mussels (Roe & Lydeard, 1998), the phylogeny and historical biogeography of squid (Anderson, 1999), and the phylogeny of giant clams (Schneider *et al.,* 1999). It has also been used as an appropriate marker for higher-level ordinal phylogenies within Mollusca (Thollesson, 1999). Mitochondrial 16S DNA codes for the larger of two ribosomal RNA strands that form the two subunits of mitochondrial ribosomes (12S and 16S). The ribosome is the site of translation of messenger RNA into protein.

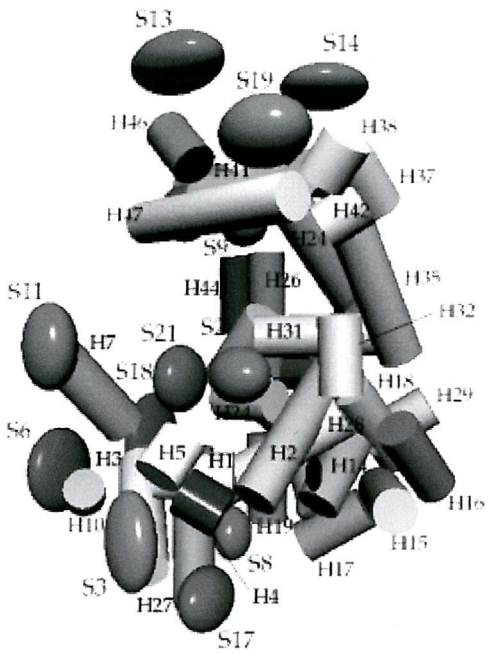


Figure 4.2. A proposed model of the structure of 16S rRNA (Fink *et al.*, 1996; Altman *et al.*, 2000). The large subunit consists of a single strand of RNA (the 16S rRNA, 1542 bases), and 21 proteins ranging in molecular weight represented by the oval structures numbered S1 to S3. The cylindrical shapes represent the position and orientation of the helixes.

Unlike mitochondrial rRNAs, nuclear ribosomal RNA genes are arranged in large clusters of many hundreds (50-5000 copies) of identical genes, containing both external and internal spacers which are transcribed with the ribosomal RNA genes as pre-rRNAs (Figure 4.3). Cleavage of the pre-rRNA molecules removes the external transcribed spacer and the internal transcribed spacers to produce the three mature rRNAs (Alberts *et al.,* 1996).

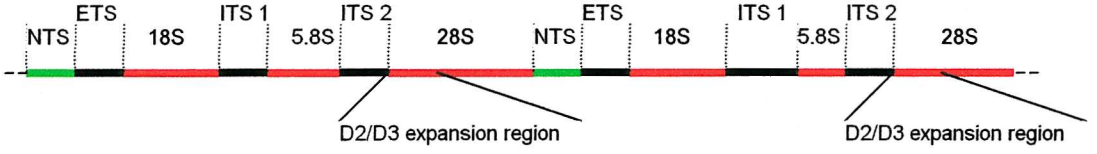


Figure 4.3. The genes encoding for eukaryotic ribosomal RNA

Eukaryotic nuclear coded ribosomes consist of a small sub-unit (SSU) and large sub-unit (LSU) that comprise the 80S ribosome. The 28S and 5.8S genes specify the rRNA of the LSU; 18S genes specify the rRNA for the SSU. 28S has been used in phylogenetic analyses at various taxonomic levels and has been established as a valid marker for ordinal level (Mariaux, 1998; Littlewood *et al.,* 2001) and generic level (Graf & O'Foighil, 2000) interrelationships within flatworms and molluscs respectively.

The primer sequences of the above markers used in the molecular work are as follows:

16S Universal Primer Palumbi (1996):
16SAR 5'-CGCCTGTTTATCAAAAACAT-3'
16SBR 5'-CCGGTCTGAACTCGATCACGT-3'

COI Universal Primer Folmer *et al* (1994)
HCO2198 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'
LCOI490 5'-GGTCAACAAATCATAAAGATATTGG-3'

28S Primers modified from Littlewood et al (2001) by Williams (in press)
LSU5 5'-TAGGTCGACCCGCTGAAYTTAAGCA-3'
LSU 900F 5'-CCGTCTTGAAACACGGACCAAG-3'
ECD2S 5'-CTTGGTCCGTGTTTCAAGACGG-3'
LSU 1600R 5'-AGCGCCATCCATTTTCAGG-3'

4.1.3 Extraction of DNA from snail tissue

For successful PCR reactions and to ensure clear reading of sequence, the template DNA extracted from the snail tissue needs to be pure (free of all endo and exogenous contaminants), of high molecular weight (integrity) (50-200kb) and, of suitable concentration (Saunders & Parkes, 1999). To release the target DNA from its intracellular membranes (nucleus and organelles such as mitochondria) the cells must first be gently lysed. This tissue digestion is mediated by the enzyme proteinase-K. Pro-K is a non-specific protease that is not inactivated by metal ions or chelating agents. Enzymatic activity is stimulated by denaturing agents (SDS or urea), with full activity over the pH range 6.5-9.5.

During tissue digestion, the DNA needs to be protected from cellular nucleases released upon cell lysis. An extraction buffer is used to stabilise and protect the total DNA, and is typically a Tris (Tris (hydroxymethyl) amino methane) based solution containing a salt, Ethylenediaminetetraacetic acid (EDTA), and detergents. Endogenous DNAses often require magnesium as a cofactor in the degradation of DNA. EDTA chelates Mg ions thereby reducing the opportunity for DNA degradation. Salt serves to stabilise free nucleic acids by creating an isotonic environment. The addition of detergents and pH optimisation of the extraction buffer also inhibits DNAse activity (Sanders & Parkes 1999).

Snail foot tissue analysed in my study is high in mucopolysaccharides that will bind to, and remain in solution with, DNA during the extraction process thereby inhibiting further reactions. The non-ionic detergent cetyltrimethylammonium bromide (CTAB) was used to liberate the DNA from the mucopolysaccharides. CTAB solubilises membranes and forms a complex with DNA, allowing selective precipitation. Sodium dodeclyl sulphate (SDS) is an anionic detergent and protein denaturant that also disassociates DNA-protein complexes and is used in many extraction protocols. RNAase may also be used to remove inhibiting RNA from the crude DNA extracts

The extraction of total cellular DNA from the cellular debris, contaminant proteins, RNA and other macromolecules such as mucopolysaccharides that have been disrupted by the Pro K,

denaturants and detergents in the extraction buffer is achieved by centrifugal separation using chloroform-isoamyl alcohol. This can also be achieved using phenol-chloroform-isoamyl alcohol extraction. Phenol is a protein denaturant, which when added to crude aqueous DNA extracts separates the proteins in an organic phase, or at the organic-aqueous interface, while DNA is dissolved in the aqueous phase. During chloroform-isoamyl alcohol extraction, the DNA is also dissolved in an aqueous phase that lies above an organic phase. Proteins and other contaminants precipitate out at the organic-aqueous phase interface.

Once total cellular DNA has been extracted and isolated in an aqueous solution, precipitation of the DNA allows a change of the solvent and resuspension of the DNA in a more suitable buffer for storage. This also enables removal of any remaining non-precipitated contaminants and concentration of the DNA to the required working molarity. A monovalent cation (0.2M) is required for a nucleic acid precipitate to form; therefore high salt concentrations facilitate the DNA precipitation by shielding the negative charge of the nucleic acid phosphate groups and allowing the aggregation of nucleic acid strands. Ethanol (at twice the DNA solution volume) or isopropanol (0.6 of the volume) with sodium acetate or sodium chloride is normally used to precipitate DNA at -20°C. Micro-centrifugation compacts the DNA into a pellet. For short DNA molecules (<200bp) $MgCl_2$ or glycogen can enhance the efficiency of ethanol precipitation by acting as a co-precipitant of the DNA. Since ethanol inhibits the solubility of DNA, once in pellet form, the DNA is dried by vacuum or on a hotblock (94°C) to remove all traces of ethanol. The pellet is then redissolved in TE or elution buffer. Vigorous mixing or vortexing to resuspend the pellet can damage the DNA, so it is best left at 4°C overnight. The DNA extraction protocol that I optimised and used is detailed in Appendix B.

4.1.4 DNA visualisation by gel electrophoresis

Once the DNA is extracted and re-suspended in storage buffer, it must be quantified to determine the quality and yield of the DNA extraction. This is necessary to determine the correct amount of starting template for PCR reactions and cycle sequencing. The DNA is first fractionated by gel electrophoresis using a suitable gel matrix for the size of fragments to be resolved. Nucleic acids are uniformly negatively charged. Double stranded (ds) DNA has a relatively good mobility through gels that is unhindered by complicating structural effects (Ausubel *et al.*, 1999). There are four main factors influencing the mobility of nucleic acids on gels: the conformation of the nucleic acid, the pore size of the gel which is the fundamental determinant of the size of fragments that can be resolved, the voltage gradient, the salt concentration of the electrophoresis buffer. Large pore agarose gels are used to resolve fragments greater than 500-1000bp with smaller pore acrylamide, or sieving agarose gels, being used for fragments less than 1000bp.

Agarose gels are used to check the success of DNA extractions and to quantify the yield of total cellular DNA from extractions and shorter PCR products. Agarose is a linear polymer extracted from seaweed. On heating, the agarose is dissolved in a suitable electrophoresis buffer (e.g. 1x TAE). Before polymerisation occurs, ethidium bromide is added at 0.5μl/ml to allow fluorescent

emission quantification of the DNA. Ethidium bromide can affect DNA mobility through the gel, and under UV enhances DNA damage (Smith, 1996). Upon cooling the gel solidifies by hydrogen bonding. The size of the pores in the polymerised gel matrix is determined by the concentration of the agarose gel. Gel concentration therefore determines DNA fragment resolution range (Table 4.2).

Table 4.2: Agarose gel concentration and suitable DNA fragment resolution range. The percentage relates to the percent of agarose to volume of buffer (w/v). Adapted from (Ausubel *et al.*, 1999)

Agarose %	DNA fragment resolution range (Kb)
0.5	30 – 1
0.7	12 – 0.8
1.0	10 – 0.5
1.2	7 – 0.4
1.5	3 – 0.2

The gel is poured onto a horizontal plate, and well combs inserted before polymerisation. Once set, the gel plate is placed in a buffer tank and filled with electrophoresis buffer. Once covered with buffer, the combs are removed carefully from the gel (Figure 4.4). DNA is loaded in to the gel wells in a loading buffer containing a dense substance (e.g.glycerol) to facilitate sinking. The loading buffer contains at least one marker dye to monitor the migration of DNA fragments along the gel matrix. Bromphenol blue and xylene cyanol are commonly used (including in my work) combined, they co-migrate with different DNA fragment sizes, approximate 0.5-1kb and 4kb fragments of DNA respectively (at 1% agarose gel concentration). A ladder of known DNA fragment sizes is loaded with the DNA samples for use in quantification calibration.

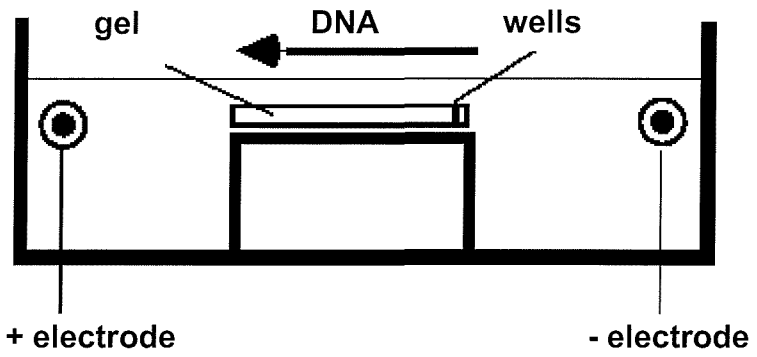


Figure 4.4. Schematic diagram of an electrophoresis gel tank.

Since nucleic acids are negatively charged, the gel needs to be set up for migration towards the positive lead of the electrophoresis tank (anode). Electrophoresis is set at 1-10v/cm of gel for optimal resolution of the DNA fragments. DNA fragments greater than 5kb are best resolved at 5v/cm of gel. Smaller fragments less than 1kb in size are optimally resolved at higher voltages/cm of gel (Smith, 1996). Under a steady current, nucleic acids position themselves end-on in the gel matrix and in the case of linear DNA molecules, migrate at a rate inversely proportion to the $\log^{10}$

of the number of base pairs. Large molecules are exponentially slower than smaller molecules due to the frictional drag through the matrix and having to squeeze through the pores.

The DNA yield is determined by measuring the fluorescent emission of a dye that labels DNA or RNA (Saunders & Parkes, 1999). The fluorescence emission is calibrated from a fragment of known size and concentration from the DNA ladder and converted into a DNA concentration. Since some dyes are specific to certain types of nucleic acids, they can also be used to remove contaminants. The amount of fluorescence is directly proportional to the amount of DNA, and therefore, using the appropriate ladder band for calibration the sample DNA can be quantified. Once the gel has run, it is placed under a UV light to induce fluorescence and a digital picture is taken and saved. For this work, Fluorescence was measured using the gel documentation system, UviDoc 008-XD system with Uvisoft Version 98 software. Ethidium bromide has a broad specificity, labelling both DNA and RNA, with a preference for dsDNA. Its sensitivity is limited to approximate 100ng/ml due to its moderate affinity for nucleic acids (Sanders & Parkes, 1999).

4.1.5 The Polymerase Chain Reaction (PCR)

PCR is an *in vitro* cycled set of temperature-determined reactions that uses the inherent properties of DNA and the polymerase enzyme *Taq* to amplify specific sections of DNA. *Taq* is a thermostable DNA polymerase isolated from *Thermus aquaticus,* a thermophilic bacterium first discovered in the hot springs of Yellowstone National Park (Brock, 1967). It was this discovery of *Taq* that enabled DNA amplification to become a viable tool in molecular biology. Since all previous known DNA polymerase enzymes were denatured at the high temperatures required for separation of the double helix DNA molecule, successful amplification required the addition of polymerase after each denaturing stage of successive cycles and consequently conferred excessive costs. PCR was pioneered in the late 1980s with the amplification of the β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anaemia (Saiki *et al.,* 1985, Saiki, *et al.,* 1988; Mullis & Faloona, 1987). Since then it has become the foundation for most molecular biology methods.

Taq polymerase is a DNA dependent DNA polymerase. All DNA polymerases add deoxyribonucleotides to the 3'-hydroxyl terminus of primed ds DNA molecules (Innis *et al.*, 1988). Synthesis can only occur in the 5'-> 3' direction with respect to synthesised strand. All DNA polymerases, however, catalyse pyrophosphorolysis, which is the reverse of polymerisation (Tabor & Richardson, 1990). Pyrophosphorolysis occurs when the 3' terminal base of a DNA molecule reacts with pyrophosphate (a by-product of DNA polymerisation) to produce a dNTP and a DNA molecule one base shorter. Although polymerisation is favoured over pyrophosphorolysis during DNA synthesis because of the high dNTP concentration and low pyrophosphate (PP_i) concentration, pyrophosphorolysis may still occur. Polymerases such as *Taq* also have a 5'->3' exonuclease activity by which ds DNA is degraded from the 5'-hydroxyl end. This exonuclease activity can excise between 1 nucleoside 5'phosphate and 10 nucleotides (Ausubel, 1999). *Taq* is a ds DNA polymerase with no 3' – 5' exonuclease enzyme activity, a fast polymerase rate and

high processivitiy (Feinberg & Vogelstein 1983). It has an optimal polymerisation temperature of approximately 72°C, conferring an extension rate of 2-4kb/min. At 94°C (the denaturing temperature of ds DNA) *Taq* has a half-life of 60min (Qiagen, 1999).

The PCR requires a double stranded (ds) DNA template, two single stranded (ss) oligonucleotide primers, a DNA polymerase, all four deoxynucleotide triphosphates (ATP, GTP, CTP and TTP), a buffer and, salts (Bebenek *et al.*, 1989). There are three phases to PCR each driven by different optimal temperatures: denaturation, primer annealing and primer extension, (Figure 4.5). The primers are short ss nucleotide sequences corresponding to specific sections of a DNA template, each one complementary to one of the double helix strands, and flanking opposite sides of the region targeted for amplification. The primers determine the ends of the final DNA fragment obtained from the PCR.

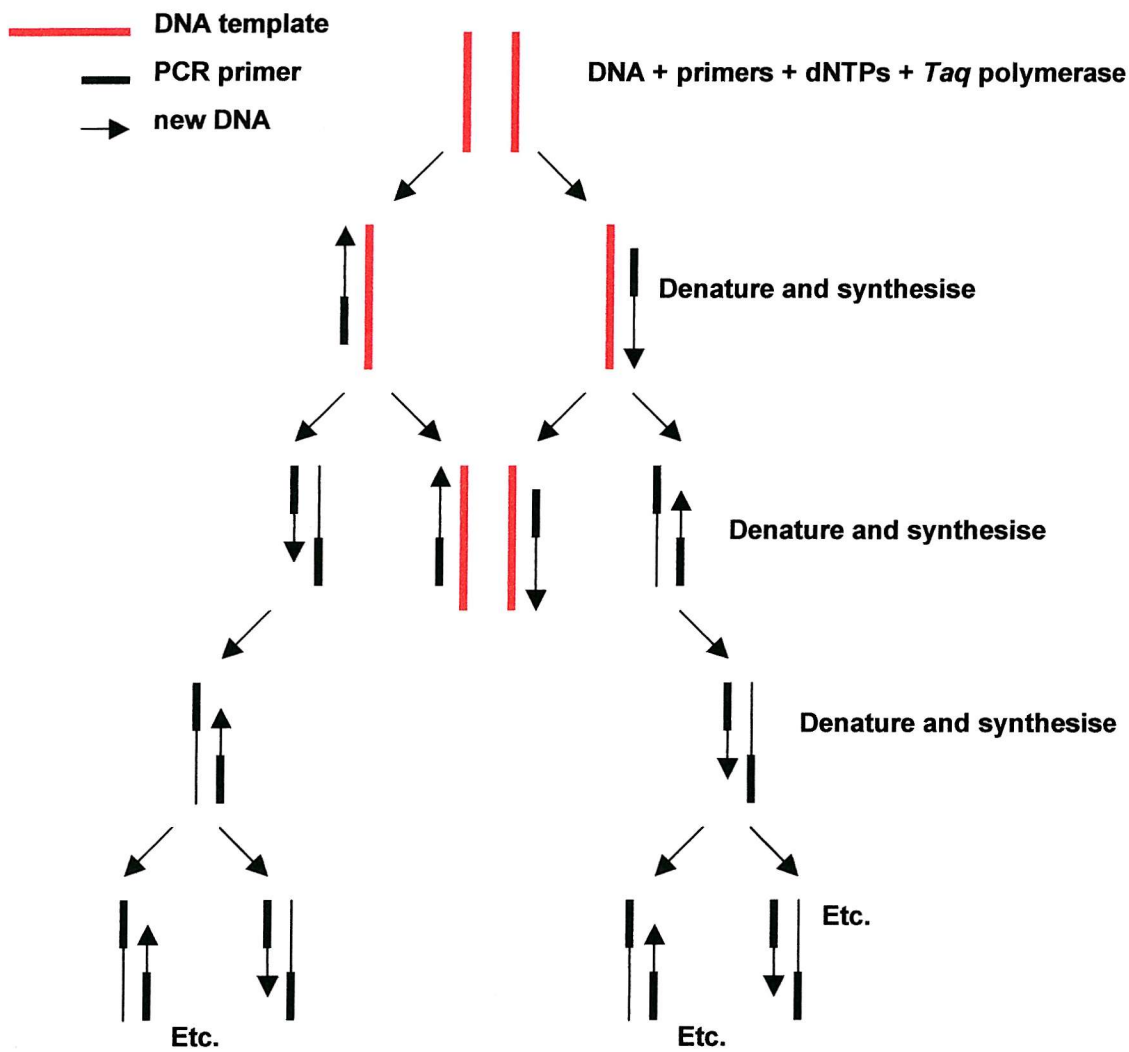


Figure 4.5: A schematic diagram of PCR, taken from Ausubel *et al.*, 1999 p15-1.

The initial denaturing stage at 94°C separates the double helix DNA template molecule into single strands and eliminates any secondary structure (Harwood, 1996). Once the ds DNA molecule has been separated, the forward and reverse primers can bind to the complementary section of the now ss template DNA upon cooling to a suitable annealing temperature (normally between 48-60°C). Hybridisation of the primers to the template usually occurs around 55°C: higher annealing temperatures (60-65°C) confer greater stringency of primer binding, and lower temperatures (37-45°C) confer reduced specificity. The optimal melting temperature T_m of a primer can be calculated from the formula

$$T_m = 2°C \times (A+T) + 4°C \times (G+C)$$

where A, T, G or C corresponds to the number of that base occurring in the primer sequence. The annealing temperature applied to the PCR is recommended at 5°C below the primer T_m. (Qiagen, 1999). The primers hybridise to opposite strands of DNA at each end of the segment to be amplified, with their 3'ends facing each other. Qiagen (1999) recommends the following be avoided when designing primers: complementarity of bases at the 3'-end of primer pairs, runs of > 3 G's or C's at 3'-end or, complementarity within primer sequence or between primer pairs, since these factors will increase the chance of forming primer dimers (Innis & Gelfand, 1990). Commercially available software for primer design includes Oligo®, PrimerPremier and PrimerSelect™. The last stage in each cycle of a PCR is the extension of new strands from the primers, mediated by *Taq* upon annealing of the primers to the template DNA. The extension phase is driven by an increase in temperature to 72°C, at which strand synthesis proceeds at a rate of 2-4kb/min. *Taq* polymerase facilitates extension of the primer by incorporation of free dNTPs to the 3'hydroxyl end of the growing primer strand, according to Watson-Crick complementary base pairing to the template DNA strand.

The initial round of DNA synthesis (always 5'-> 3') produces sequences of indeterminate length, beginning with one of the two primers. These strands hybridise to the primers during the second cycle to produce single stranded DNA of the exact length between the two primer ends. These discrete strands quickly become the predominant product, and therefore the template in subsequent cycles. After 30 cycles the PCR produces an exponential 2^{28} fold increase in the product. Once all cycles have been completed, a final extension phase at 72°C for 5-15min facilitates completion of any unfinished DNA synthesis.

DNA denaturation, primer annealing and primer extension are not three separate processes occurring at different temperatures during PCR. They are a dynamic process in which each stage progresses to the next (denaturing -> annealing -> extension) before the next cycle. Each process has an optimal temperature above and below which it may start or continue to work (McDowell, 1999). Every primer and template combination needs to be optimised for successful PCR amplification product. Excluding primer sequence, discussed above, there are seven variables

affecting PCR that can be optimised: annealing temperature, template concentration, primer concentration, magnesium chloride ($MgCl_2$) concentration, dNTP concentration, extension time and the number of cycles. Addition or exclusion of hot start *Taq* or a proof reading enzyme can also affect the amplification and reproducibility of a PCR (Table 4.3).

Table 4.3: Factors affecting PCR amplification and reproducibility (adapted from McDowell, 1999). The effects of increasing/decreasing or including/excluding various factors on the sensitivity, fidelity and specificity of the PCR reaction. Key: + include; - exclude; ⇑ increase; ⇓ decrease; [ ] concentration.

Factor	Adjustment	Effect on		
		Sensitivity	Specificity	Fidelity
Annealing T°C	⇑	⇓	⇑	
	⇓		⇓	
[Template]	⇑		⇓	
	⇓			
[*Taq*]	⇑		⇓	
	⇓		⇑	
[Primer]	⇑			
	⇓			
[$MgCl_2$]	⇑	⇑	⇓	
	⇓	⇓	⇑	
[dNTP]	⇓			⇑
	⇑			⇓
Extension *t*	⇑			
	⇓			
Cycle No.	⇑	⇑	⇓	
	⇓	⇓	⇑	
Hot Start	+	⇑	⇑	
	-	⇓	⇓	
Proof reading enzyme	+			⇑
	-			⇓

Specificity is fundamentally determined by the priming sequence uniqueness within the template DNA. Universal primers can increase amplification product, but increase mispriming and amplification of non-target sequences. Excessive DNA template can also lead to the production of non-specific products, $10^4 – 10^5$ target copies are considered sufficient for good amplification. If the DNA is degraded, a small target (100-300bp) that is more likely to stay intact is recommended (McDowell, 1999). Increasing amounts of degraded DNA can be inhibitory to amplification due to the increased number of interrupted targets and cross hybridising fragments that can serve as templates for *Taq*, thereby reducing amplification of the intended target.

Magnesium chloride is essential for successful PCR since DNA polymerases are magnesium (Mg) dependent. Mg acts as an enzyme co-factor to *Taq* polymerase during strand synthesis, and therefore $MgCl_2$ concentration is paramount to PCR optimisation. Excess $MgCl_2$ can reduce the specificity of a reaction; too little can reduce *Taq* enzyme activity and therefore yield. In addition, dNTPs bind to magnesium with a stoichiometry of 1:1, and is therefore an important consideration in calculating the quantity of free $MgCl_2$ remaining available for the polymerase reaction. A $MgCl_2$ concentration of 50-200mM/dNTP is optimal for most reactions (McDowell, 1999). Excess *Taq* can also decrease primer-annealing specificity by increasing the chance of producing non-specific

products, these are indicated by artefactual bands and smearing on gel analysis. However, the most critical factor ensuring optimal results during PCR is the primer annealing temperature. Optimising the annealing temperature and Mg concentration will minimise the production of erroneous products by limiting the potential for non-specific priming events.

There are also many factors that affect the reliability and validity of PCR, including the presence or absence of inhibitors and enhancers. Pure template DNA is key to successful PCR, since many reagents used in extraction protocols can be inhibitory to DNA amplification. The following compounds are all inhibitory to PCR at the levels indicated in parenthesis: Phenol (0.5%), SDS (0.01%), ammonium acetate (150mM), ethanol (5%); isopropanol (1%), potassium acetate (0.2M), EDTA (1mM), ethidium bromide (1%) and CTAB (0.01%). Proteinase K may digest the *Taq* DNA polymerase (Bickley & Hopkins 1999). Once completed, the PCR product is 'cleaned' for use in subsequent sequencing reactions. Commercial kits are used to recover the PCR product fragments from the amplification reaction to produce purified DNA. The PCR reactions and cycle conditions that I used for the amplification of 16S, COI and 28S are given in Appendix B.

4.1.6 DNA Sequencing

The automated, fluorescence-based dideoxynucleotide thermal cycle DNA sequencing method used today is based on the dideoxynucleotide chain termination reaction of Sanger *et al.* (1977). The Sanger dideoxy sequencing method is also known as the enzymatic method, partly due to its use of enzymes, and partly to distinguish it from the sequencing method developed concurrently during the late 1970's by Maxam & Gilbert (1977; 1980), which was chemically based. There are two stages to DNA sequencing. First, the thermal cycle sequencing reaction similar to PCR in temperature driven stages, during which all possible lengths of DNA fragments are produced from the template DNA. Each fragment has a fixed 5' end primer sequence, and is marked at one end by either a radioactive isotope or fluorescent dye.

During the cycle sequence reaction the primer DNA molecule anneals to the complementary DNA template and extension is mediated by a derivative of *Taq* polymerase or other suitable DNA polymerase (e.g. T7, Helentjaris & McCreery, 1996) in the presence of deoxynucleotide triphosphates (dNTPs) and dideoxynucleotide triphosphates (ddNTPs). During normal DNA polymerisation (addition of dNTPs) nucleotides are covalently linked by the formation of a phosphate ester (phosphodiester bond) between the 3'-hydroxyl group on the sugar residue of the last base of the nucleic acid and the 5'-phosphate group of the incoming nucleotide (Alberts *et al.,* 1996). DNA polymerase activity can be inhibited by ddNTPs because they lack the 3'-hydroxyl group; thus they prevent further nucleotides being added to the DNA molecule, terminating chain elongation. Dideoxy sequencing relies on the ability of DNA polymerase to utilize ddNTPs as polymerisation substrates (Tabor & Richardson, 1995). If ddNTPs corresponding to all four bases (A, G, C, T) are present, either in the same or separate sequencing reactions, the end product is DNA fragments ending at every base along the template sequence.

The second stage of DNA sequencing is the visualisation of the different length DNA fragments on a high-resolution polyacrylamide gel, from which the actual DNA sequence is determined. A polyacrylamide gel with high urea concentration as a denaturant is essential to visualisation of DNA sequence fragments. The denaturing polyacrylamide gel separates dsDNA, removes any secondary structure, and confers powerful resolution of short fragments (<500bp) of ss DNA. The ability to fractionate fragments differing by only 1 nucleotide is fundamental to all DNA sequencing protocols.

When Sanger *et al.,* (1977) pioneered the use of chain terminators for DNA sequencing, the mechanism of ddTTP inhibitory activity on DNA polymerase was already known (discovered by Atkinson *et al.,* 1969). The only commercially available ddNTP was ddTTP (Russell & Moffatt, 1969; Geider, 1974), when Sanger *et al.,* adapted ddA, prepared by McCarthy *et al.,* (1966), and converted it to ddATP by the methods of Tener (1961) and Hoard & Ott (1965). They then applied these methods to prepare ddGTP and ddCTP for the first time. Originally Sanger *et al.*, (1977) used two types of terminating triphosphates: dideoxy derivatives and arabinonucleosides. The latter is a stereoisomer of ribose in which the 3'-OH group is orientated in a trans position relative to the 2' group, thus preventing polymerisation in a similar way to ddNTPs. The Sanger method relied on four separate cycle sequencing reactions, each containing a primer, the DNA template, one ddNTP and all four dNTPs, one of which was labelled with ^{32}P. Each reaction thus produced radioactively labelled DNA fragments ending in a particular base, A, G, C, or T. The reactions were run in four separate adjacent lanes on an acrylamide gel, the fragments fractionated by electrophoresis according to size. The resulting patterns of bands were read as a sequence when an image of the gel was transferred to film by autoradiography.

Partial automation of DNA sequencing and the use of fluorescent detection were developed by Smith *et al.,* (1986) providing a safer and quicker method of DNA sequencing, and forming the basis of the fluorescence sequencing protocol used today. Based on the Sanger dideoxy method, each of the four reactions, and therefore termination bases, were distinguished by a different colour flourophore covalently attached to the primer sequence as an alternative to identifying the sequence by radioisotope. The sequence reaction products were then combined and visualised on one gel lane. Each flourophore had a different absorption and emission maxima upon excitation by two different laser lines and thus each base could be distinguished in real-time during electrophoresis, and the information retrieved automatically by computer. The fluorescent sequence fragments could now be resolved temporally rather than spatially as in the Sanger dideoxy sequencing method.

Until Prober *et al.,* (1987), DNA fragments from the thermal cycle sequence reaction had been labelled by a radioactive isotope or fluorescent dye linked to the primer sequence or incorporated internally in the sequence fragment. Thus four separate reactions were required. Prober and his co-workers developed novel chain terminating ddNTPs with distinct fluorescent tags that facilitated the identification of the terminal nucleotide of DNA sequence cycle fragments. The cycle

sequencing for all four bases could now be performed within the same reaction and visualised on one gel lane as with the Smith *et al.,* (1986) protocol. Prober *et al.,* (1987) designed a fluorescent detection system that matched the emission characteristics of the fluorescent dyes and an automated computerised retrieval of the sequence identification in real time. The four fluorescent tags developed (SF-505, SF-512, SF-519 and SF-526) were modified from the parent dye succinyl-flourescein-505 (SF – 505, 505 being the emission maximum in nm). Each dye possesses distinct emission maxima spectra close enough for efficient excitation by one emission line, provided by an argon ion laser beam (488nm). This contrasted with Smith *et al.*, (1986) who used two laser excitation lines because of the greater disparity between emission maxima of their chosen dyes. The tags possessed a carboxylic acid, not required in fluorescence and were therefore used to covalently attach nucleotides to the dye.

The advantage of incorporating the fluorescent label at the end of the DNA fragment attached to the ddNTP is that only genuine chain termination events are recorded. This enables the generation of all four sets of reactions to be performed simultaneously and subsequently sequenced on one gel lane. Furthermore, the terminal fluorescent tags confer no change on DNA charge or mobility. Prober *et al.,* (1987) provided the basis for multiple sample analysis. Fully automated fluorescence based on dideoxy DNA sequencing is now an established protocol in molecular biology. The associated advanced technology and reagents are now widely commercially available.

4.1.7 DNA sequencing methodologies applied in this work

During my molecular work for this thesis, cycle sequencing was performed using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kits (Perkin Elmer). The extension products were purified using the DyeEx™ Spin Kit (Qiagen, October, 2000), or once kits were exhausted by ethanol precipitation (Applied Biosystems, 2000). The products were separated and detected on an ABIPrism377™ sequencer (Perkin Elmer) according to the instructions of the manufacturers for use. During gel electrophoresis of cycle sequence DNA products on the ABIPrism377™ sequencer, I used fluorescence-labelled DNA fragments. These are detected and distinguished by the different emission spectra (and therefore colour) of each terminator nucleotide in the response to excitation by the laser beam as it scans across the gel.

The ABI Big Dye kit consists of a Terminator Ready Reaction Mix™ containing Big Dye terminators, dNTPs, Ampli *Taq* FS, r*Tth* pyrophosphatase, $MgCl_2$ and Tris-HCl pH 9.0 buffer. The Big Dye terminators are labelled with a flourescein donor dye linked to a dichlororhodamine (drhodamine) acceptor dye. The excitation maxima of each dye is that of the flourescein donor and the emission spectra that of the d-rhodamine acceptor (see Table 4.4).

Table 4.4 Big Dye Terminator acceptor dyes

Terminating base	Acceptor dye	Colour in ABI Prism 377 gel	Max emission spectrum λ (nm)
A	dRG6	Green	570
C	dROX	Red	620
G	dR110	Blue	540
T	dTAMRA	Yellow	600

The sequencing enzyme AmpliTaq DNA Polymerase FS is a variant of Taq DNA polymerase with a point mutation for greater acceptance of ddNTPs, and a point mutation that virtually eliminates the 5' to 3' exonuclease activity of the Taq enzyme. r*Tth*_pyrophosphatase, a thermally stable inorganic pyrophosphatase is incorporated into the polymerase enzyme. (ABI Prism®BigDye™Terminator Protocol v2.0 – Applied Biosystems). Inorganic pyrophosphatase is used in sequence reactions to stabilise the forward reaction by catalysing the hydrolysis of pyrophosphate to two inorganic phosphate molecules (PP_i -> $2P_i$), thereby inhibiting pyrophosphorolysis (Fuller *et al.,* 1996).

Nucleotide analogues are used extensively in DNA sequencing (Smith *et al.,*1986; Ansorg *et al.,* 1987; Prober *et al.,* 1987; Brumbaugh *et al.,* 1988; Kambara *et al.,* 1988), such as the use of dITP instead of dGTP (Tabor & Richardson, 1987; Mizusawa *et al.,* 1986). The dNTP mix incorporated in the TRMM contains dITP instead of dGTP to minimise band compression effects, and dUTP instead of dTTP for enhanced T terminator incorporation and T pattern.

During the automated sequencing protocol, DNA samples were loaded onto a polyacrylamide gel using Rapid Load™ membrane comb gel-loading system with Microstop Red™ denaturing loading buffer, according to manufacturers instruction for use (Webscientific Ltd). Upon completion of the sequence run, the gel image produced was tracked either automatically or manually to ensure the best sequence read was extracted. The data output from the ABIPrism DNA sequencing software (DNA Sequencing Analysis Software Version 3.3, Applied Biosystems) is an electropherogram of the sequence read and a text file of the sequence in nucleotide bases. Both output formats were used to edit the sequence for phylogenetic analyses (see Chapters 5 and 6). All protocols and solution formulations I used in the molecular work conducted for this thesis are detailed in Appendix B.

4.2 Phylogenetic data analysis

4.2.1 Introduction

DNA sequences provide the ultimate level of evolutionary information. The genes passed from parent to offspring determine an individual, and are in turn a reflection of the ancestry of an individual (Maddison, 1996). DNA replication is a non-random process; it is driven by strictly controlled mechanisms, and DNA behaves in a regulated manner (e.g. substitution rates and complimentary base pairing). The evolutionary information inherent in DNA sequences is obtained by transforming observed differences between sequences into models of evolutionary

relationships, presented as tree diagrams. Since the behaviour of DNA is understood, models of evolution (substitution matrixes) are imposed on sequences to convert the observed distances (variation between sequences) into measures of actual evolutionary change (Avise, 1994). These measures of actual evolutionary change between groups of taxa reflect their ancestral relationships. The ancestral relationships and states provide insight into evolutionary processes such as speciation.

4.2.2 DNA sequence editing

Sequence editing and alignment methods are detailed in chapter 5 and 6. Once DNA sequences have been retrieved from the ABIPrism 377™ sequencer the software Chromas (v2.23; Copyright © Technelysium Pty Ltd http://ww.technelysium.com.au/) is used to view the electropherograms of both reverse and forward sequences to ensure a robust sequence read and obtain the beginning and end of the gene sequenced by deduction from complementary base pairing of the opposite strand. The primer sequence is removed and the edited sequence exported as a text file (usually of FASTA format, see Appendix B). Before alignment a Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, (1990) is conducted via the NCBI web page http://www.ncbi.nlm.nih.gov/BLAST/. This ensures the integrity of the DNA sequence; that the correct gene has been sequenced and that it is derived from the correct source and not a result of contamination. The BLAST*n* (nucleotide to nucleotide) searches for sequences that align to the query sequences within GenBank. The BLAST*n* output is a summary of the aligned sequences (Figure 4.6).

BLAST*n* gives statistical analysis of each sequence comparison (alignment between the query sequence and GenBank homologue); an E-value and Bit score (Altschul *et al.*, 1996). The E-value is the probability of the alignments of the GenBank sequence with the query sequence being due to chance. The BLAST*n* can thereby provide homologous sequences to expand your data set and provide potential outgroup taxa sequences.

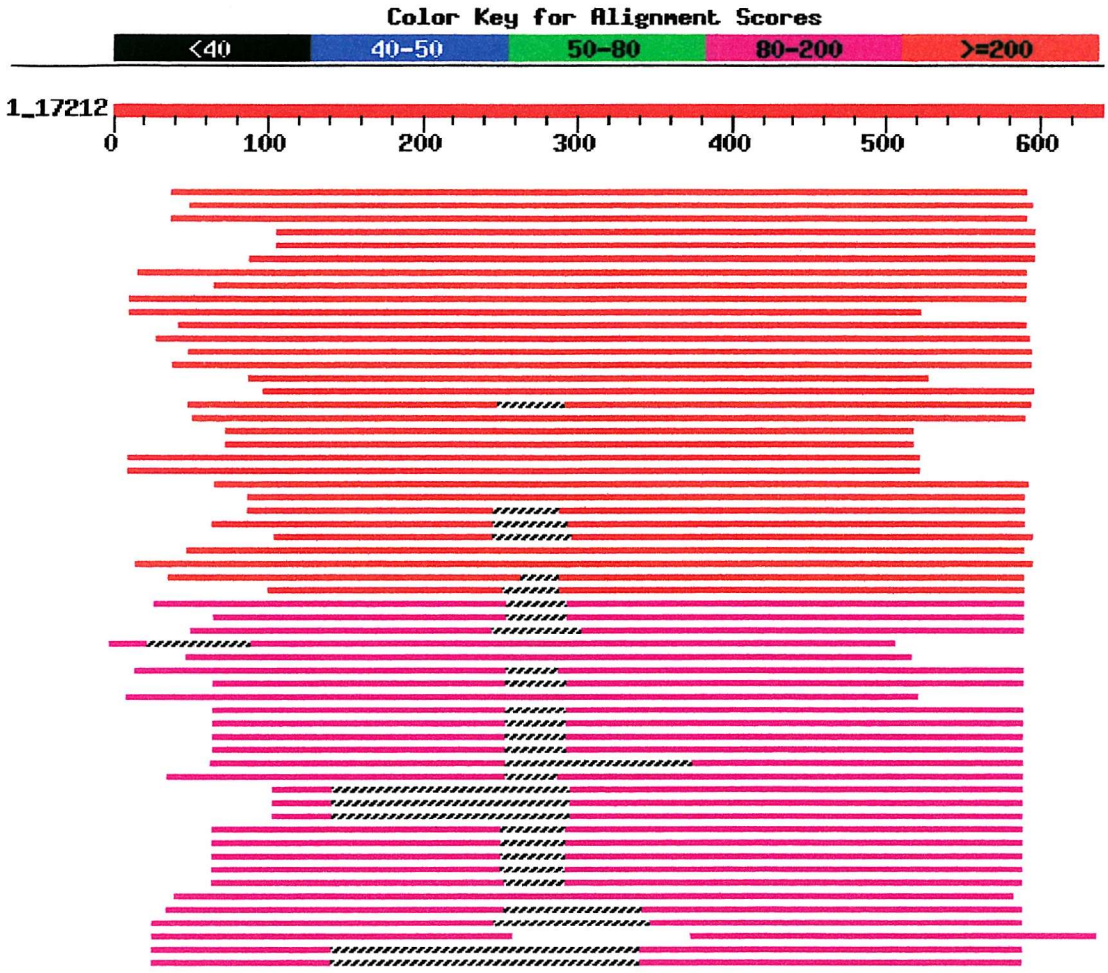


Figure 4.6 An example of the BLAST*n* alignment search output produced by the NCBI website (http://www.ncbi.nlm.nih.gov/). The alignment scores represent the number of complementary base pairs, of a COI partial sequence of *Osilinus edulis* collected from Lanzarote, shared with GenBank sequences. Each horizontal bar links to the actual base pair pairwise alignment between the query sequence and GenBank homologue.

4.2.3 Multiple sequence alignment

A sequence alignment is a hypothesis on the evolutionary history of the sequences (Phillips *et al.,* 2000). The quality and validity of any sequence based phylogenetic analysis is determined by the quality of the input file; therefore confidence in the sequence alignment is essential. The object of an alignment is to create the most efficient statement of initial homology (Phillips *et al.,* 2000). The DNA sequences to be included in any phylogenetic analysis need to be combined in a single text file of the appropriate format (e.g. FASTA format). These are the input files for the alignment software program ClustalX (Thompson *et al.,* 1997). ClustalX provides an interface for multiple sequence alignment. A complete alignment is achieved in three stages. First pairwise alignments are made, in which all sequences are compared to each other and their genetic distances calculated. Second, a phylogenetic tree (dendogram) is constructed that describes groups of similar sequences, based on the genetic distances calculated during the pairwise alignment. Lastly, a multiple alignment is constructed, using the phylogenetic tree as a guide.

Pairwise and multiple alignment parameters within ClustalX can be altered to improve an alignment. The main parameters used in this work include the gap open penalty, gap extension penalty, the delay divergent sequences switch and the transition weight parameter. The gap open penalty and gap extension penalty parameters are used to control the presence and size of gaps within an alignment. The gap open parameter can be set between 1 and 100 and represents the cost assigned to opening a gap within an alignment, therefore a higher penalty value will result in less frequent gaps within an alignment. The gap extension penalty can be set between 1 and 100 and represents the cost assigned to extending a gap by 1 residue; therefore a higher penalty value will result in shorter gaps.

The delay divergent sequences switch determines the order of pairwise alignments based on sequence percentage identity. Thereby, closely related sequences are aligned before more distantly related sequences. The threshold at which the delay divergent sequences switch is set, determines the percentage identity at which the alignment of two sequences is delayed. For example, if set at 50%, sequences less than 50% identical to any other sequences are aligned later. Higher delay divergent sequences values are useful for the alignment of more closely related sequences, lower scores for the alignment of distantly related sequences. The transition weight parameter gives transitions (purine to purine A<->G and pyrimidine to pymrimdine C<->T substitutions) a weight between 0 and 1 (1 conferring a match score on transitions, 0 a mismatch). The pairwise and multiple alignment parameters implemented in the ClustalX alignment of the 16S and COI sequences are given in Appendix B.

Each ClustalX alignment produces by default an alignment file and dendogram file. Additional output format options can be specified, including nexus format alignment files and nexus tree files. The nexus files are subsequently used as PAUP* v4b2 (Phylogenetic Analysis Using Parsiomony - * and other methods) input files. The software TreeView (Page, 1996) is used to view and perform simple edits of ClustalX dendograms. Sequence alignments produced by ClustalX can be further edited and manipulated using the phylogenetic software MacClade (Maddison & Maddison, 1999) which also produces output files compatible with PAUP*4.0b10 (Swofford, 1999). All phylogenetic analysis was performed using PAUP*4.0b10 and is detailed in the methodologies of chapters 5, 6 and 7. An overview of tree building methods, substitution models, hierarchical likelihood ratio tests, search strategies and confidence limits on phylogenies is briefly given below.

4.2.4 Tree building methods: optimality criterion and clustering algorithms.

A sequence alignment is a description of the variation between DNA sequences. The next requirement is to develop models for the observed patterns. An optimality criterion or clustering algorithm must be decided upon using the criteria that determines which tree best describes your data. These are based on either distances or discrete data (see Table 4.5). Distance methods convert aligned sequences into a pairwise matrix upon which the tree building is based (e.g. Neighbour joining and minimum evolution). Discrete character methods consider each nucleotide

site directly (e.g. maximum likelihood and parsimony). The latter method is preferable since no sequence information is lost. For an overview of tree building methods see Page & Holmes (1998) pp 172-227.

Table 4.5: Summary of commonly used tree building methods (after Page & Holmes, 1998).

Tree building methods		Data type	
		Distances	Nucleotide sites
	Clustering algorithm	UPGMA Neighbour Joining	
	Optimality criterion	Least Squares Minimum Evolution	Maximum Parsimony Maximum Likelihood

Clustering algorithms provide a fast tree building method, and are often used to produce starting trees for further analyses. There are two main clustering methods: Neighbour Joining (NJ) (Nei & Saitou, 1987) and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) (Michener and Sokal, 1957). UPGMA is the simplest tree building method, based on operational taxonomic units (OTU's). UPGMA employs a sequential clustering algorithm, in which local topological relationships are identified in order of similarity, and the phylogenetic tree is built in a stepwise manner (Sneath & Sokal, 1973). NJ is a clustering method that attempts to approximate the least squares tree, and allows for unequal rates of change among branches (Avise, 1994, see also Li, 1997). NJ was used in my work to build starting trees for use in further analysis. There are four main optimality criteria: minimum evolution, least squares, maximum parsimony and maximum likelihood. These are discussed briefly below.

Minimum evolution is a distance analysis based on least squares calculations (Lewis, 2000) (Figure 4.7). The best tree is found by the least squares branch lengths; those values of a, b, c, d, e that maximise the fit between the branch length (p_{ij}) and pairwise distance (d_{ij}). Therefore $P_{ij} = d_{ij}$ for all comparative sequences if the tree topology is correct. This is the equivalent of minimising the sum of the squares (Lewis, 2000; see also Rzhetsky & Nei, 1993). The minimum evolution criterion discards the SS and uses estimated branch lengths; the opposite is true of the least squares criterion.

Also distance based; according to the *least squares* optimality criteria, the best tree is the tree with the smallest sum of squares (SS) score (Lewis, 2000), Equation 1:

Equation 1: $$SS = \sum_{i-j} \frac{(d_{ij} - p_{ij})^2}{dij^2}$$

Where *dij* = pairwise distance estimated between sequence *i* and sequence *j* and *Pij* = pairwise distance between *i* and *j*, obtained by adding up paths across a tree (see Figure 4.7).

(A)

Taxa sequence	1	2	3
2	d_{12}		
3	d_{13}	d_{23}	
4	d_{14}	d_{24}	d_{34}

(B)

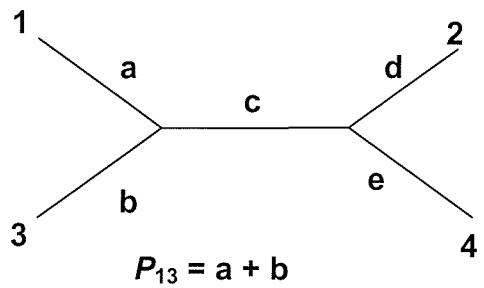


Figure 4.7. The pairwise distances (*dij*) and branch lengths (*Pij*) used in least squares and sum of squares calculations. (A) An example of a sequence distance matrix to illustrate *dij*. (B) A four taxon trees with generic branch lengths to illustrate *Pij*.

Parsimony aims to find the ancestral states that require the least amount of evolutionary change, thereby establishing the minimum number of character changes to explain your data set. According to Lewis (2000) '*Given 2 trees, the one minimising the number of required character state changes is best*'. There are three types of parsimony (Table 4.6), but output from these three different types of parsimony analysis cannot be compared (unlike the models of substitution used in maximum likelihood analysis). For a critical appraisal of this approach see Cunningham *et al.* (1998).

Table 4.6. The three types of parsimony and the corresponding DNA substitution weightings implemented.

Type of parsimony	Substitution weightings				
FITCH	All changes are weighted equally				
Transversion	Unequal weightings – Transitions are unseen (0). Transversions = (1).				
General (weighted)	The base change step matrix is decided by the user:				
		A	C	G	T
	A	-	*a*	*b*	*c*
	C	*a*	-	*d*	*e*
	G	*b*	*c*	-	*f*
	T	*d*	*e*	*f*	-

For a detailed explanation of the *likelihood* optimality criterion, see Huelsenbeck & Crandall (1997). According to the maximum likelihood optimality criterion, '*given two trees the one maximising the probability of the observed data is best*' (Lewis, 2000). Likelihood analysis is based on equation 2:

Equation 2: $L = Pr(D|H)$

This is the probability of data D, given hypothesis, H. The hypothesis is the probabilities imposed by a chosen model of substitution (discussed below in section 4.2.5). Maximum likelihood analysis produces trees with a log-likelihood score, which is the natural log of the overall likelihood score. The overall likelihood is the sum of each site likelihood. Each site likelihood is determined by the ancestral state that gives the best likelihood score for a data set under the assumed substitution model. This analysis recreates trees of all the possible ancestral sequence states for each site in a set of sequences. This makes maximum likelihood analysis computationally expensive with medium to large data sets. Given a four taxa tree, one site has 16 possible ancestral states. There is an exponential growth of the number of possible trees with the number of taxa included in the analysis.

4.2.5 Substitution models

The role of substitution models is to attempt to convert observed distances into measures of actual evolutionary change (for review see Rzhetsky & Nei, 1995). The General Time Reversible (GTR) family of DNA substitution models is widely used in phylogenetic analyses. It consists of 64 nested DNA substitution models (Posada & Crandall, 1998), the simplest of which is the Jukes-Cantor model (Jukes & Cantor, 1969) of equal substitution rate between all bases illustrated by the rate matrix summarised in Table 4.7. The most complex is the GTR + I + Γ (see Lanave *et al.*, 1984; Tavare, 1986; Rodriguez *et al.,* 1990) which has 6 substitution rates (rmatrix), unequal base frequencies, with variants I + Γ. I is the proportion of invariable sites. Γ is the gamma distribution; among site substitution rate variation (Table 4.7). The GTR +I+ Γ model of DNA substitution provides the best likelihood but with a greater potential standard error.

Table 4.7. DNA evolution models. Defined by the DNA substitution probability matrix and base frequencies. α = rate of substitution (JC model) α = rate of transition/site β = rate of transversion/site πi = frequency of *ith* base averaged over all sequences. a – e = rate of substitution.

Model	Probability matrix						Base frequencies
			A	C	G	T	
Jukes-Cantor (JC).	P*t*	A	-	α	α	α	F = [¼ ¼ ¼ ¼]
		C	α	-	α	α	
		G	α	α	-	α	
		T	α	α	α	-	
Kimura 2-parameter(K80) Kimura (1980)	P*t*	A	-	β	α	β	F = [¼ ¼ ¼ ¼]
		C	β	-	β	α	
		G	α	β	-	β	
		T	β	α	β	-	
Felsenstein 1981(F81)	P*t*	A	-	π Cα	πG α	πT α	F = [πA πC πG πT]
		C	π Aα	-	πG α	πT α	
		G	πA α	πC α	-	πT α	
		T	π Aα	πC α	πG α	-	
Hasegawa-Kishino-Yano (HKY85) Haseqawa *et al.,* (1985)	P*t*	A	-	πC β	π Gα	πT β	F = [πA πC πG πT]
		C	π Aβ	-	πG β	πT α	
		G	π Aα	πC β	-	π Tβ	
		T	π Aβ	πC α	πG β	-	
General Time Reversible (GTR):	P*t*	A	-	πCa	πGb	πTc	F = [πA πC πG πT]
		C	πAa	-	πGd	πTe	
		G	πAb	πCd	-	πTf	
		T	πAc	πCe	πGf	-	

The best model is therefore the simplest model that does not provide a significantly worse fit than the GTR +I+ Γ model, since this will be computationally faster with less potential error. The hierarchical likelihood ratio test (hLRT) is used to decide the best DNA substitution model to use for a data set. It provides an objective way of reaching a compromise (Goldman *et al.,* 2000).

4.2.6 Hierarchical Likelihood ratio tests (hLRTs)

Since the DNA substitution models of the GTR family are nested, so are comparisons between two different evolutionary hypotheses. The likelihood ratio test uses a starting tree obtained from neighbour joining analysis or maximum parsimony to estimate the likelihood scores of all substitution models given the data and starting tree topology. The likelihood score from each model is compared to its nested more complex alternative (Equation 3).

Equation 3: $LRT = 2([-\ln L_{null}] - [-\ln L_{alternative}])$

Where the *null* model is a (simpler) special case of the *alternative* (more complex) model. L equals the likelihood score of the tree topology under the substitution model and data. The Chi^2 test is used to determine if LRT is statistically significantly, with degrees of freedom determined by the difference in the number of free parameters. The ModelTest software (Posada & Crandall 1998) is used to implement hLRTs and determine the best DNA substitution model and parameters to use in likelihood analysis. For a detailed explanation of hLRT, see Felsenstein

(1981), Huelsenbeck & Crandall (1997), Huelsenbeck & Rannala (1997), and Swofford *et al.*, (1996).

4.2.7 Search strategies

Once a tree building optimality criteria has been chosen, a method of finding the optimal tree is required. In many cases, it is not computationally feasible to search all possible trees to get the best tree according to a chosen optimality criterion. Therefore, several methods of exploring the tree space exist (Page & Holmes 1998). There are three main search strategies:

Algorithmic strategies are compatible with any optimality criterion (although it is not technically a search). These methods provide a highly efficient method of attaining a single tree. There are two methods of building trees algorithmically. Star decomposition starts with one internal node and joins pairs of nodes to find the optimal value given a hypothesis. Stepwise addition starts with a three-sequence tree, it then finds the optimal branch position of each subsequently added taxa. Both algorithmic methods can be used to establish a starting tree for other search methods (for review see Page & Holmes 1998).

Exact strategies search all possible trees to produce the globally optimal tree. Exact searches using the maximum likelihood criteria are limited to small data sets of maximum 12 sequences. Alternatively, in the case of a branch-and-bound search (Fukunaga and Narendra, 1975) (only compatible with maximum parsimony analysis) an exhaustive search is simulated. This requires a starting tree derived from a fast tree building method such as neighbour joining. The starting tree branch configurations are rearranged. Tree topologies with better scores than the starting tree are retained and the process repeated. Providing the starting tree is a reflection of the correct topology, this is a very effective method of finding the best tree (Lewis, 2000).
Heuristic methods explore a subset of all possible trees with the object of finding the optimal tree (for review see Page & Holmes, 1998). This is achieved by the implementation of branch-swapping or genetic algorithms (Lewis, 2000). The disadvantage of this approach is that once a sub-optimal tree island is reached, the optimal tree cannot be derived (Lewis 2000). There are three main branch swapping methods:

•*Nearest Neighbour Interchange* (NNI): This method executes small branch swapping steps to create local rearrangements to produce an optimal tree rearrangement. This strategy however does have tendency to produce sub-optimal trees due to the greater likelihood of 'tree islands' (Lewis, 2000).
• *Subtree Pruning/Regrafting* (SPR): Because this strategy allows global rearrangement, it is more systematic and thorough than the NNI thereby minimising the occurrence of sub-optimal tree islands.
• *Tree Bisection/Reconnection (TBR):* Similar in approach to SPR, TBR provides the most thorough branch swapping strategy.

4.2.8 Confidence limits on phylogenies

Sample replication is often unfeasible in phylogenetic analyses. Therefore, the statistical tool of bootstrapping is employed to estimate sampling error (Felsenstein, 1985a, 1985b). Bootstrapping is applied to estimate the reliability of individual clades in a tree (Huelsenbeck & Hillis, 1996). Instead of sampling replicates from a population, bootstrapping resamples each site in the existing data set at random with replacement, to produce pseudoreplicates. The psuedoreplicate is subsequently used to build a tree according to a specified optimality criterion and search strategy. The variation among estimates from the pseudo-replicates provides a measure of sampling error. For example, 100 replicates could produce three trees with the frequencies 90/100 6/100 and 4/100. It is however, often unfeasible to show the frequencies and topologies of all trees produced from bootstrap analysis of large data sets. To summarise the data, a majority-rule consensus tree is compiled with each node being given a frequency of occurrence among bootstrap trees (Margush & McMorris, 1981). Felsenstein (1985a) advises well supported nodes are those with more than 95% bootstrap values (for review on boostrapping as a tool for assessing confidence in phylogenetic analyses, see Sanderson 1990, Hillis & Bull 1993 and Huelsenbeck & Rannala, 1997).

4.3 Morphological methods

Sample preservation, radula dissection and Scanning Electron Micrograph stub preparation methods are given below (chapter 7). The cladistic character coding methods and parameters implemented in parsimony analysis of discrete morphological characters, performed using PAUP*4.0b10, are also detailed below in chapter 7.

Chapter 5

Molecular phylogeny of selected genera from the Gibbulini tribe based on mitochondrial 16S partial sequences

5.1 Introduction

In order to correctly resolve phylogenetic relationships, it is essential that an appropriate outgroup is chosen (Kitching *et al.,* 1998; Reid, 1999). The use of a too distantly related outgroup taxa can result in increased homoplasy between ingroup and outgroup (Winnepenninckx *et al.*, 1998) and hence falsely unite distantly related genotypes. The main aim of this analysis was therefore firstly to establish the most appropriate outgroup (the sister taxa to the *Osilinus* clade) for the resolution of interspecific relationships within *Osilinus* in subsequent analyses. Secondly, it serves to test if all species currently classified within the genus *Osilinus* form a monophyletic group, and consequently to establish if the current classification is correct (Rosen, 1979; Eldredge & Cracraft, 1980; Nelson & Platnick, 1981; Nixon & Wheeler, 1990, 1992). Of particular interest is the relationship between *Osilinus kotschyi* in the Indian Ocean, and the Atlantic and Mediterranean *Osilinus* species.

5.2 Materials and Methods

5.2.1 Taxon selection and sample tissue collection

Twelve taxa of Gibbulini were used in the analyses; these comprised six *Osilinus* species and eight species representing the genera *Gibbula, Austrocochlea, Monodonta* and *Oxystele* (Table 5.1).

Table 5.1. Taxa used in outgroup analysis with identification number (sample number and sequence identification), location and source. * Denotes samples included in the subset analysis.

Taxa	ID	Location	Source
Austrocochlea constricta (Lamarck, 1822)	216/404*/218*	Sydney Harbour. Australia	NHM London
Monodonta labio (Linnaeus, 1758)	402*/403*	Cambodia	NHM, London
Oxystele tigrina (Anton, 1989)	174/177/395*	Knysna Heads, S. Africa	S.J.Hawkins 10/2000
Oxystele variegata (Anton, 1839)	149*/391/393	Hermanus, S. Africa	S.J.Hawkins 10/2000
Oxystele sinensis (Gmelin, 1791)	390*	Hermanus, S. Africa	S.J.Hawkins 10/2000
Gibbula umbilicalis (da Costa, 1778)	127/128*/129	Porthguerra, UK	J. Preston 11/1999
Osilinus kotschyi (Philippi, 1849)	198/202*/400/401*	Khor Al Hamra, UAE	BMNH, London
Osilinus edulis (Lowe, 1842)	182*/183	Seixal, Madeira	J. Preston 10/2001
Osilinus articulatus (Lamarck, 1822).	093*	Calahonda, Spain.	S.Gofas 07/2002
	106	Torre Guardiaro, Spain.	
Osilinus atratus (Wood, 1828)	067*/069	Las Galletas, Tenerife	J. Preston 08/2000
Osilinus turbinatus (Born, 1780)	096	Torre Guardiaro, Spain.	S. Gofas 07/2002
	121	Coral Bay, Cyprus	S.J.Hawkins 09/2000
Osilinus lineatus (da Costa, 1778)	001	Anza, Morocco	J. Preston 08/2000
	243*	Basque Coastline	Rob Alcock 10/2001

5.2.2 *Molecular methods*

For details of the molecular laboratory techniques see Chapter 4. Amplification and sequencing of the partial 28S rDNA variable regions D1 to D6 (Littlewood *et al.,* 2000) was successful for all taxa (Table 5.1). This nuclear gene region, however, contained no sequence variation between any of the outgroup taxa or *Osilinus* species. The 28S gene region was phylogenetically uninformative for resolving genus-level relationships in the gibbuline taxa considered here.

5.2.3 *Phylogenetic analysis*

A 478bp fragment of the 16S gene was sequenced for 29 individuals. Sequences were initially aligned using ClustalX (Thompson *et al.,* 1997) with a gap-opening penalty of 50 (1-100) and gap-extension penalty of 10 (1-100) enforced on both pairwise and multiple alignment parameters. The delay divergent sequence switch was set to 80% and the transition weight to 0.25. The alignment was double-checked by eye and ambiguous sequences or sequence residue regions were realigned using an appropriate gap-opening penalty. The alignment is given in Appendix C. All phylogenetic analyses were performed using the software package PAUP* version 4.0b10 (Swofford 1999). Distance and parsimony analyses were performed on all 29 sequences. Neighbour-joining analysis was used to obtain a starting tree for minimum evolution (distance - general time-reversible) analysis with the tree-bisection-reconnection branch-swapping algorithm. Negative branch lengths were allowed but set to zero for tree-score calculation, zero-length branches not collapsed. No topological constraints were enforced and trees were unrooted. The minimum-evolution search was subsequently repeated as a bootstrap analysis with 500 replicates (Felsenstein, 1985a).

Exact (branch-and-bound) parsimony was performed on all 16S sequences with all characters equally weighted for all positions and gaps treated as missing data. The initial upper bound (starting tree) was computed heuristically, with the addition sequence set at furthest and branches collapsed if maximum length equalled zero. Trees were unrooted with no topological constraints enforced. The most parsimonious trees were subsequently used as starting trees in a second branch and bound search repeated under identical settings, except for the initial upper bound score set by that of the starting trees. A heuristic parsimony search was also performed on all 29 16S sequences with equal weights for all positions and transformation types. The starting tree was obtained by stepwise addition with simple addition sequence: tree-bisection-reconnection (TBR) branch swapping with branches collapsed if maximum length equalled zero. No topological constraints were enforced and trees were unrooted. The heuristic search was repeated as a bootstrap analysis, (100 replications) with the starting tree obtained via stepwise addition with 10 random addition sequence replicates. Ideally a larger number of replicates (1000 – 10,000) would be performed, however this was limited by available processing power. The suite of trees produced by the analyses, including the consensus bootstrap tree values and distance tree topologies, were used to determine a subset of sequences for further maximum-parsimony and maximum-likelihood analysis.

All 12 Gibbulini taxa included in the original data set were represented by 16 sequences to allow for computationally feasible further analysis. Of the 478 base pairs included in this 16S sequence analysis, 87 base pairs represented 2 ambiguous, low scoring alignment fragments analogous from bp 206-247 and 317-361 (underlined in Appendix C). Consequently the initial maximum-parsimony analysis was replicated with no bases excluded, and with 87 low scoring bases excluded. This was to establish if exclusion of ambiguous alignment positions resulted in a loss of phylogenetically informative characters, or a significant change in tree topology.

Maximum-parsimony analysis was performed on the established subset of sequences under various character weightings and transformation types on both the excluded and non-excluded data sets, to provide a suite of starting trees. The parsimony optimality criterion settings imposed on all analyses were kept constant. The Goloboff fit criterion (Goloboff, 1993) was enforced where k=2, uninformative characters were excluded from the total score and branches collapsed if the maximum length equalled zero. Heuristic searches were performed at constant search settings of 100 random sequence-addition replicates with starting trees obtained via stepwise addition with 10 trees held at each step. The tree-bisection-reconnection (TBR) branch-swapping algorithm was in effect (branches collapsed if maximum branch length equalled zero) with no topological constraints enforced and trees were unrooted.

The non-excluded data set was analysed under these search settings with all characters assigned equal weights for all positions and transformation types, and with transversion/transition weight ratios of 2:1, 5:1, 10:1 and 100:1. The transformation weightings were enforced using a user defined step matrix (Table 5.2).

Table 5.2: User defined step matrix for transversion/transition ratio 5:1. The purine nucleotides are represented by A = adenine, and G = Guanine. Pyrimidine nucleotides are represented by C = cytosine and T = thymine. Transitions (purine to purine or pyrimidine to pyrimidine) are weighted one. Transversions (purine to pyrimidine and *vice versa*) are assigned a weight of five.

	A	C	G	T
A	-	5	1	5
C	5	-	5	1
G	1	5	-	5
T	5	1	5	-

For the excluded data set, parsimony analysis was performed with all characters assigned equal weights for all positions and transformation types (unordered), and with transversion/transition weight ratios 2:1 and 5:1. The tree topologies arising from parsimony analysis on the non-excluded and excluded data sets were compared for phylogenetic resolution and consequently the appropriate data set chosen for maximum-likelihood analysis.

Since any model of substitution employed in phylogenetic analysis makes assumptions about the process of evolutionary change (Felsenstein, 1988), it is important to have confidence that the chosen model of DNA substitution best fits the data set being analysed (Goldman, 1993). Therefore, the likelihood score of all maximum-parsimony starting trees was calculated with all parameters estimated under several nucleotide substitution models within the General Time Reversible (GTR) family of nested models (Rodriguez, *et al.,* 1990; Posada & Crandall, 1998). The likelihood score of all the excluded MP suite of starting trees was estimated under the following substitution models: JC (Jukes and Cantor, 1969), K80 (Kimura, 1980), F81 (Felsenstein, 1981), HKY85 (Hasegawa *et al.*, 1985) and GTR (Rodriguez *et al.*, 1990) with variants Γ (the shape parameter of the gamma distribution) and I (proportion of invariable sites) estimated separately or combined. A total of 48 tree plus model plus parameter combinations were compared. The hierarchical Likelihood ratio test statistic (hLRT) (Kendall & Stuart, 1979; Posada & Crandall, 1998) was subsequently used to evaluate the comparison between likelihood scores for all models estimated on the best scoring parsimony tree (Equation 5.1).

Equation 5.1

If

$$LRT = 2([-\ln L_{null}] - [-\ln L_{alternative}])$$

Where:

-lnL *null* is the likelihood under the null hypothesis (simple model)

-lnL *alternative* is the likelihood under the alternative hypothesis (more complex, parameter rich model)

The *null* model is a special case of the *alternative* model
The degrees of freedom equals the difference in the number of free parameters

The hierarchical Likelihood Ratio Test (hLRT) was then used to decide the most appropriate model to describe the data set. A more extensive comparison of the GTR family of nucleotide substitution models was achieved using ModelTest 3.0 (Posada & Crandall, 1998) that also employs hLRT to compare model fit to a data set. The Modeltest software (Posada & Crandall, 1998) generates a neighbour-joining starting tree from the data set and computes the score for all 64 nested GRT models. Again the best model is chosen when the addition of new parameters no longer results in a significant change in the tree likelihood score.

A maximum-likelihood heuristic search was then performed on the excluded data set using the parameters estimated from the likelihood score of the best scoring maximum-parsimony tree under the chosen model of substitution. The likelihood score of this tree was subsequently re-estimated

under all the GTR models imposed on the maximum-parsimony trees to determine if a more appropriate model could be used or any further models rejected. This process was repeated until the parameters stabilized. The likelihood score of best maximum-parsimony tree under the chosen model was compared to the likelihood score with a molecular clock enforced to establish if the sequence data behaved in a clock-like manner. Once the best model and parameter estimates had been established, these were employed in a ML search and the best tree was taken as the correct topology for the outgroup data set. In a final comparison, a maximum-likelihood heuristic search was performed under the same assumptions, parameters with equal base frequencies and estimated base frequencies, and the likelihood scores of each tree compared. Bootstrapping was used to estimate the sampling error of the outgroup data set and establish a level of confidence in the estimated phylogeny. 100 bootstrap replicates were performed under a maximum-likelihood heuristic search using the model and parameters estimated as best fitting the data set, and a maximum-parsimony branch and bound search.

5.3 Results

5.3.1 Nucleotide Base Composition

The mitochondrial genome is typically rich in the nucleotide bases adenine (A) and thymine (T) (e.g. Simon *et al.,* 1994). This is reflected in the nucleotide base composition of the outgroup taxa used in the analysis which showed a consistent base bias (Table 5.3). The average percentage nucleotide base composition amongst the outgroup taxa is A = 31.2%, G = 13.3%, C = 21.9%, T = 33.6%. G + C content was low (35.2%) and A + T content was high (64.8%) at levels comparable to other molluscan groups (e.g. Lydeard *et al.*, 1996).

Table 5.3. Percentage nucleotide base compositions of the 16S mt DNA partial gene sequences of the Gibbuline outgroup and ingroup taxa.

Taxa	Percentage Nucleotide Base Composition of taxa			
	A	G	C	T
Osilinus kotschyi	30.4	13.2	22.0	34.4
Osilinus lineatus	29.8	13.6	23.3	33.3
Osilinus edulis	29.0	12.8	22.7	35.5
Osilinus turbinatus	31.4	13.9	21.7	33.0
Osilinus atratus	30.5	13.5	22.0	34.2
Osilinus articulatus	31.1	13.5	20.4	35.0
Oxystele variegata	33.1	13.4	21.5	32.0
Oxystele tigrina	32.1	14.1	21.4	32.4
Oxystele sinensis	33.8	13.0	20.0	33.2
Austrocochlea constricta	31.3	12.9	21.8	34.0
Monodonta labio	28.4	13.9	25.2	32.5
Gibbula umbilicalis	32.6	12.2	21.3	33.9
Average	**31.2**	**13.3**	**21.9**	**33.6**

Significant differences in base frequencies across taxa undermines many tree-building methods (Lockheart *et al.*, 1994; Lydeard *et al.*, 1996), although a Chi-square (χ^2) test on the homogeneity of base frequencies across the outgroup taxa revealed no significant differences (χ^2 = 21.8, df=84, P = 1.0).

5.3.2 *Pairwise distance matrix*

The average GTR pairwise distance matrix for all all 29 16S mtDNA sequences used in the analysis is given in Table 5.4.

Table 5.4. The average GTR pairwise distance matrix for all 29 16S mtDNA sequences used in the analysis.

	O.lin	*O.artic*	*O.atrat*	*O. edul*	*O.turb*	*O.kots*	*G.umb*	*A.con*	*M.lab*	*Ox.sin*	*Ox.var*	*Ox.tig*
O.lin (2)	0.007											
O.artic (2)	0.088	0.007										
O. atrat (2)	0.074	0.069	0.007									
O. edul (2)	0.121	0.140	0.110	0.007								
O.turb (2)	0.102	0.100	0.073	0.090	0.002							
O.kots (4)	0.197	0.171	0.170	0.184	0.177	0.006						
G.umb (3)	0.124	0.117	0.114	0.144	0.133	0.165	0.008					
A.con (3)	0.231	0.219	0.212	0.227	0.229	0.217	0.215	0.003				
M.lab (2)	0.219	0.211	0.220	0.216	0.222	0.215	0.198	0.191	0.002			
O.sins (1)	0.230	0.214	0.202	0.213	0.221	0.249	0.197	0.235	0.222	-		
O.varta (3)	0.238	0.237	0.226	0.215	0.232	0.278	0.244	0.252	0.260	0.122	0.003	
O.tig (3)	0.216	0.213	0.204	0.216	0.212	0.250	0.204	0.248	0.246	0.058	0.142	0.002

Number of samples denoted in parenthesis. Species abbreviations: *O.lin = Osilinus lineatus. O.artic = Osilinus articulatus* ; *O.atrat = Osilinus atratus; O. edul = Osilinus edulis; O.turb = Osilinus turbinatus; O.kots = Osilinus kotschyi; G.umb = Gibbula umbilicalis; A.con = Austrocochlea constricta; M.lab = Monodonta labio; Ox.sin = Oxystele sinensis; Ox.var = Oxystele variegata; Ox.tig = Oxystele tigrina.*

5.4 Phylogenetic analysis

5.4.1 *Entire data set*

A neighbour-joining analysis was performed on the entire outgroup taxa data set to produce a starting tree for minimum evolution. The minimum evolution heuristic search computed 6633 rearrangements and found a single best tree with a sum of tree branch lengths score of 0.910 (Figure 5.1). Based on morphological similarity (chapter 7) the European *Osilinus* were initially assumed to be a monophyletic group, the ingroup. All other genera including *O. kotschyi* were initially included in the outgroup since these is no *a priori* reason to assume any of the potential outgroups is most distant. In the resultant tree topology (Figure 5.1B), the outgroup is paraphyletic to the European *Osilinus* ingroup. In Tree A (Figure 5.2) the European species *G. umbilicalis* is included in the ingroup (*Osilinus atratus* + *O. lineatus* + *O. articulatus* + *O. edulis* + *O. turbinatus* + *Gibbula umbilicalis*), the outgroup is still paraphyletic. If all *Osilinus* species are defined as the ingroup, with all remaining taxa defined as the outgroup taxa, the tree cannot be rooted with respect to a monophyletic ingroup. No matter where the true root is, it is impossible to make all *Osilinus* monophyletic. Therefore the current classification of *O. kotschyi* must be incorrect. The minimum evolution search was repeated as a bootstrap analysis (1000 replicates) (Tree A, Figure 5.1).

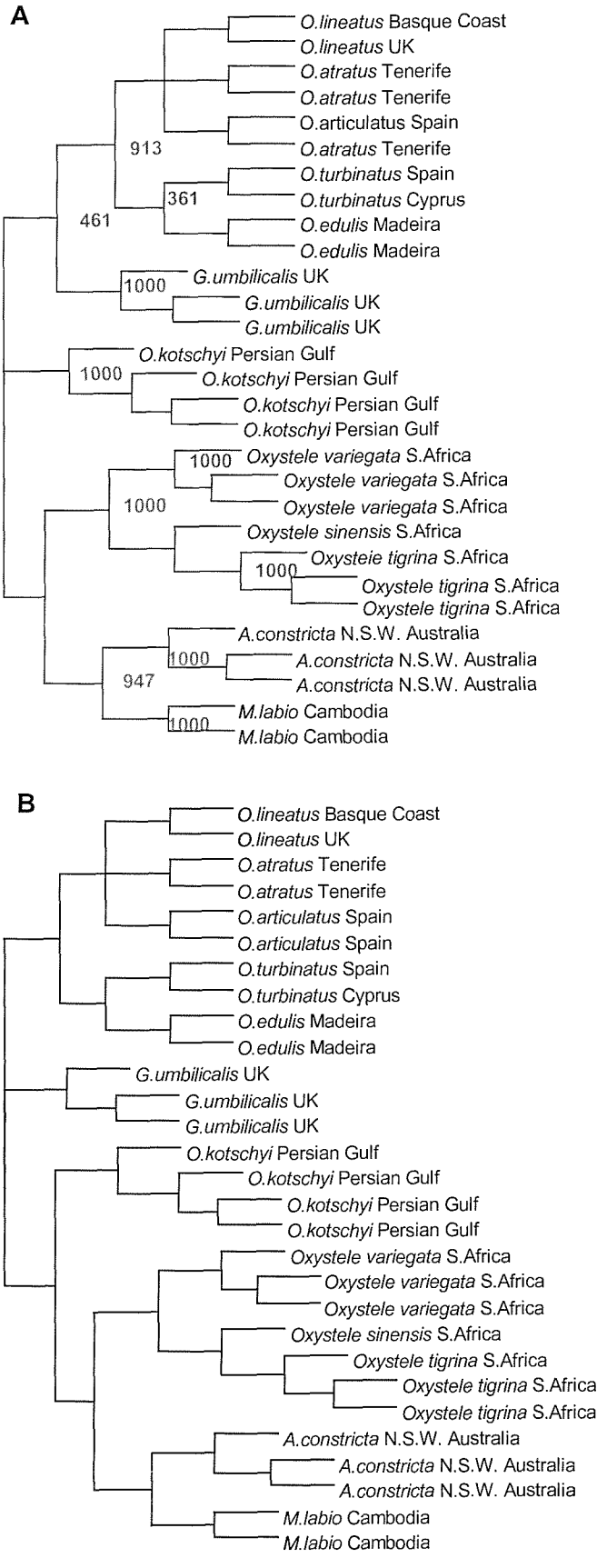


Figure 5.1. 16S mtDNA minimum evolution analysis distance trees. All trees rooted with the as outgroup monophyletic to the ingroup. (A) Mediterranean and Atlantic *Osilinus* + *Gibbula* defined as the monophyletic ingroup. Boostrap values are to the right of nodes. (B) Mediterranean and Atlantic *Osilinus* defined as the monophyletic ingroup.

The exact branch and bound parsimony search performed on all 29 sequences produced four best trees of total length (TL) 439. These trees were used as starting trees in a repeat branch and bound search defining the upper bound at 439. No superior scoring trees were found and the same four tree topologies were retained as the most parsimonious estimate of phylogeny. The same four tree topologies were also produced from the heuristic parsimony search with 26512 rearrangements tried. The four most parsimonious tree topologies found under all the above maximum-parsimony analyses are therefore considered as the globally optimal trees (see Figure 5.2).

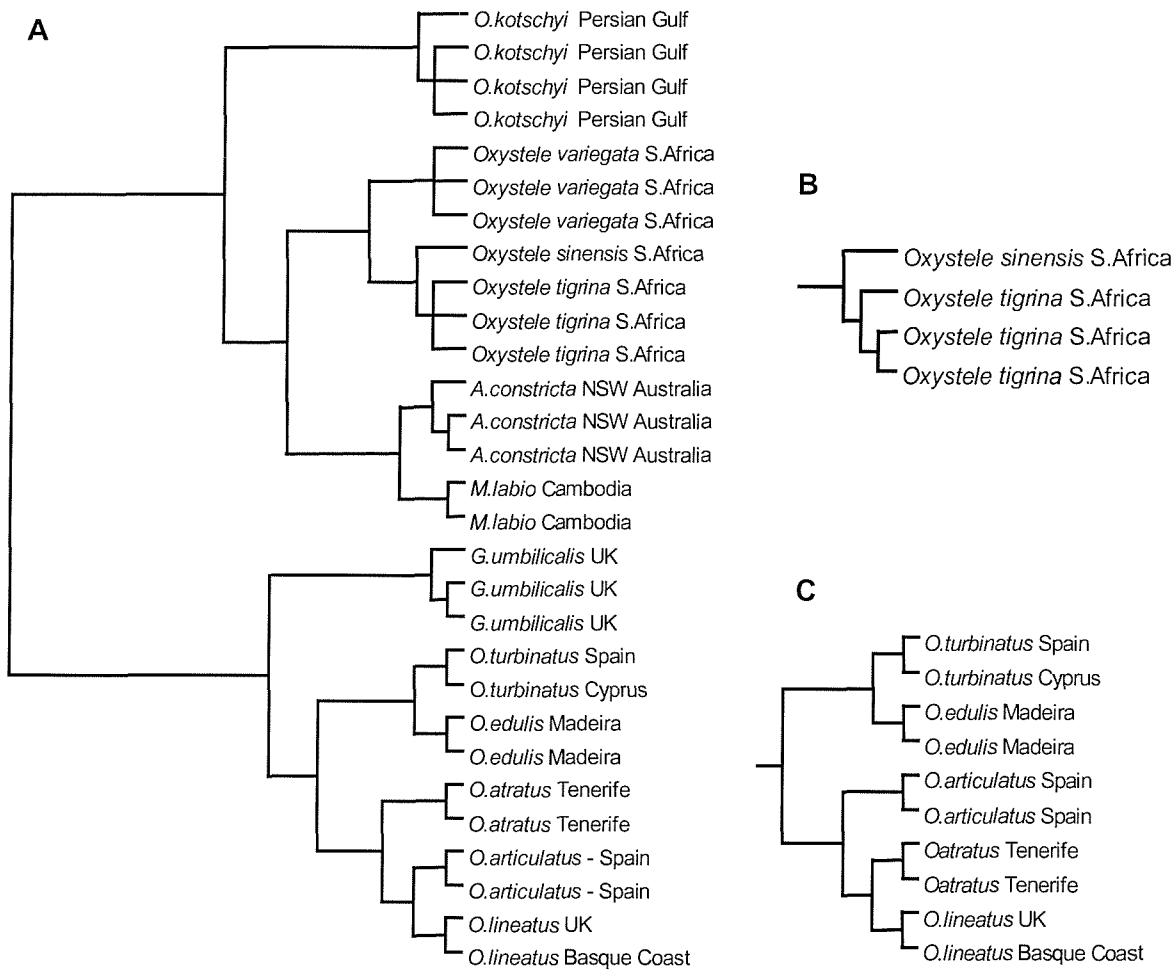


Figure 5.2. The 16SmtDNA maximum-parsimony exact branch-and-bound consensus tree with alternate twigs. The search produced four equally parsimonious trees, TL 439. All four trees shared the same interspecific topology between outgroup taxa (A) differing only within the intraspecific *O. tigrina* clade (B). Two alternative tree topologies of interspecific relationships in European *Osilinus* were represented (A) and (C).

The trees in Figure 5.2 are rooted with the outgroup clade (*Monodonta, Austrocochlea, Oxystele* and *Osilinus kotschyi*) monophyletic to the ingroup clade (Atlantic and Mediterranean *Osilinus* and *Gibbula*). All four best parsimony trees agree with the minimum evolution trees in that *Osilinus* is not a monophyletic group with respect to all inclusive species, and *Gibbula umbilicalis* is the sister taxa to the Mediterranean/Atlantic *Osilinus* species. All four topologies retain the same relationships within the *Monodonta*, *Austrocochlea, Oxystele* and *Osilinus kotschyi* clade with respect to the Atlantic and Mediterranean *Osilinus* and *Gibbula* ingroup clade. Within the ingroup clade, *Gibbula* remains the sister taxa to the monophyletic Atlantic and Mediterranean *Osilinus* group. Two tree topologies are represented concerning interspecific relationships within the Atlantic and Mediterranean *Osilinus* group. The first (Figure 5.2A) places *O. atratus* as basal to *O. lineatus* and *O. articulatus*. The second topology (Figure 5.2C) places *O. articulatus* as basal to *O. lineatus* and *O. atratus*. All four trees placed *O. edulis* and *O. turbinatus* as a separate sister clade to *O. lineatus, O. atratus* and *O. articulatus*. The only other variation between the trees lies within the intraspecific relationship of *Oxystele tigrina* (see Figure 5.2B).

The heuristic parsimony search was repeated as a bootstrap analysis with 100 replicates. The bootstrap 50% majority-rule consensus tree is shown in Fig 5.3. Tree (A) shows the relative branch lengths. Tree (B) is rooted with the outgroup monophyletic to the Mediterranean/Atlantic *Osilinus* + *Gibbula* clade. The bootstrap analysis confers high support (90%) for the Atlantic/Mediterranean *Osilinus* species and *G. umbilicalis* as a monophyletic group relative to the excluded taxa. In the distance tree (Figure 5.2A) *Gibbula umbilicalis* is closer to *Osilinus* than the other outgroup species suggesting it is the best outgroup for subsequent analyses of intraspecific relationship of the Mediterranean/ Atlantic *Osilinus* (Chapter 6). *G. umbilicalis* has 100% bootstrap support as a distinct clade, as does *Osilinus kotschyi* and all-inclusive sequences of the genera *Oxystele*, *Austrocochlea* and *Monodonta*. Similar (87%) bootstrap support is conferred on *O. kotschyi, Oxystele, Monodonta* and *Austrocochlea* as a monophyletic clade relative to the Atlantic/Mediterranean *Osilinus* and *Gibbula* ingroup clade. The above tree topologies and bootstrap support values were used to decide a subset of sequences for further maximum-parsimony and maximum-likelihood analysis. The sequences comprising the subset are highlighted in Figure 5.3 (B).

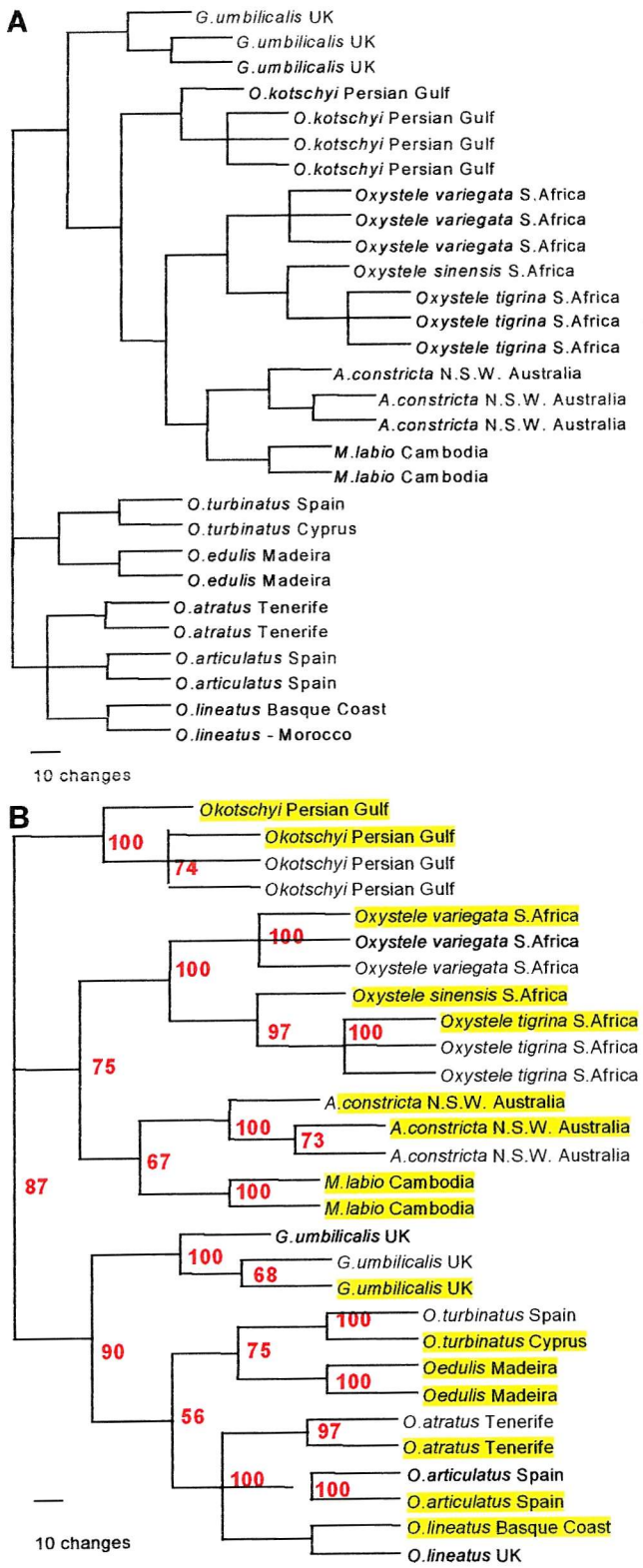


Figure 5.3. Maximum-parsimony 50% majority rule consensus bootstrap tree. with relative branch lengths expressed. Tree (A) is rooted with the outgroup monophyletic to the Mediterranean/ Atlantic *Osilinus* clade. Tree (B) is rooted with the outgroup monophyletic to the Mediterranean/ Atlantic *Osilinus* + *Gibbula* clade. Bootstrap support values are placed at nodes. Sequences used in the subset analysis are highlighted.

5.4.2. *Subset analysis*

Maximum-parsimony

Of the 470 total characters included in the analysis, 293 bases were constant, 24 variable bases were parsimony-uninformative and, 153 bases were parsimony-informative. With the 87 low scoring alignment residues excluded, of the remaining 383 bases included in the analysis 289 bases were constant, 12 variable bases were parsimony uninformative and 82 bases parsimony informative. The score and number of trees found under the maximum-parsimony analysis performed on the non-excluded and excluded data sets are detailed in table 5.5. The maximum-parsimony analysis performed with no positions excluded produced a single most parsimonious tree for all character weightings and transformation types enforced. Two tree topologies were represented differing within the interspecific Atlantic and Mediterranean *Osilinus* clade (Figure 5.4). All trees placed *Gibbula umbilicalis* as the sister taxa to the monophyletic Atlantic and Mediterranean *Osilinus* sp. clade, but regarded *Osilinus* as a paraphyletic clade inclusive of the Indian Ocean species *Osilinus kotschyi.*

Table 5.5. Total length and number of trees found under various transversion/transitions (TV/Ts) ratios for the non-excluded and excluded data sets.

Character weights and types	All positions		Excluded data set	
	No. trees	Tree Score	No. trees	Tree Score
Equal weights for all positions and transformation types	1	424	3	193
Equal weights for all positions TV/Ts weight ratios of 2:1	1	606	2	265
Equal weights for all positions TV/Ts weight ratios of 5:1	1	1148	1	480
Equal weights for all positions TV/Ts weight ratios of 10:1	1	2048		
Equal weights for all positions TV/Ts weight ratios of 100:1	1	18248		

The excluded data set produced three equally parsimonious tree topologies of total length (TL) 193 under equal weights and transformation types (Figure 5.5). All three trees diverge within the Atlantic/Mediterranean *Osilinus* clade but retain the relative outgroups topology produced under the non-excluded MP analysis. Parsimony analysis on the excluded data set under the 2:1 TV/Ts weight ratio produced two equally parsimonious tree topologies of TL 265. These had the same Atlantic/ Mediterranean *Osilinus* relationships exhibited by tree topologies A and C under equal weights for all positions and transformation types (Figure 5.5). Parsimony analysis under the 5:1 Tv/Ts weight ratio produced a single best tree of TL480 of the same topology produced under the 5:1 Tv/Ts. weight ratio enforced on the non-excluded data set (Figure 5.4).

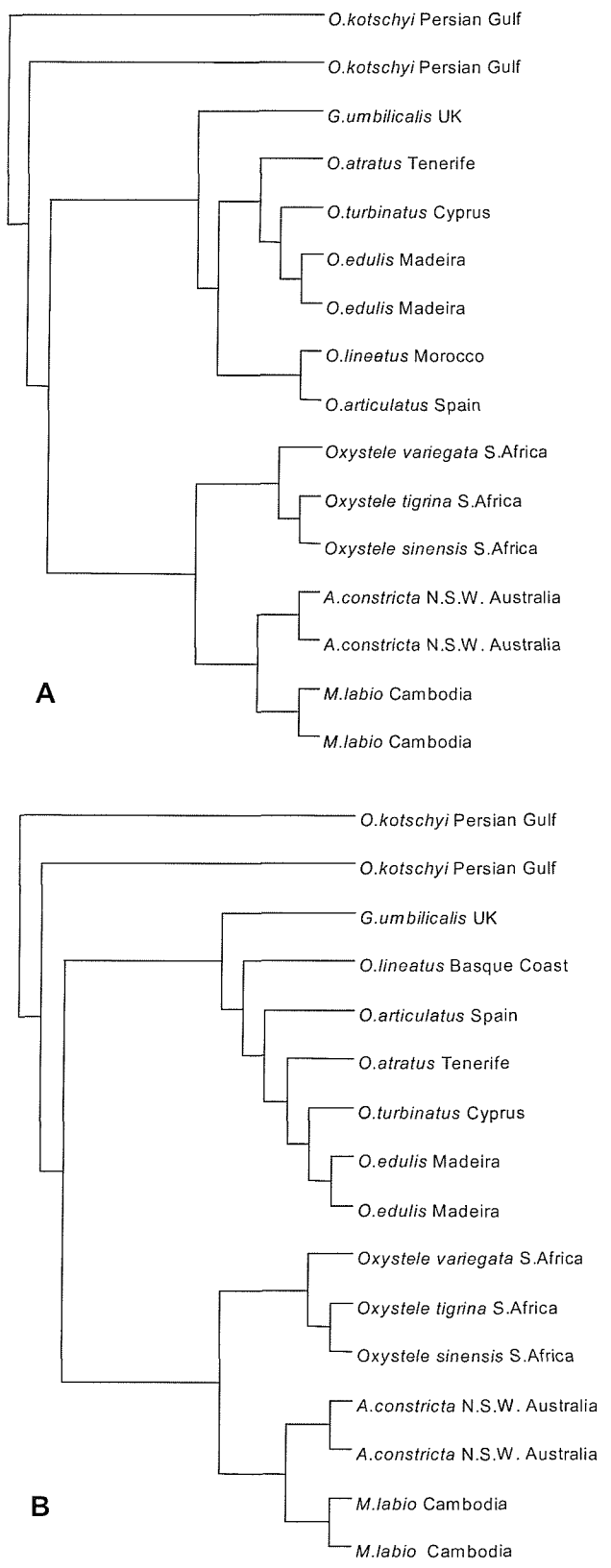


Figure 5.4. Tree topologies produced under two types of maximum-parsimony analyses. (A) Tree topology produced under equal weights for all positions and transformation types, and equal weights for all positions and TV/TS weight ratio 2:1. (B) Tree topology under equal weights for all positions and TV/TS weight ratios 5:1, 10:1 and 100:1.

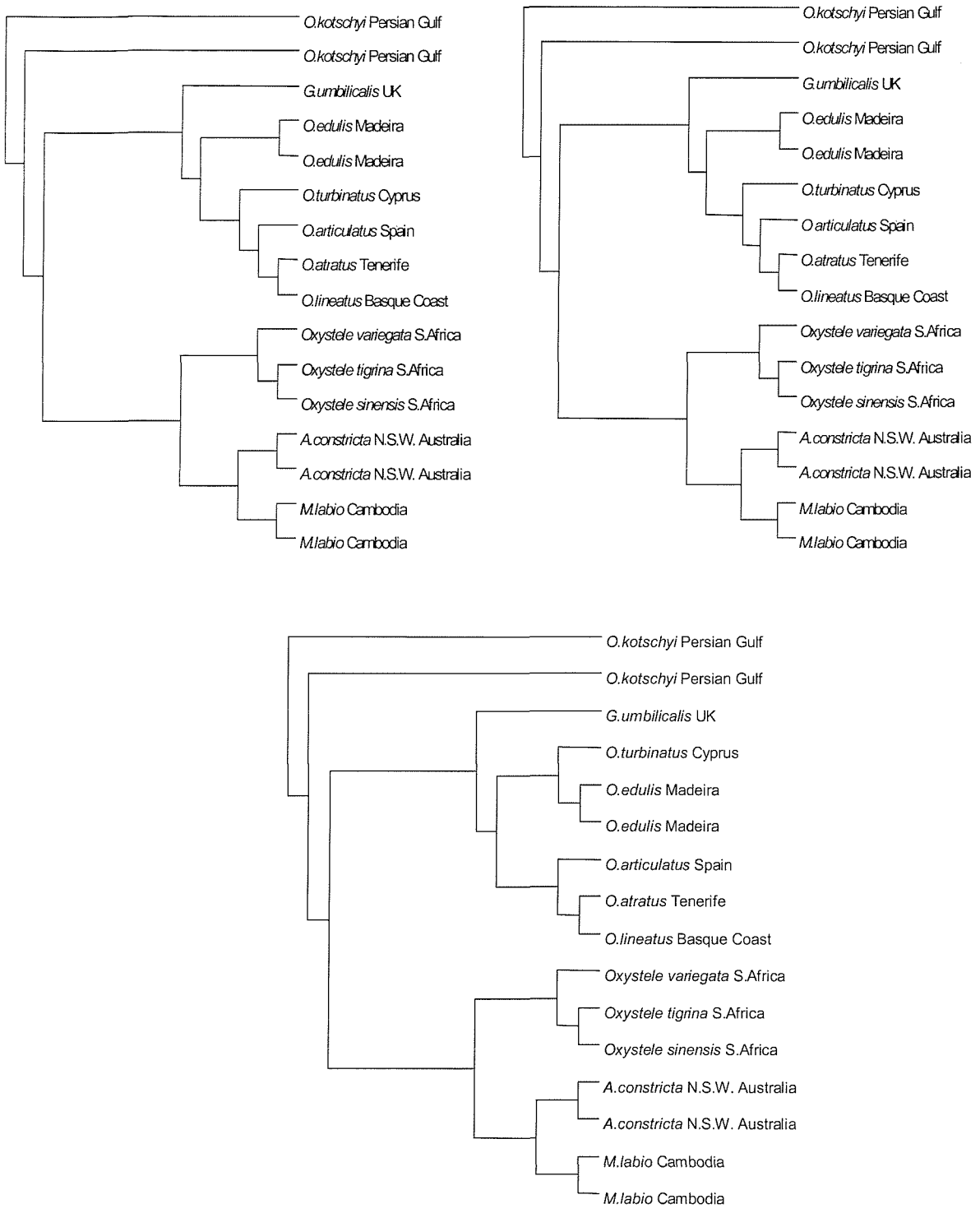


Figure 5.5. The three most parsimonious tree topologies (TL 193) found under equal weights and transformation types for the excluded outroups data set.

It is noteworthy that the relationship between the Atlantic /Mediterranean *Osilinus* clade and the remaining outgroup taxa remained constant on all trees throughout all Maximum-parsimony analysis performed. For the purposes of the outgroup analysis, changes in tree topology between species within the Atlantic/Mediterranean *Osilinus* clade do not matter if the clade remains monophyletic with regard to the outgroup taxa. The excluded data set was therefore selected for further analysis above the non-excluded data set as it consistently produced better scoring Maximum-parsimony trees, with no loss of taxonomic resolution in tree topology.

Likelihood analysis

The likelihood score was calculated for all trees produced under the maximum-parsimony analysis on the excluded data set with all parameters estimated where applicable, table 5.6.

Table 5.6. Likelihood score of excluded maximum-parsimony suite of starting trees generated form the excluded data set.

Substitution model	Transformation weightings					
	1:1			2:1		5:1
	Tree 1	Tree 2	Tree 3	Tree 1	Tree 2	Tree 1
JC+G+I	1500.59	1503.75	1502.00	1596.07	1594.92	16.4.64
F81+G+I	1473.46	1476.92	1474.91	1576.75	1575.50	1584.00
K2P+G+I	1463.64	1467.46	1464.57	1563.19	1562.36	1569.54
HKY+G+I	1423.94	1427.54	1424.79	1540.34	1539.45	1545.18
GTR	1513.63	1518.25	1515.83	1515.83	1513.64	1520.96
GTR+I	1417.14	1420.82	1418.47	1418.47	1417.14	1419.33
GTR+G	1417.36	1417.92	1416.16	1416.15	1414.36	1416.79
GTR+G+I	1410.78					

A likelihood ratio test was performed on the tree likelihood scores of maximum-parsimony tree 1 (Figure 5.6) to compare the significance of the difference in tree likelihood scores between the substitution models (see Table 5.7).

Table 5.7: Likelihood ratio test values (bold) and P > 0.001 $\chi 2$ critical values (*italics*) for comparisons of the maximum parsimony tree 1 likelihood score under different substitution models (see Table 5.6 & Figure 5.6). All null models could be rejected.

		***Alternative* model**					
		F81	K2P	HKY85	GTR	GTR + G	GTR+G+I
***Null* model**	JC	**54.26** *16.27*					
	F81		**19.64** *13.82*				
	K2P			**79.4** *16.27*			
	HKY85				**26.3** *18.47*		
	GTR					**96.27** *10.83*	
	GTR + G						**13.6** *10.83*

The most appropriate model chosen was GTR+G+I with a likelihood score of –*ln*L 1410.78, with the variant parameters estimated at I = 0.585112 and Γ = 0.677304 and the estimated substitution rate matrix (Table 5.8).

Table 5.8. DNA substitution rate matrix estimated by likelihood analysis of the best maximum-parsimony tree under the GTR+G+I model. The nucleotide bases are represented by A = adenine, G = guanine, C = cytosine and T = thymine.

	A	C	G	T
A	-	1.85×10^{-07}	5.42	1.67
C		-	6.38×10^{-07}	4.99
G			-	1
T				-

The Model test hLRT was also ran on the excluded data set and similarly selected the GTR+Γ+ I model with the following estimated parameters: I = 0.582452; Γ = 0.582640; substitution rate matrix (Table 5.9).

Table 5.9. DNA substitution rate matrix estimated by likelihood analysis of the neighbour joining tree generated by the ModelT-test software. The nucleotide bases are represented by A = adenine, G = Guanine, C = cytosine and T = thymine.

	A	C	G	T
A	-	4.23×10^{-14}	5.82	1.47
C		-	1.17×10^{-07}	5.60
G			-	1
T				-

The heuristic likelihood search performed on the excluded data set using the parameters estimated from likelihood analysis of the maximum-parsimony tree 1 (Figure 5.5) under the GTR + G + I model of substitution produced a single best tree (Figure 5.6). The maximum-likelihood tree retained the same tree topology as the best maximum-parsimony tree 1 (Figure 5.5).

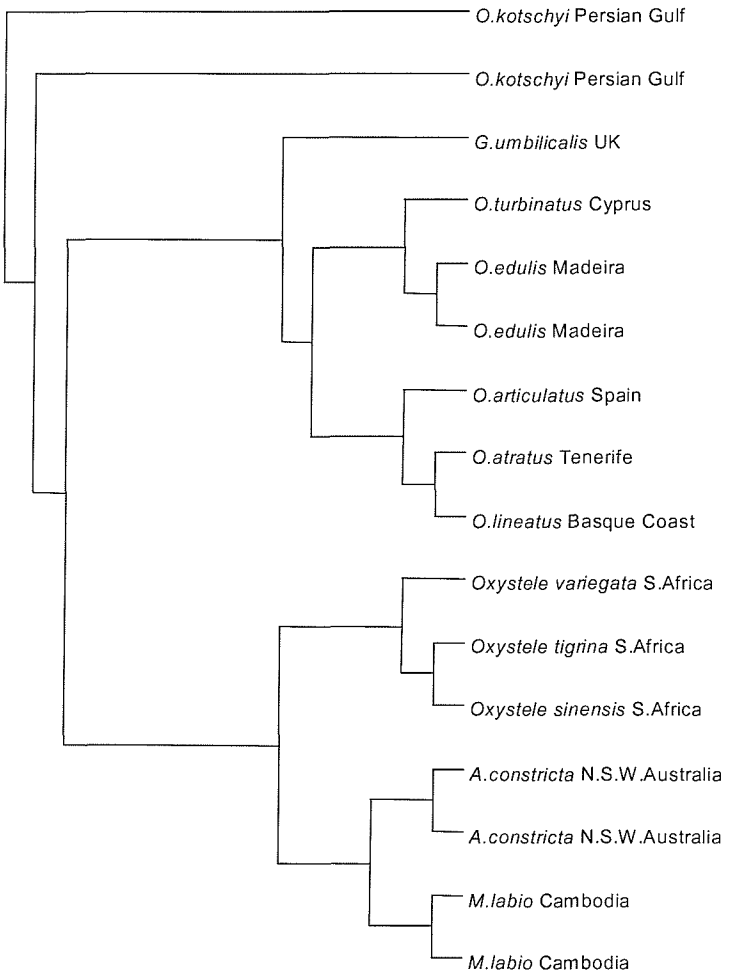


Figure 5.6. The best maximum-likelihood tree produced under heuristic search using parameters estimated from the likelihood tree score of the best maximum-parsimony tree under the GTR+G+I model of substitution.

The likelihood scores of the maximum-likelihood tree estimated under the same GTR models of substitution are listed in Table 5.10. A likelihood ratio test performed on the GTR+G vs. GTR+G+I model established that no simpler models could be accepted.

Table 5.10. Maximum-likelihood tree scores under various GTR DNA substitution models

Model	JC	F81	K80	HKY85	GTR	GTR+G	GTR+I	GTR+G+I
ML tree score	1594.91	1574.49	1562.96	1540.00	1513.64	1414.36	1417.14	1410.78

LRT = 2(GTR+G – GTR+ G+ I)

2(1414.36 – 1410.78) = 7.16

D.F. = 1

P> 0.01 χ^2 critical value = 6.63 - *Null model (GTR+G) rejected*

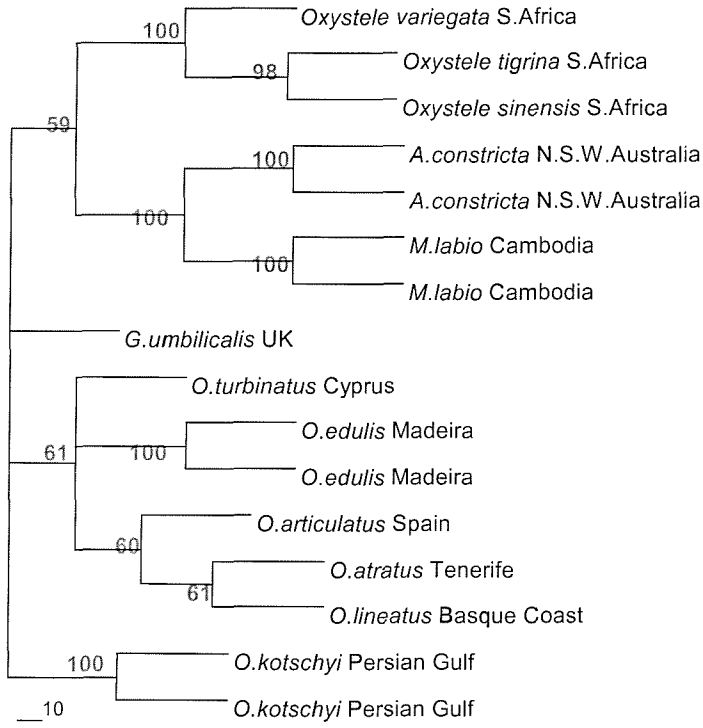
Therefore, the GTR+G+I model was retained as the best fitting model given the data set. Furthermore the parameters estimated in the likelihood analysis under the GTR +G+I model remain unchanged with I = 0.585112; Γ = 0.677304 and the substitution rate matrix (Table 5.11).

Table 5.11. DNA substitution rate matrix estimated by likelihood analysis of the maximum-likelihood tree under the GTR model. The nucleotide bases are represented by A = adenine, G = Guanine, C = cytosine and T = thymine.

	A	C	G	T
A	-	1.85×10^{-07}	5.42943	1.67653
C		-	6.38×10^{-07}	4.995983
G			-	1
T				-

The accepted maximum-likelihood estimate of phylogeny (Figure 5.6) confirms earlier findings under the distance and parsimony analysis. The Atlantic/Mediterranean *Osilinus* clade remains monophyletic with *Gibbula* as the basal sister taxa. The South Atlantic and Indo-Pacific group comprising *Oxystele*, *Monodonta* and *Austrocochlea* also form a monophyletic clade with respect to the remaining taxa. *Osilinus* forms a paraphyletic group inclusive of *Osilinus kotschyi*.

Bootstrap analysis performed with 100 replicates on the excluded data set under maximum-parsimony branch and bound search and a heuristic likelihood search produced the bootstrap 50% majority trees in figure 5.7. The basic tree topology is retained from earlier bootstrap analysis under minimum evolution and maximum-parsimony. The Atlantic/Mediterranean *Osilinus* group remains monophyletic, albeit with consistently low bootstrap support of 58% and 61% for the maximum-parsimony and maximum-likelihood bootstraps respectively. 98% and 100% bootstrap support is conferred on *O. kotschyi* as a distinct clade, basal to all the Mediterranean/North-east Atlantic species, including *Gibbula*. The phylogenetic relationships between *Oxystele*, *Austrocochlea* and *Monodonta* also remain consistent with earlier analysis.



A

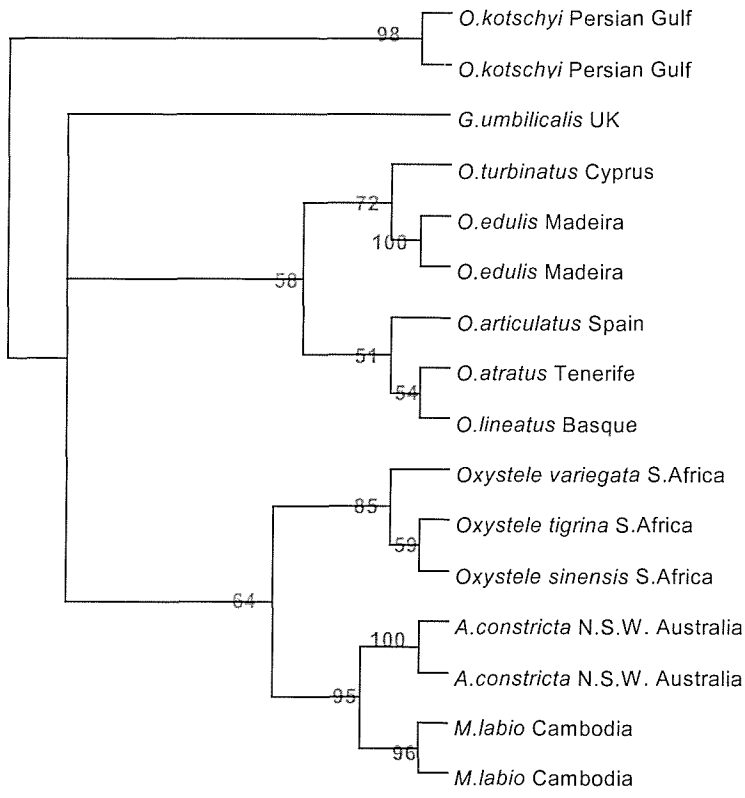


Figure 5.7. 50% majority-rule bootstrap consensus trees (A) Maximum-parsimony branch and bound tree depicting relative branch lengths. (B) Maximum-likelihood tree. Bootstrap support values are placed to the left of nodes.

5.5 Discussion

The purpose of this chapter was two fold: The main aim was to establish appropriate outgroup taxa for subsequent phylogenetic analyses of interspecific relationships within *Osilinus*. Secondly, this chapter aimed to establish if all *Osilinus* species constitute a monophyletic group. This was required to clarify if the re-classification of *O. kotschyi* within this genus was actually a reflection of its sister-taxa status to the Atlantic-Mediterranean *Osilinus* (Herbert, 1994). Both these aims were achieved through the mtDNA 16S phylogenetic analyses of selected gibbuline outgroup taxa.

In all the analyses (minimum-evolution, maximum-parsimony and maximum likelihood) the Atlantic/Mediterranean *Osilinus* form a monophyletic group. The included species of the genus *Oxystele, Austrocochlea* and *Monodonta* genera also form a separate monophyletic group in all analyses. The ingroup and outgroup taxa cannot, however, be drawn as reciprocally monophyletic. This is true when the ingroup is limited to the Atlantic/ Mediterranean *Osilinus* species, and when all *Osilinus* species are included in the ingroup. Reciprocal monophyly is only achieved in the maximum-parsimony branch-and-bound consensus tree (Figure 5.2) when *G. umbilicalis* is included in the ingroup, and *O. kotschyi* is excluded. The Atlantic and Mediterranean *Osilinus* and *O. kotschyi* do not, therefore, constitute a monophyletic group. Based on monophyly, and therefore shared common ancestry as a criterion of taxonomic division (Rosen, 1979), *O. kotschyi* appears misclassified within the genus *Osilinus*.

The position of *O. kotschyi* within the phylogeny is unresolved. The maximum-parsimony analysis (Figure 5.2) places *O. kotschyi* as basal to the outgroup taxa *Oxystele, Austrocochlea* and *Monodonta*. In the subset analysis *O. kotschyi* is basal to all included taxa (Figure 5.4 and 5.5). This is also true of the maximum-likelihood tree (Figure 5.6). In the 50% majority-rule consensus tree, for both the maximum-parsimony and maximum-likelihood analyses (Figure 5.7 and 5.8), there is a lack of resolution regarding the relationship of *O. kotschyi* and *G. umbilicalis* to the Atlantic/ Mediterranean *Osilinus* clade. Phylogenetic analyses, including representatives of all living genera of the Gibbulini, would be necessary to resolve the intergeneric evolutionary relationships, and consequently resolve the correct genus classification of *O. kotschyi*. The phylogenies presented in this chapter, therefore, cannot be used to infer hypotheses of the evolutionary relationships between the outgroup taxa.

This chapter does, however, identify the most appropriate outgroup taxa for resolving interspecific relationships of the Atlantic/ Mediterranean *Osilinus* species. *G.umbilicalis* repeatedly occurs as the sister taxon to the monophyletic Atlantic/ Mediterranean *Osilinus* clade (Figures 5.1, 5.2, 5.3, 5.4, 5.5 & 5.6) albeit with low bootstrap support (Figure 5.7). The European species *Gibbula umbilicalis* is also the most closely related of the included outgroup taxa to the Atlantic–Mediterranean *Osilinus* species according to the mtDNA 16S pairwise distances (Table 5.4). This chapter has therefore provided a basis for selection of an outgroup taxon by which the interspecific *Osilinus* phylogeny can be rooted.

Chapter 6

Phylogenetic analysis of interspecific relationships within the genus *Osilinus* using the mitochondrial DNA genes 16S and Cytochrome Oxidase I

6.1 Introduction

The most important consideration in phylogenetic or population genetic studies is the selection of markers appropriate for the level of taxonomic hierarchy or relatedness under investigation (Simon *et al.,* 1994). The degree of sequence variation and rate of evolution in a chosen marker gene must be suitable for the amount of genetic divergence among the subject taxa, in order to resolve phylogenetic relationships (e.g. Kumazawa & Nishida, 1993; Graybeal 1994). Highly conserved genes or gene fragments (such as the small and large subunit ribosomal RNA genes 18S and 28S respectively) are required for the resolution of evolutionary patterns of taxa related at the level of order and beyond (e.g. Mallatt & Winchell, 2002). Such genes are, however, often uninformative for resolving relationships within a genus (see chapter 5) or within species complexes, because of the absence of sequence variation between relatively recently diverged lineages (Simon, 1991). Conversely, relatively rapidly evolving genes/gene fragments are required for resolving shallow phylogenies. But they are not useful for distantly related taxa, because of the loss of phylogenetic information as the sequence becomes saturated by base changes (Avise, 1989; Hillis & Dixon, 1991; Simon, 1991).

Estimates of sequence divergence rates have been established from known divergence times of taxa for both nuclear genes (Brower & DeSalle, 1994) and mitochondrial genes (Simon *et al.*, 1994; see also Graybeal, 1994). Mitochondrial (mt) DNA is maternally inherited, haploid and does not undergo allelic segregation or recombination. Hence, it has a quarter of the effective population size of nuclear genes and the effects of genetic drift are exaggerated (Birky *et al.,* 1983). Mt DNA also has a higher mutation rate than nuclear DNA (Avise 1994). Because of these factors, mt DNA is a useful tool for resolving relationships of closely related taxa.

The mitochondrial genes 16S and COI were therefore considered suitable for the phylogenetic analysis of interspecific relationships within the genus *Osilinus.* These genes have very comparable divergence rates within recently diverged lineages (Whitfield & Cameron, 1998) of approximately 2% per million years (My), if a clock-like rate of evolution is assumed (see Klicka & Zink, 1997). This is reflected in the scientific literature: many phylogenetic studies utilise both genes in sequence analysis of the same set of samples (e.g. Anderson, 2000). Although mt DNA is effectively a single locus (Buonaccorsi *et al.,* 2001), the 16S and COI genes can offer more phylogenetic information when analysed both separately (Miyamoto & Fitch, 1995) and as a combined data set (see Kluge, 1989).

Variation within the mitochondrial 16S rRNA gene has been widely used to resolve intraspecific and interspecific relationships of many marine organisms. These include: marine bacteria (Naganuma *et al.,* 1997; Rappe *et al.,* 1998; Mullins *et al.,* 1995; Methe, 1998; Holmes *et al.,* 1997), phytoplankton (Paul *et al.,* 1999), Hydrozoa (Scheirwater 2000), Copepoda (Bucklin *et al.,* 1992, Miller *et al.,* 1999), deep-sea amphipods (France & Kocher, 1996), clawed lobster (Tam & Kornfield 1998), the decapod shrimp species *Penaeus* (Gutierrez-Millan *et al.,* 2001), the deep-sea bivalve *Deminucula atacellana* (Chase *et al.,* 1998), species of the octopodid genus *Pareledone* (Allcock & Piertney, 2002), holothurian larvae (Medeiros-Bergen, 1995), Brachiopoda (Luter & Cohen, 2002) and fish including the N. Atlantic redfish *Sebastes sp.* (*S.marinus, S.mentella, S.fasciotus* and *S.viviparous:* Sundt & Johansen 1998) and red mullet *Mullus* sp. (Mamuris *et al.,* 2001). Within Gastropoda, extreme genetic divergence was exhibited within the 16S mitochondrial gene between bathymetrically separated populations of the deep-sea species *Frigidoalvania brychia* (Quattro *et al.,* 2001). This large rRNA subunit gene also revealed complex speciation patterns within the land snail genus *Albinaria* (Douris *et al.,* 1998).

There is no absolute rule, however, regarding the taxonomic rank at which a certain gene is useful (Simon *et al.,* 1994). The 16S rRNA gene has also been used as an appropriate marker for the resolution of higher phylogenetic relationships. 16S has been successfully used to resolve evolutionary relationships between the genera *Solea, Microchirus, Monochirus* and *Buglossidium* of the Atlantic-Mediterranean Soles tribe (Tinti *et al.,* 2000) and in the wrass tribe Labrini (Hanel *et al.*, 2002). Resolution of generic relationships at the family level has also been achieved using the 16S gene within the mud crabs (Panopeidae; Schubart *et al.,* 2000) and Chilean limpets (Lottiidae; Espoz & Castilla, 2000).

As for 16S, the mitochondrial COI gene has been widely applied to investigate macroevolution (e.g. interspecific phylogenies) and microevolution (e.g. population structure) patterns and processes within marine taxa, (see Table 6.1). As a coding region, the COI gene can also provide phylogenetic information at different hierarchical levels by examining different codon positions (Folmer *et al.,* 1994).

Table 6.1. A summary of the applications of the COI gene to macroevolution and microevolution patterns and processes within marine taxa.

Taxa	Application	Author
Macroevolution		
Halichondrid sponges	Host and symbiont phylogenies	Erpenbeck *et al.*, (2002)
Polychaete *Streblospio*	Interspecific developmental patterns	Schulze *et al.*, (2000)
Atlantic copepod *Calanus*	Species identification	Hill *et al.*, (2001)
Hawaiian isopod family Philosciidae	Speciation patterns	Rivera *et al.*, (2002)
Shrimp genus *Penaeus*	Phylogeny reconstruction	Baldwin (1998)
Mole crab genus *Emerita*	Genetic divergence	Tam *et al.*, (1998)
Abalone genus *Haliotis*	Species identification	Elliott *et al.*, (2002)
Portuguese Oyster *Crassostrea angulata*	Investigation of evolutionary origin	Foighil *et al.*, (1998)
North Atlantic gastropod *Crepidula*	Phylogeny reconstruction and population structure	Collin, (2001)
Sea star genus *Coscinasterias*	Global phylogeny reconstruction	Waters & Roy (2003)
Sea star subgenus *Hexasterias*	Resolution of the species rich phylogeny	Hrincevich *et al.*, (2000)
Starfish genus *Linckia*	Identifying species boundaries	Williams (2000)
Sea urchin genus *Diadema*	Global speciation patterns	Lessios *et al.*, (2001)
Sea urchin genus *Echinometra*	Speciation patterns	McCartney (2000)
Gastropod genus *Tegula*	Speciation patterns	Hellberg (1998)
Transequatorial fish subgenus *Gonustius*	Phylogeny reconstruction	Burridge & White (2000)
Microevolution		
Various red algae species	Intraspecific relationships	Zuccarello *et al.*, (1999)
Shrimp species *Haptosquilla pulchella* and *H.glyptocercus*	Population recovery after the Krakatau eruption	Barber *et al.*, (2002)
N. Atlantic horseshoe crab *Limulus polyphemus*	Genetic population structure	Pierce *et al.*, (2000)
Chinese mitten crab *Eriocheir sinensis*	Invasion pathways	Hanfling *et al.*, (2002)
Indian Ocean mud crab *Scylla serrata*	Intraspecific variation	Fratini & Vannini (2002)
Marine bivalve *Lasaea*	Genetic diversity of lineages	Park & Foighil (2000)
Hydrobia ulvae and *H.ventrosa* (Gastropoda)	Biogeographical patterns of intraspecific diversity	Wilke & Davis (2000)
Sea urchin species *Stronglyocentrotus purpuratus*	Recruitment	Flowers *et al.*, (2002)

This chapter is concerned with the 16S, COI and combined 16S and COI phylogenies of the *Osilinus* species *O. edulis, O. lineatus, O. atratus, O. articulatus* and *O. turbinatus*. The 16S mtDNA phylogeny of all species is estimated using genetic distance (UPGMA) analysis, maximum-parsimony and maximum-likelihood analyses. In addition the phylogeography of *O. edulis, O. lineatus* and *O. atratus* is examined using haplotype frequency combined with current biogeographical distribution of populations. Neighbour-joining analysis, maximum-parsimony analysis and maximum-likelihood analysis are also employed to estimate the COI and combined 16S and COI phylogenies of the North Atlantic and Mediterranean *Osilinus* species *Osilinus edulis, O. lineatus, O. atratus, O. articulatus* and, *O. turbinatus* (excluding the Indo-Pacific species *Osilinus kotschyi*). The gibbuline trochid *Gibbula umbilicalis* is used as a suitable outgroup species in all the phylogenetic analyses, (see chapter 5). *O. kotschyi* has been included as an outgroup for the 16S mtDNA sequence analyses.

Specifically, I aim firstly to establish whether the species designations within *Osilinus* assumed in the analysis reflect biological species and are monophyletic groups (clades), or if the current taxonomic species classifications conceal indications of sibling species or species complexes. Both *O. edulis* and *O. atratus* have been proposed as species complexes encompassing potential sibling species (Gosling, 1994), as discussed in Chapter 1. Secondly, I aim to investigate if the evolutionary pattern of the 16S, COI and combined mtDNA phylogenies provides useful phylogeographic information that can be used to hypothesise about the processes driving the speciation of *Osilinus*.

6.2 Materials and Methods

6.2.1 Sampling of taxa

The *Osilinus* and *Gibbula* specimens used for the molecular work described in this chapter were collected from sampling locations along the northeast European and African continental coast and within the Mediterranean, on the Atlantic Islands and in the Indian Ocean (Persian Gulf). The collection sites were chosen to represent the known distribution of each speces (as discussed in chapter 3). The number of samples successfully sequenced for each geographic location for all the species are given in Table 6.2. The map references in this table refer to Figures 3.2 and 3.3 in Chapter 3. Detailed description of fieldwork and sampling design is also given in Chapter 3. All individuals used in the analysis were collected specifically for this project by the author and collaborators unless otherwise indicated. Once collected the snails were placed in flowing sea water for 5 days to purge gut contents, then relaxed in 8% $MgCl_2$ solution and preserved in 95% ethanol as described (see Chapter 4). Seven species are represented in the analysis: *Gibbula umbilicalis, Osilinus kotschyi, Osilinus lineatus, Osilinus atratus, Osilinus articulatus, Osilinus edulis* and *Osilinus turbinatus*.

Table 6.2. Sample locations and sizes for the Gibbulini species *Gibbula umbilicalis, Osilinus lineatus, Osilinus atratus, Osilinus articulatus, Osilinus edulis* and *Osilinus turbinatus.*

Map Ref.	Location	Latitude	Longitude	*O.kot*	*G.um*	*O.lin*	*O.atr*	*O.art*	*O.edu*	*O.tur*
1	UK - Lyme Regis	N50°43.267'	W002°56.113'		[2]	2 [2]				
2	UK - Porthguerra	50°19.223'	W005°13.503'		[5]	2 [2]				
3	Basque Coastline	N43°17.28'	W002°58.08'		4 [7]	3 [6]			1 [3]	
4	Spain - Torre Guadiaro, Cadiz	N36°14.40'	W005°17.28'					[4]		[5]
5	Spain - Calahonda, Malaga	N36°29.06'	W004°44.08'					2 [2]		2 [4]
6	Spain - Ponta Sol, Malaga	N36°23.13'	W005°12.14'							
7	Spain - Puerto duguesa, Malaga	N36°21'	W005°14'						1	2
8	Cyprus - Coral Bay	N34°46.57'	W032°24.20'							2
9	Greece – Corinth	N37°56.33'	W022°57.13'							1 [5]
10	Morocco - Anza	N30°26.767'	W009°39.663'			[4]			[5]	
11	Morocco - Tifnit	N30°11.619'	W009°38.357'			2 [6]			3 [1]	
12	Morocco - mourane	N30°30.374	W009°41.238'			2 [6]			3 [3]	
13	Morocco - Cap Ghir	N30°38.676'	W009°53.357'			2 [6]			2 [1]	
14	Tenerife - Las Galletas	N28°06.366'	W016°37.302'				3 [5]		2 [4]	
15	Tenerife - Los Gigantes	N28°13.444'	W016°50.135'						3 [4]	
16	Tenerife - La Rambla	N28°25.589'	W016°30.071'						2 [4]	
17	Tenerife - El Medano	N28°01.512'	W016'32.126'						2 [2]	
18	Tenerife - Santa Cruz	N28°26.239'	W016°09.102'						[1]	
19	Fuerteventura - Coleta del Cotillo	N28°40.264'	W014°10.462'				1 [2]		[1]	
20	Fuerteventura - Punta de la Caletones	N28°14.628'	W014°12.735'						[1]	
21	Fuerteventura - nr Punta Paloma	N28°10.471'	W014°11.536'				[2]			
22	Fuerteventura - nr Roja Mountain	N28°39.441'	W013°49.888'				1 [2]		2	
23	Fuerteventura - Castillo Beach	N28°13.256'	W013°46.724'				[2]		[2]	
24	Fuerteventura - Playa de Ugan	N28°16.243'	W014°13.333'						2	
25	Lanzarote - La Santa	N29°06.977'	W013°39.573'				[2]		[2]	
26	Lanzarote - Costa Teguise	N28°58.927'	W013°30.879'				2 [2]		[2]	
27	Lanzarote - Jameous de Agua	N29°09.485'	W013°25.876'				1 [2]		2 [2]	
28	Lanzarote - La Golfa	N28°58.950'	W013°49.901'				2 [1]		1 [2]	
29	Gran Canaria - Cuintanella, San Andres	N28°08.372'	W015°34.077'				1		[1]	
30	Gran Canaria - Playa de Balito	N27°44.245'	W015°40.426'						2 [1]	
31	Gran Canaria - Playa del Burrero	N27°57.028'	W015°19.372'				[2]		2 [2]	
32	Gran Canaria - Playa de la Aldea	N27°58.311'	W015°50.053'						[2]	
33	Gran Canaria - San Cristobal	N28°10.213'	W015°46.298'				[2]		1 [2]	
34	Gran Canaria - Playa de Sardina	N28°04.587'	W015°25.264'						[1]	

Map Ref.	Location	Latitude	Longitude	*O.kot*	*G.um*	*O.lin*	*O.atr*	*O.art*	*O.edu*	*O.tur*
35	Madeira - Prainha Beach	N32°44.28'	W016°37.18'						1 [1]	
36	Madeira - Ponta do Sol	N32°40.07'	W017°05.06'						1 [2]	
37	Madeira - Sao Vincente	N32°48.45'	W017°00.44'						1 [1]	
38	Madeira - Seixal	N32°49.35'	W017°07.33'						1 [1]	
39	Portugal - Viana do Castelo	N41°41.25'	W008°50.14			2				
40	Portugal - Povoa de Varzim	N41°22.41'	W008°45.31'		1	2				
41	Portugal - Figueira da Foz	N40°08.55'	W008°50.54'			2			1	
42	Portugal - Ericeira	N38°57.59'	W009°25.07'			2				
43	Portugal - Sesimbra	N38°26.48'	W009°04.16'		1	2				
44	Portugal - Porto Cova	N37°50.08'	W008°48.53'			2			3	
45	Portugal - Sagres	N37°01.14'	W008°56.58'			1			1	
46	Portugal - Burgua	N37°04.08'	W008°46.12'			2				
47	UAE - Khor Al Hamra	N25°40.52'	E055°47.03'	[4]						
	TOTAL			4	9	30	11	2	40	7

The numbers are the sample sizes used in the COI sequence analysis, and in parenthesis the sample sizes used in the 16S sequence analysis. *G.um* = *Gibbula umbilicalis*, *O.kot* = *Osilinus kotschyi*, *O.lin* = *Osilinus lineatus*, *O.atr* = *Osilinus atratus*, *O.arc* = *Osilinus articulatus*, *O.edu* = *Osilinus edulis*, and *O.tur* = *Osilinus turbinatus*.

6.2.2 *Molecular methods*

All individuals used in the analysis were removed from their shells. The soft tissue was preserved in 95% ethanol at –20°C and the corresponding shells were individually marked. Small sections (2-5 mg) of foot tissue were removed from each individual for DNA extraction and sequence analysis. The DNA was extracted using a modified CTAB protocol (Hillis & Moritz *et al.,* 1996; Marko, 1998; details in Chapter 4). A 479 base pair (bp) fragment of the 16S gene was amplified by PCR for 151 individuals from 35 locations (Table 6.2) using the universal 16S primers 16SAR and 16SBR (Palumbi, 1996). A 534 bp fragment of the COI gene was amplified by PCR for 100 individuals from 47 locations (Table 6.2) using the universal COI primers LCOI490 and HCO2198 developed by Folmer *et al.* (1994). The fluorescence-based dideoxynucleotide thermal cycle DNA sequencing method was used to produce DNA sequence fragments from the PCR products that were visualised on an ABI377 automated sequencer (Applied Biosystems, Warrington, Cheshire). Detailed sequencing methodology is given in Chapter 4.

6.2.3 *Sequence editing and alignment*

GenBank Blastn searches were run for representative 16S and COI *Osilinus* and *Gibbula* sequences (Altschul *et al.,* 1990; Madden *et al.,* 1996). Firstly this confirmed that the correct gene fragment had been sequenced and that no contamination had taken place. It also identified possible outgroup taxon sequences.

The ABI sequence files were viewed and edited using the software Chromas (version 2.23; Copyright © 2002 Technelysium Pty Ltd http://www.technelysium.com.au/). To ensure an accurate sequence read, both forward and reverse strands were sequenced. Between two and five individuals were sequenced for every location represented in the analysis to ensure no sample misidentification had occurred or sample mix up taken place during the molecular protocols. Edited chromas sequences were exported from Chromas as FASTA format files. Various text files were subsequently created from the sequences exported from Chromas as input files for the alignment software program ClustalX (Thompson *et al.*, 1997). Sequences were initially aligned using ClustalX with a gap-opening penalty of 50 (1-100) and gap extension penalty of 5 (1-100) enforced on both pairwise and multiple alignment parameters. The alignment was then checked by eye and ambiguous sequences or sequence residue regions were realigned using an appropriate gap-opening penalty (for example, for a region of excessive transformations to explain the alignment, a lower gap penalty of 5 was enforced). The final alignment files were exported as NEXUS format files for input into the phylogenetic software package PAUP* (version 4.0b10; Swofford 1999). The COI, 16S and combined alignments used in this analysis are given in Appendices D, E and F respectively. The alignment files were examined and identical sequences removed to create a data set of consensus sequences for use in phylogenetic analysis.

6.2.4 *Sequence saturation*

Saturation curves are important in determining the phylogenetic usefulness of sequence variation within a gene. For any given observed divergence levels (sequence variation), genes in which only a few sites are 'free to vary' (sensu Palumbi 1989) will be more saturated with nucleotide substitutions (i.e. contain more superimposed changes) than sequences in which more sites are freely variable. A saturated sequence-divergence accumulation curve is strongly convex near the origin, reaching a rapid low-level plateau as additional substitutions are superimposed and therefore unobserved. In contrast, recently diverged sequences or genuinely highly conserved sequences exhibit a linear divergence accumulation curve.

Saturation curves were calculated from observed and expected genetic distances. Observed distances are calculated and plotted against the inferred number of differences. The inferred number of differences is calculated form the genetic distances values calculated under an imposed model of evolution (e.g.: K2P in this analysis). The observed distances are derived from the absolute number of differences divided by the base-pair length of the gene fragment (number of characters). If the observed versus inferred number of differences is greater than 0.25 then the sequence is probably saturated and therefore not phylogenetically informative. It can also result in the underestimation of divergence (Suzanne Williams *pers. comm.*).

6.2.5 *Phylogenetic analysis*

Neighbour-joining analysis was performed on the selected 16S sequences and repeated as a bootstrap analysis with 1000 replications. Maximum-parsimony analysis was performed on 16S sequences under various character types and weightings. Subsequently a smaller sequence data set was chosen to enable computationally feasible analysis to be performed: 12 representative sequences were selected on the basis of the haplotype frequency and geographical distribution for a maximum-parsimony branch-and-bound search. Sequences of the most common and rare haplotypes of each Atlantic/Mediterranean *Osilinus* species plus one sequence each of *Gibbula umbilicalis* and *O.kotschyi* were included.

The neighbour-joining (NJ) tree was subsequently used as a starting tree for computation of the likelihood tree scores under 56 models of DNA substitution. The tree likelihood scores of the NJ tree were compared for goodness of fit using the hierarchical likelihood ratio test (hLRT) statistics to establish the best model and parameters for application in subsequent maximum-likelihood analysis (Posada & Crandall, 1998). The chosen substitution model and estimated parameters were subsequently employed in a heuristic likelihood search of all 16S sequences (96 in total). The tree likelihood score of the best maximum-likelihood tree was re-estimated using all DNA substitution models estimated on the NJ tree to establish if any simpler models could be accepted, further models rejected or the parameters improved. A molecular clock was enforced on the best maximum-likelihood trees under the chosen model of substitution and the tree likelihood score re-estimated to establish if the data behaved in a clock-like manner. Finally, a heuristic maximum-

likelihood bootstrap search (100 replicates with replacement) was performed on the 12 sequences chosen for the maximum-parsimony subset analysis.

Neighbour-joining analysis was performed on the selected COI sequences and repeated as a bootstrap analysis with 1000 replications. A heuristic maximum-parsimony search was also performed on the COI sequences with *Gibbula umbilicalis* designated as the outgroup. This maximum-parsimony heuristic search was repeated as a bootstrap analysis with 100 replications. A subset of sequences was decided upon representing all locations allowing a more computationally intensive maximum-parsimony analysis (under various character weighting schemes). This procedure produced a suite of starting trees for likelihood ratio test analysis. The likelihood score of all the starting suite trees was estimated by 56 models of DNA substitution and the best model of the data determined using the likelihood ratio test (Posada & Crandall, 1998). The best model of DNA substitution was then applied in a maximum-likelihood search of the entire data set and repeated as a bootstrap analysis with 100 replications.

The 16S and COI sequences of 24 samples used in the previous analyses were combined to create a 999bp mt DNA gene fragment for phylogenetic analysis. Neighbour-joining analysis was performed and repeated as a bootstrap analysis (100 replicates) subject to two distance models. A maximum-parsimony branch-and-bound search was also performed and repeated as a bootstrap analysis (100 replicates). The Modeltest software (Posada & Crandall, 1998) was used to determine the best model of DNA substitution to employ in a maximum-likelihood search, which was subsequently assumed in a heuristic search. The maximum-likelihood heuristic search was repeated with a molecular clock enforced and the tree likelihood scores compared to establish if the combined data set behaved in a clock-like manner. Finally a maximum-likelihood bootstrap analysis was performed (100 replicates) assuming the best substitution model and parameters.

6.2.6 Haplotype distribution and abundance

The taxonomic status of both *Osilinus edulis* and *O. atratus* is controversial (see Chapter 1). The mtDNA 16S sequences for *O. edulis, O. atratus* and *O. lineatus* were therefore examined and all unique intraspecific haplotypes identified. The number and presence of each unique haplotype was subsequently plotted against geographic location to identify potential phylogeographic patterns of haplotype abundance and distribution.

6.3 Results

6.3.1 Consensus sequences

Of the 151 16S partial gene sequences, 96 were unique. The remaining 55 were identical to one or more 16S sequences (Table 6.3).

Table 6.3. Consensus mtDNA 16S *Osilinus* sequences (the map references refer to Figures 3.2 and 3.3 in Chapter 3).

Consensus sequence			Identical sequences		
Species	Location	Map Ref.	Species	Location	Map Ref.
008 *O. lineatus*	Morocco - Anza		050 *O. lineatus*	Cap Ghir, Morocco	
038 *O. lineatus*	Morocco - Tifnit	11	030 *O. lineatus*	Morocco - Imourane	12
			007 *O. lineatus*	Morocco – Anza	10
			235/250/251 *O. lineatus*	Basque coastline	3
			140 *O. lineatus*	UK - Porthguerra, Cornwall	2
			132/133 *O. lineatus*	UK - Lyme Regis, Dorset	1
069 *O. atratus*	Tenerife - Las Galletas	14	259/260 *O. atratus*	Lanzarote - Jameous de Agua	
			299 *O. atratus*	Fuerteventura - Punta Paloma	21
			321 *O. atratus*	Fuerteventura - Coleta de Cotilla	19
			308 *O. atratus*	Fuerteventura - Castillo Beach	23
			287/291 *O. atratus*	Lanzarote - La Santa	
			332 *O. atratus*	Lanzarote - La Golfa	
			279 *O. atratus*	Lanzarote - Costa Teguise	
			323 *O. atratus*	Fuerteventura - Coleta de Cotilla	19
			365 *O. atratus*	Gran Canaria - San Cristoban	33
106 *O. articulatus*	Spain - Torre Guadiaro, Cadiz	4	110 *O. articulatus*	Spain - Torre Guadiaro, Cadiz	4
225 *O. edulis*	Basque Coastline	3	041 *O. edulis*	Morocco – Tifnit	11
044 *O. edulis*	Morocco - Tifnit	11	081 *O. edulis*	Tenerife - Santa Cruz	18
282 *O. edulis*	Lanzarote - Costa Teguise		035 *O. edulis*	Morocco – Imourane	12
310 *O. edulis*	Fuerteventura - nr Punta de la Caletones	20	351 *O. edulis*	Gran Canaria - Playa de Balito	30
346 *O. edulis*	Gran Canaria - San Andres	29	053 *O. edulis*	Morocco - Cap Ghir	13
			031 *O. edulis*	Morocco – Imourane	12
			263 *O. edulis*	Lanzarote - Jameous de Agua	
			354 *O. edulis*	Gran Canaria - Playa de Balito	30
			317 *O. edulis*	Fuerteventura - Coleta de Cotilla	19
			309 *O. edulis*	Fuerteventura - Punta de la Caletones	20
060 *O. edulis*	Fuerteventura - nr Roja Mountain	22	272 *O. edulis*	Tenerife - El Medano	17
			072 *O. edulis*	Tenerife - Las Galletas	14
			264 *O. edulis*	Lanzarote - Jameous de Agua	
			355 *O. edulis*	Gran Canaria - Playa del Burrero	31
			380 *O. edulis*	Gran Canaria – Sardina	34
196 *O. edulis*	Madeira - Ponta do Sol	36	192 *O. edulis*	Madeira - Prainha Beach	35
			184 *O. edulis*	Madeira - Sao Vincent	37
			181 *O. edulis*	Madeira – Seixal	38
			054 *O. edulis*	Morocco - Cap Ghir	13
018 *O. edulis*	Morocco – Anza	10	032 *O. edulis*	Morocco – Imourane	12
325 *O. edulis*	Lanzarote - La Golfa		043 *O. edulis*	Morocco – Tifnit	11
			294 *O. edulis*	Lanzarote - La Santa	
077 *O. edulis*	Tenerife - Los Gigantes	15	059 *O. edulis*	Tenerife - El Medano	17
096 *O. turbinatus*	Spain - Torre Guadiaro, Cadiz	4	097/099/100/107	Spain - Torre Guadiaro, Cadiz	4
			101/102/105	Spain - Puerto duguesa, Malaga	7
			112	Spain - Calahonda, Malaga	5
131 *G. umbilicalis*	UK - Porthguerra	2	145 *G. umbilicalis*	UK - Lyme Regis	1
			338 *G. umbilicalis*	UK – Lyme Regis	28
			374 *G. umbilicalis*	UK - Porthguerra	33
			245/406 *G. umbilicalis*	Basque coastline	3
			128/129 *G. umbilicalis*	UK - Porthguerra	2

The species name and sampling location of all identical sequences are listed next to the arbitrarily chosen identical consensus sequence used as a representative in the molecular analysis. The numbers refer to the DNA sequence identification.

Of the 100 COI sequences 74 were unique, the remaining 24 were identical to one or more COI sequences (Table 6.4).

Table 6.4. Consensus COI *Osilinus* sequences.

Consensus Sequence		Map	Identical sequences		Map
ID/Species	Location	Ref.	ID/Species	Location	Ref.
053 *Osilinus edulis*	Morocco - Cap Ghir	13	059 *O. edulis*	Tenerife - El Medano	17
			096 *O. edulis*	Spain - Puerto Duguesa, Malaga	7
			071 *O. edulis*	Tenerife - Las Galletas	14
			022 *O. edulis*	Morocco – Imourane	12
			041 *O. edulis*	Morocco – Tifnit	11
			072 *O. edulis*	Tenerife - Las Galletas	14
042 *Osilinus edulis*	Morocco - Tifnit	11	465 *O. edulis*	Portugal - Sesimbra	43
064 *Osilinus edulis*	Tenerife - La Rambla	16	080 *O. edulis*	Tenerife - Los Gigantes	15
351 Osilinus edulis	Gran Canaria - Playa de Balito	30	021 *O. edulis*	Morocco – Imourane	12
114 *Osilinus turbinatus*	Spain - Calahonda, Malaga	5	103 *O. turbinatus*	Spain - Calahonda, Malaga	5
028 *Osilinus lineatus*	Morocco - Imourane	12	047 *O. lineatus*	Morocco - Cap Ghir	13
			048 *O. lineatus*	Morocco - Cap Ghir	13
469 *Osilinus lineatus*	Portugal – Porto Cova	44	422 *O. lineatus*	Portugal – Viana do Castelo	39
			423 *O. lineatus*	Portugal – Viana do Castelo	39
			430 *O. lineatus*	Portugal – Ericeira	42
			439 *O. lineatus*	Portugal – Ericeira	42
			446 *O. lineatus*	Portugal – Figueira da Foz	41
			480 *O. lineatus*	Portugal - Sagres	45
490 *Osilinus lineatus*	Portugal – Playa Burgua	46	037 *O. lineatus*	Morocco – Tifnit	11
			459 *O. lineatus*	Portugal - Sesimbra	43
036 *Osilinus lineatus*	Tifnit, Morocco	11	479 *O. lineatus*	Portugal - Sagres	45
277 *Osilinus atratus*	Lanzarote – Costa Teguise	26	268 *O. atratus*	Fuerteventura – nr Roja mountain	22
			332 *O. atratus*	Lanzarote – La Golfa	28
			345 *O. atratus*	Gran Canaria – San Andres	29

The species name and sampling location of all identical sequences are listed next to the arbitrarily chosen identical consensus sequence used as a representative in the molecular analysis.

6.3.2 Homologous sequences in GenBank

Representative mtDNA COI and 16S *Osilinus lineatus* sequences were submitted in a GenBank nucleotide Blast*n* search. The alignment scores are defined by the similarity of the Genbank sequence to the query sequence. The COI Blastn search produced 42 homologous COI sequences with a raw alignment score of >200. The 16S *O. lineatus* sequence Blastn search found three homologous 16S sequences of score >200. Of the 15 COI GenBank alignment sequences with the highest scores, 12 corresponded to the suborder Littorinimorpha, eight of which were from the superfamily Rissooidea. The highest scoring homologue sequence was of *Pyrgulopsis turbatrix*, a caenogastropod of the Rissooidea. The remaining three homologues were Trochidae COI sequences, representing two *Tegula* spp. and *Norrisia norrisi*. This is a reflection of the limited number of COI Trochidae sequences submitted to GenBank, complete in the three listed in the blast*n* alignment results. In contrast all three high scoring 16S homologous sequences belonged to species from the Trochidae: *Pseudotalopia sakurai*, *Gibbula cineraria* and *Calliotropis* sp., sharing 87-96% identity with the *O. lineatus* sequence. The remainder of the alignments had an 80-200 raw alignment score. These results confirm that the correct species and gene fragments had been sequenced.

6.3.3 Pairwise distance matrix

The average pairwise p-distance matrix for all 16S (96) and COI (74) sequences is given in Tables 6.5 and 6.6 respectively.

Table 6.5. The average pairwise p-distances matrix for all 95 16S sequences used in this analysis, (sample sizes in parenthesis).

	O.lin	*O. atratus*	*O.artic*	*O.ed*	*O.turb*	*O.kots*	*G.umb*
O. lineatus (20)	0.008						
O. atratus (12)	0.077	0.029					
O. articulatus (4)	0.093	0.064	0.005				
O. edulis (45)	0.119	0.101	0.125	0.009			
O. turbinatus (2)	0.111	0.070	0.093	0.085	0.002		
O.kotschyi (4)	0.193	0.160	0.161	0.180	0.183	0.008	
G. umbilicalis (9)	0.157	0.132	0.133	0.156	0.150	0.178	0.001

Table 6.6. The average pairwise p-distances matrix for all 76 COI sequences used in this analysis, (sample sizes in parenthesis).

	O.lin	*O. atratus*	*O.artic*	*O.ed*	*O.turb*	*G. umb*
O. lineatus (19)	0.014					
O. atratus (8)	0.109	0.006				
O. articulatus (2)	0.143	0.145	0.002			
O. edulis (31)	0.123	0.128	0.134	0.009		
O. turbinatus (2)	0.121	0.133	0.138	0.109	0.013	
G. umbilicalis (9)	0.163	0.176	0.181	0.171	0.154	0.006

O.lin = Osilinus lineatus, O.artic = Osilinus articulatus, O.ed = Osilinus edulis, O.turb = Osilinus turbinatus, O.kots = Osilinus kotschyi, G.umb = Gibbula umbilicalis

The pairwise distance between *O.kotschyi* and the Atlantic/Mediterranean *Osilinus* species is greater than all comparable 16S pairwise distances between *G. umbilicalis* and the Atlantic/Mediterranean *Osilinus* species. The distance between *Gibbula umbilicalis* and all Atlantic/Mediterranean *Osilinus* species exceeds that of all inter-*Osilinus* comparisons for both 16S and COI pairwise matrixes. Considering all pairwise comparisons, the interspecific variation exceeds the intraspecific variation, for most by an order of magnitude.

The greatest COI inter-*Osilinus* pairwise distance was between *O. articulatus* and *O. atratus* (0.145) reflected in the rest of the *O. articulatus* comparisons. These exceed all other inter-*Osilinus* pairwise distances. This suggests that according to the COI phylogeny, *O. articulatus* is basal to the monophyletic Atlantic/Mediterranean *Osilinus* clade. Excluding *O.kotschyi* comparisons, *O. articulatus* and *O. edulis* exhibited the greatest 16S inter-*Osilinus* pairwise distance (0.125). This was not, however, reflected in the remainder of the *O. articulatus* 16S comparisons.

The greatest 16S intraspecific variation was exhibited by *Osilinus atratus* (0.029), the least intraspecific variation by *G. umbilicalis* (0.001). Of the *Osilinus* spp., *O. turbinatus* showed the lowest *Osilinus* intraspecific variation (0.002) followed by *O. articulatus* (0.005), *O.kotschyi, O. lineatus* and *O. edulis* all exhibited comparable intraspecific variation of 0.008, 0.008 and 0.009

respectively. An eight base pair deletion was observed in all 12 *O. atratus* 16S sequences (positions 208-221) relative to all others. A three bp deletion was also observed in all nine *G. umbilicalis* 16S sequences (positions 323-325). A 14bp insertion was observed in all four *O.kotschyi* sequences (positions 225-232) relative to all other sequences.

O. lineatus (0.014) exhibited the greatest COI intraspecific variation, closely followed by *O. turbinatus* (0.013). Lower genetic variation was observed in *O. edulis* (0.009). The *O. atratus* and *G. umbilicalis* sequences shared average COI intraspecific pairwise distances (0.006); *O. articulatus* exhibited the lowest COI intraspecific variation (0.002) most likely a reflection of the very small sample size (2) from a single location (Ponta do Sol, Malaga, Spain).

6.3.4 Sequence saturation curve

The observed distance was plotted against the inferred distance (calculated under the Kimura's 2-Parameter model of DNA substitution; Kimura, 1980) to produce 16S and COI mtDNA saturation curves. These determined whether or not the gene fragments were saturated with substitutions, and therefore phylogenetically useful for resolving interspecific relationships within *Osilinus* (Figures 6.1 and 6.2). Both the 16S and COI saturation curves exhibit a positively correlated linear relationship between the observed and inferred sequence distances. All pairwise distances are less than 0.25, demonstrating that neither gene fragment is saturated with base pair substitutions (Williams, *pers comm.*). They are therefore suitable for use in phylogenetic analysis at this level of taxonomic relatedness. Both saturation curves have an obvious break in the slope at the base of the saturation curve. No sequences were observed or inferred to have a distance of 0.03 – 0.06, or 0.28 – 0.1 in all 16S and COI pairwise comparisons respectively. This gap represents the difference in intraspecific and interspecific pairwise comparisons. The data points in the lower portion of the curve (16S, 0-0.03; COI 0-0.28) represent exclusively intraspecific pairwise distances. Those greater than 0.06 and 0.1 (16S and COI respectively) in the upper portion of the curve correspond exclusively to interspecific pairwise distances.

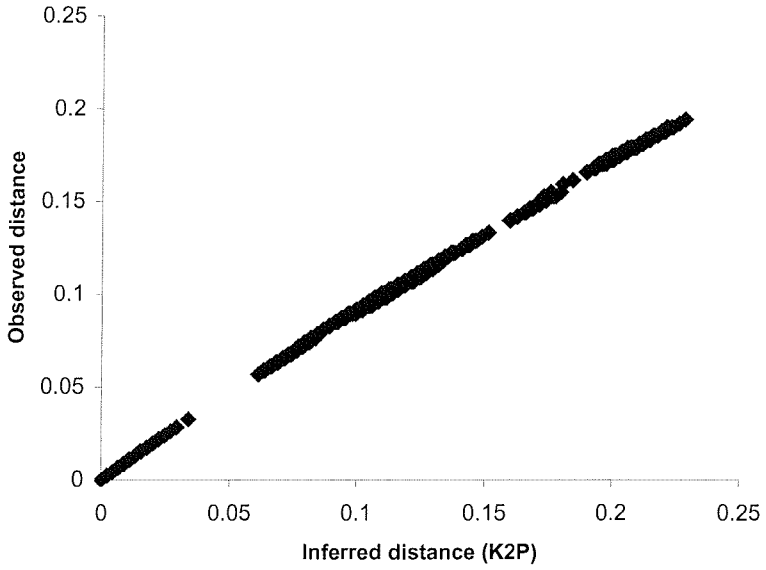


Figure 6.1. Saturation Curve for the 16S partial gene sequenced for *Osilinus* and *Gibbula* species

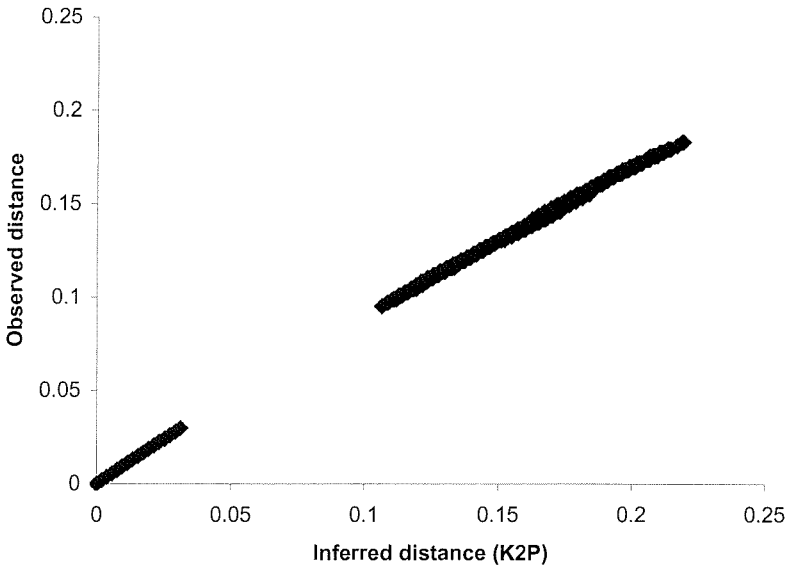


Figure 6.2. Saturation curve for the COI partial gene sequenced for *Osilinus* and *Gibbula* species

6.3.5 *Nucleotide base frequencies*

The nucleotide frequency bias for both the 16S and COI mt DNA gene fragments was constant between all species included in this analysis (Table 6.7). A Chi-square (χ^2) test on the homogeneity of base frequencies across the *Osilinus* taxa and *Gibbula umbilicalis* revealed no significant differences (16S: χ^2 = 46.69, df=285, P = 1.0, COI: χ^2 = 42.41, df=219, P = 1.0). The adenine and thymine nucleotide richness exhibited by the 16S partial gene sequenced for the gibbuline genera analysed in Chapter 5 was reflected within this data set with total average frequencies of 0.30 and 0.34 respectively. Similarly cytosine frequency remained low across all *Osilinus* species, averaging 0.13. The average nucleotide base frequencies for the COI sequences was adenine = 0.26, cytosine = 0.21, guanine = 0.17 and thymine = 0.36 (Table 6.6). Similarly, the COI sequence was AT-rich, the Guanine frequency was, however, consistently low across all COI sequences, averaging 0.17, in contrast to the low cytosine frequencies (0.13) exhibited in the 16S sequences

Table 6.7. Mean base frequencies for all species included in the 16S and COI sequence analyses.

	16S				COI			
Species	A	C	G	T	A	C	G	T
O. lineatus	0.30	0.14	0.23	0.33	0.25	0.22	0.17	0.36
O. atratus	0.30	0.13	0.22	0.34	0.23	0.22	0.19	0.36
O. articulatus	0.31	0.13	0.21	0.35	0.22	0.21	0.20	0.37
O. edulis	0.29	0.13	0.23	0.35	0.26	0.21	0.18	0.35
O. turbinatus	0.31	0.14	0.22	0.33	0.26	0.20	0.18	0.36
G. umbilicalis	0.33	0.12	0.21	0.34	0.26	0.20	0.17	0.37
O.kotschyi	0.30	0.13	0.22	0.34	-	-	-	-
Total Average	0.30	0.13	0.23	0.34	0.26	0.21	0.17	0.36

A = adenine, G = guanine, C = cytosine and T = thymine

6.3.6 *Transition/Transversion ratio*

The number of observed transitions (TS) was plotted against the number of observed transversions (TV) for all 9216 16S and 5476 COI pairwise comparisons within the *Osilinus* and *Gibbula* sequences (Figure 6.3 and 6.4 respectively). The average 16S TS/TV was 1.67, the median 1.55. For COI, the average TS/TV ratio was 3.307 and the median 3.38, indicating that transitions occur more frequently than transversions, as expected of non-saturated sequences. In the 16S sequences the highest number of transversions observed for any pairwise comparisons exceeded that of transitions by 12 (57 versus 45 respectively). In the COI sequences the reverse was true; the number of observed transistions exceeded that of transversions by 18 (49 TV *vs* 67 TS). The trend observed in the COI sequences was expected since transistions occur at a much higher rate than transversions in mitochondrial genes (Brown *et al.,* 1982; Moriyama & Powell, 1997; Peer *et al.,* 2002).

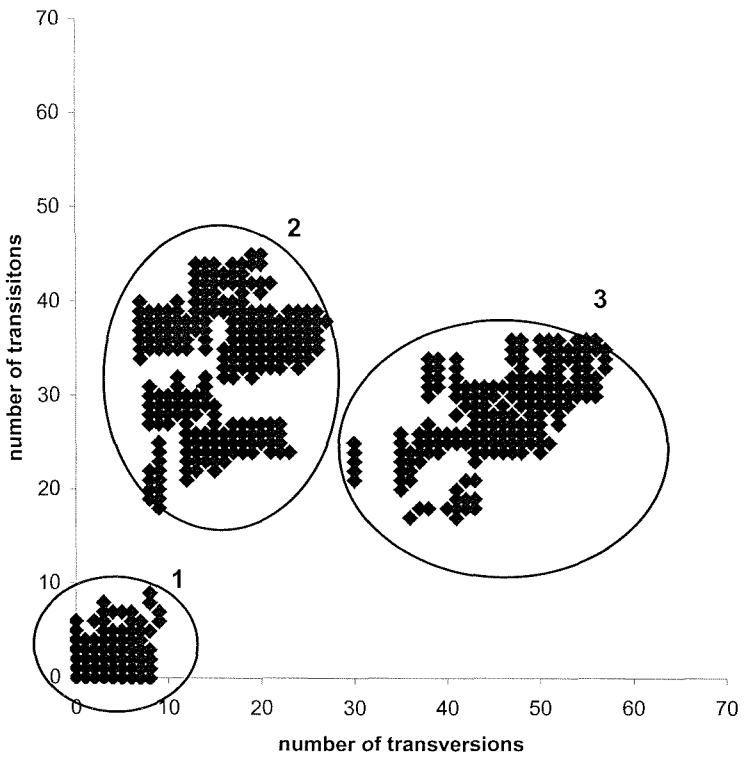


Figure 6.3. Observed number of transitions plotted against number of observed transversions from all mt DNA 16S pairwise comparisons within *Osilinus* and *Gibbula*. See text for discussion of the data clusters (circled 1-3)

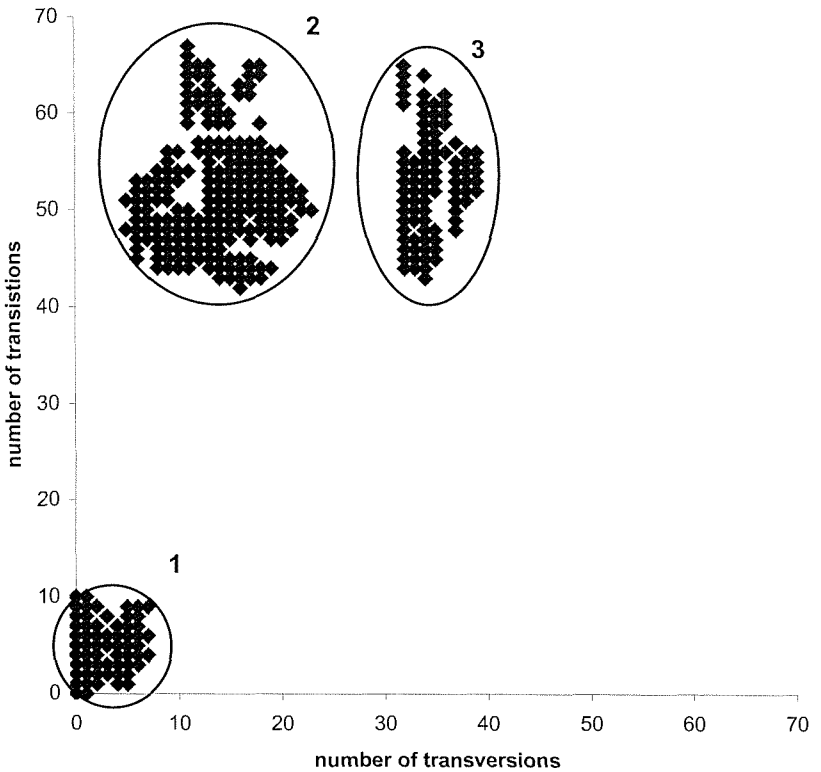


Figure 6.4. Total number of transistions plotted against total number of transversions from all mtDNA COI sequence pairwise comparisons within *Osilinus* and *Gibbula*. See text for discussion of data clusters (circled 1-3)

There is a clear clustering pattern in both the 16S and COI TS/TV graphs. In the 16S TS/TV plot (Figure 6.3) the three data clusters are circled and numbered: Data cluster 1 (delimited by 0-9 TS, 0-10 TV) represents only intraspecific pairwise comparisons. Data cluster 2 (delimited by 18-45 TS, 7-27 TV) represents only interspecific pairwise comparisons within the Mediterranean /Atlantic *Osilinus* species (i.e. excluding *Osilinus kotschyi*). Data cluster 3 (delimited by 18-36 TS, 35-57TV) represents only interspecific pairwise comparisons between the outgroup species (*Gibbula umbilicalis*) and the Indian Ocean *O.kotschyi* with the Atlantic /Mediterranean *Osilinus* species. A similar trend is true for COI: in the TS/TV plot (Figure 6.4) three data clusters (circled 1, 2 and 3) similarly correspond to intraspecific comparisons within *Osilinus* and *Gibbula* (delimited by 0-10 TS; 0-7 TV), interspecific pairwise comparisons between *Osilinus* spp. (delimited by 42-68 TS; 5-23 TV), and interspecific comparisons between *G. umbilicalis* and *Osilinus* spp. (delimited by 42-65 TS; 32-69 TV) respectively.

6.3.7 16S haplotype distribution and abundance

The haplotype distribution and abundance analysis of *O. edulis, O. lineatus* and *O. atratus* 16S sequences reveals distinct geographic patterning. The variable bases of all the unique haplotypes within the *O. edulis* sequence data are listed in Table 6.8 relative to the most common haplotype (Eduhap1). The geographic distribution of 16S haplotypes for *O. edulis* reveals that no haplotype is represented in all geographic locations (Figure 6.5). The Canary Islands are represented by individuals sampled from Lanzarote, Fuerteventura, Gran Canaria and Tenerife, between which haplotypes were shared. Of the 29 haplotypes identified from the Canary Is, 25 were unique to the islands; individuals sampled from the Moroccan coastline shared the remaining four haplotypes. Of the remaining 10 haplotypes sampled from Morocco, eight were unique, one was shared with individuals from Madeira, and one shared with individuals from the Basque region, north Spain. Four of the five Madeira haplotypes were endemic to the island and two of the three haplotypes samples from the Basque region were geographically unique. The most common haplotypes exhibited the greatest biogeographical range, represented by individuals from more than one geographic location.

O. atratus was collected only from the Canary Islands; no specimens were found on Madeira despite extensive searching (Figure 6.6). The variable bases of all the unique haplotypes within the *O. atratus* sequence data are listed in Table 6.9 relative to the most common haplotype (Atrahap1). Of the 11 haplotypes identified, only one was found on all four islands. This haplotype was also the most abundant. The remaining ten haplotypes revealed strong geographic patterning. One haplotype was unique to Lanzarote and three haplotypes were unique to each of the islands Fuerteventura, Gran Canaria and Tenerife. The sample size is too small to make any conclusions regarding population structure or gene flow. It indicates, however, that future work on the population genetics of *O. atratus* on the Canary Islands may reveal that the island populations are isolated, or possess the genetic structure expected of semi-isolated populations with limited gene flow.

The geographic distribution of the *O. lineatus* 16S haplotypes spans the continental coastline from the southwest of the United Kingdom and Ireland, to the Basque Coast and Morocco (Figure 6.7). The variable bases of all the unique haplotypes within the *O. lineatus* sequence data are listed in Table 6.10 relative to the most common haplotype. Of the 20 unique haplotypes identified, 17 were unique to the Moroccan samples. The most common *O. lineatus* 16S haplotype (Linhap1) was shared by all geographic locations. As a result of the haplotype analysis, a smaller data set of 16S sequences was chosen for the subset analysis based on inclusion of the most common and rare haplotypes.

Table 6.8. ***Osilinus edulis.*** **16S mitochondrial DNA diversity (see Figures 3.2 and 3.3 for sampling locations). The variant bases relative to the most common haplotype observed are shown with haplotype frequency (*f*).**

Haplotype		SamplingLocations								Total	*f*
		Tenerife	Lanzarote	Fueteventura	Gran Canaria	Canary Is	Morocco	Madeira	Basque		
Eduhap 1	A - G G T T T A G A T T G C A C G G T C T T A A T T T C T T A G - G C G G G T G - G - A	0	1	2	2	5	2	0	0	7	0.14
Eduhap 2	T T C G	1	0	0	0	1	0	0	0	1	0.02
Eduhap 3	C T - G C	1	0	0	0	1	0	0	0	1	0.02
Eduhap 4	T - -	0	0	0	1	1	0	0	0	1	0.02
Eduhap 5	T -	0	1	0	0	1	0	0	0	1	0.02
Eduhap 6	T - - - - - -	0	0	0	0	0	1	0	0	1	0.02
Eduhap 7	T - G	1	0	0	0	1	0	0	0	1	0.02
Eduhap 8	T -	1	0	0	0	1	0	0	0	1	0.02
Eduhap 9	T -	0	2	0	0	2	1	0	0	3	0.06
Eduhap 10	- C T -	0	0	0	0	0	1	0	0	1	0.02
Eduhap 11	C T -	0	0	0	0	0	1	0	1	2	0.04
Eduhap 12	C - T -	0	0	0	0	0	1	0	0	1	0.02
Eduhap 13	T T -	2	0	0	0	2	0	0	0	2	0.04
Eduhap 14	T -	0	1	0	0	1	1	0	0	2	0.04
Eduhap 15	- T -	0	1	0	0	1	0	0	0	1	0.02
Eduhap 16	T - A -	1	0	0	0	1	0	0	0	1	0.02
Eduhap 17	A -	0	0	0	0	0	0	0	1	1	0.02
Eduhap 18	C C -	0	0	0	0	0	1	0	0	1	0.02
Eduhap 19	C -	0	0	0	0	0	2	0	0	2	0.04
Eduhap 20	A T T -	0	0	0	0	0	0	1	0	1	0.02
Eduhap 21	A T T -	0	0	0	0	0	0	1	0	1	0.02
Eduhap 22	T T -	0	0	0	0	0	0	1	0	1	0.02
Eduhap 23	T C -	0	0	0	0	0	1	0	0	1	0.02
Eduhap 24	T -	0	0	0	0	0	1	4	0	5	0.1
Eduhap 25	T A -	0	0	0	0	0	0	1	0	1	0.02
Eduhap 26	C T - -	1	0	0	0	1	0	0	0	1	0.02
Eduhap 27	A C -	0	1	0	0	1	0	0	0	1	0.02
Eduhap 28	- C	0	0	1	0	1	0	0	0	1	0.02
Eduhap 29	- -	1	0	0	0	1	0	0	0	1	0.02
Eduhap 30	C -	1	0	0	0	1	0	0	0	1	0.02
Eduhap 31	C -	1	0	0	0	1	0	0	0	1	0.02
Eduhap 32	G -	0	0	0	1	1	0	0	0	1	0.02
Eduhap 33	A -	0	0	1	0	1	0	0	0	1	0.02
Eduhap 34	A - C G	0	0	0	0	0	1	0	0	1	0.02
Eduhap 35	A -	0	0	1	0	1	0	0	0	1	0.02
Eduhap 36	A C -	0	0	0	1	1	0	0	0	1	0.02
Eduhap 37	C -	0	0	0	0	0	0	0	1	1	0.02
Eduhap 38	A -	1	0	0	0	1	0	0	0	1	0.02
Eduhap 39	-	2	1	1	2	6	0	0	0	6	0.12
Eduhap 40	T T -	1	0	0	0	1	0	0	0	1	0.02
Eduhap 41	T T C - C	1	0	0	0	1	0	0	0	1	0.02
Eduhap 42	T -	1	0	0	0	1	1	0	0	1	0.02
Eduhap 43	G -	0	0	1	1	2	0	0	0	2	0.04
Eduhap 44	G T A -	0	1	0	0	1	0	0	0	1	0.02
Eduhap 45	G C T C C A C C G	0	0	0	0	0	1	0	0	1	0.02

Figure 6.5 A

Haplotype	Madeira	Basque	Canary Is	Morocco
Eduhap 20	1			
Eduhap 21	1			
Eduhap 22	1			
Eduhap 25	1			
Eduhap 24	4			1
Eduhap 11		1		1
Eduhap 17		1		
Eduhap 37		1		
Eduhap 14			1	1
Eduhap 42			1	1
Eduhap 9			2	1
Eduhap 6				1
Eduhap 10				1
Eduhap 12				1
Eduhap 18				1
Eduhap 23				1
Eduhap 34				1
Eduhap 45				1
Eduhap 1			5	2
Eduhap 19				2
Eduhap 2			1	
Eduhap 3			1	
Eduhap 4			1	
Eduhap 5			1	
Eduhap 7			1	
Eduhap 8			1	
Eduhap 15			1	
Eduhap 16			1	
Eduhap 26			1	
Eduhap 27			1	
Eduhap 28			1	
Eduhap 29			1	
Eduhap 30			1	
Eduhap 31			1	
Eduhap 32			1	
Eduhap 33			1	
Eduhap 35			1	
Eduhap 36			1	
Eduhap 38			1	
Eduhap 40			1	
Eduhap 41			1	
Eduhap 44			1	
Eduhap 13			2	
Eduhap 43			2	
Eduhap 39			6	

Figure 6.5 B

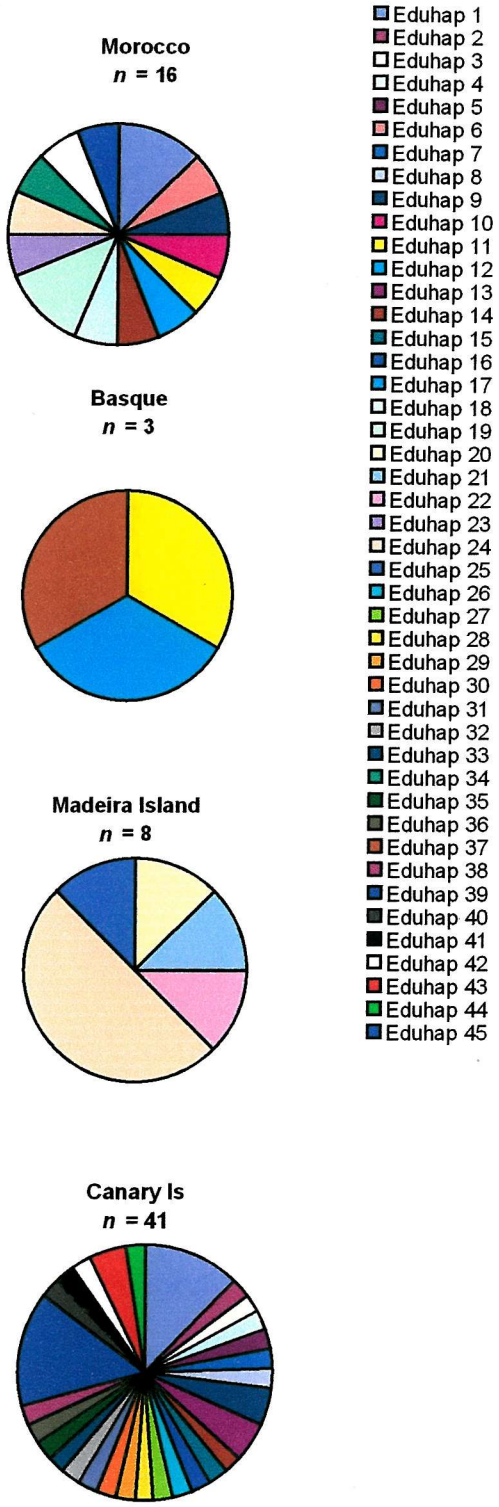


Figure 6.5. ***Osilinus edulis*: A. Haplotype distribution and abundance of the partial 16S mtDNA sequence. The number of samples representing each haplotype is listed according to geographica location (represented by different degrees of shading). B. Genetic biodiversity of each major sampling location illustrated by pie charts of the 16S haplotypes. C. Genetic biodiversity of each Canary Island illustrated by pie charts of the 16S haplotypes.**

Figure 6.5 C

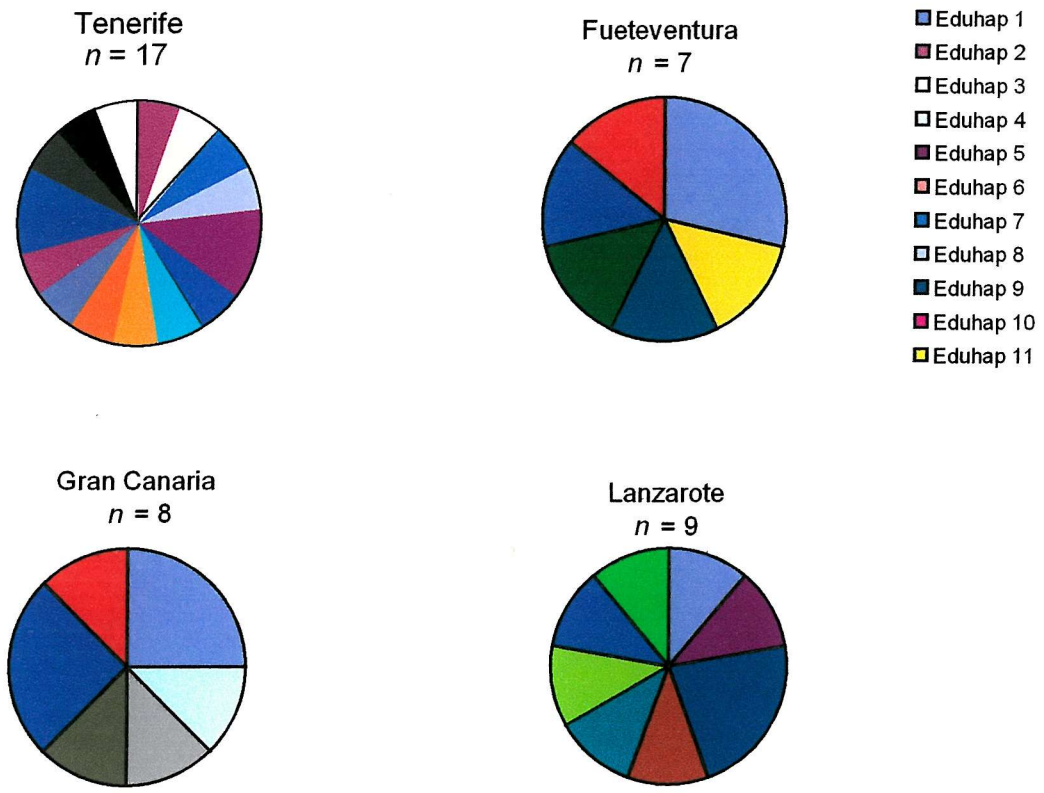

Tenerife
n = 17
Fueteventura
n = 7
Gran Canaria
n = 8
Lanzarote
n = 9
Eduhap 1
Eduhap 2
Eduhap 3
Eduhap 4
Eduhap 5
Eduhap 6
Eduhap 7
Eduhap 8
Eduhap 9
Eduhap 10
Eduhap 11

Table 6.9. ***Osilinus atratus.*** **16S mitochondrial DNA diversity (see Figures 3.2 and 3.3 for sampling locations). The variant bases relative to the most common haplotype observed are shown with haplotype frequency (*f*).**

Haplotype		Sampling location				Total	*f*
		Gran Canaria	*Tenerife*	*Fuerteventura*	*Lanzarote*		
Atrahap 1	G T A G C C T C C G C A A	1	1	4	6	12	0.545
Atrahap 2	T	0	0	0	1	1	0.045
Atrahap 3	T \	0	0	1	0	1	0.045
Atrahap 4	A T	0	0	1	0	1	0.045
Atrahap 5	T	1	0	0	0	1	0.045
Atrahap 6	G	0	0	1	0	1	0.045
Atrahap 7	G	1	0	0	0	1	0.045
Atrahap 8	T	1	0	0	0	1	0.045
Atrahap 9	G T	0	1	0	0	1	0.045
Atrahap 10	A C	0	1	0	0	1	0.045
Atrahap 11	C T A	0	1	0	0	1	0.045

Figure 6.6 A

Haplotype	Lanzarote	Fuerteventura	Gran Canaria	Tenerife
Atrahap 1	6	4	1	1
Atrahap 2	1			
Atrahap 3		1		
Atrahap 4		1		
Atrahap 5		1		
Atrahap 6			1	
Atrahap 7			1	
Atrahap 8			1	
Atrahap 9				1
Atrahap 10				1
Atrahap 11				1

Figure 6.6 B

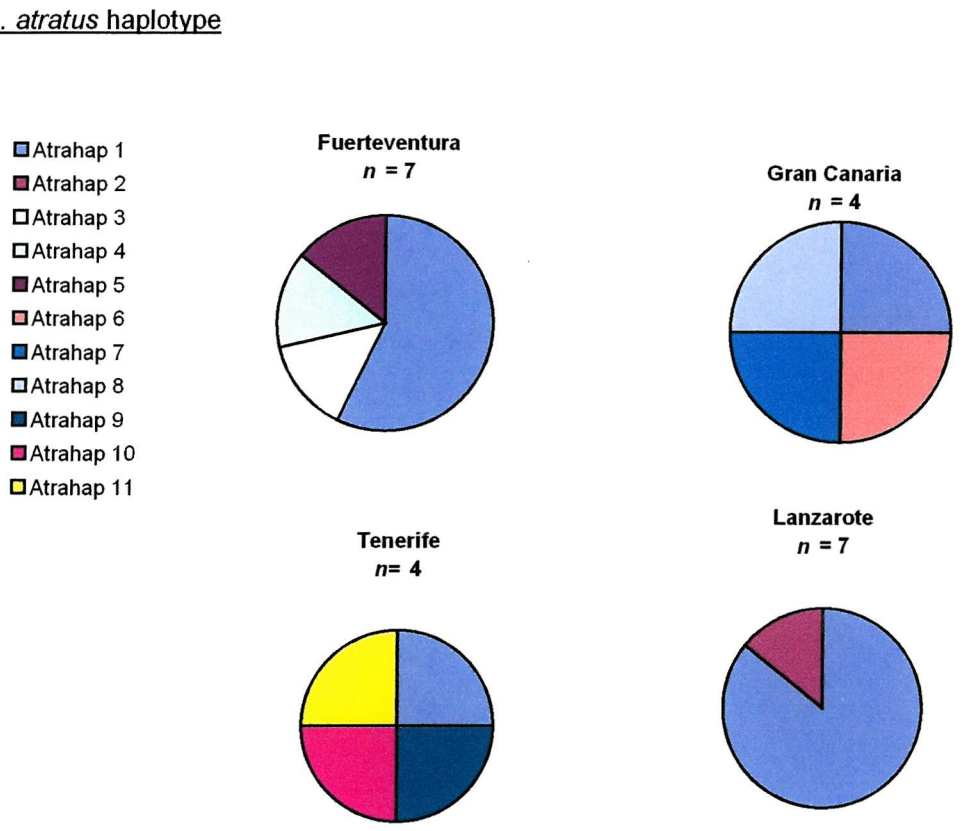


Figure 6.6. *Osilinus atratus*: A. Haplotype distribution and abundance of the partial 16S mtDNA sequences. The number of samples representing each haplotype is listed according to geographical location (represented by degrees of shading). B. Genetic diversity of each sampling location illustrated by pie charts of the 16S haplotypes.

Table 6.10. *Osilinus lineatus.* 16S mitochondrial DNA diversity (see Figures 3.2 and 3.3 for sampling locations). The variant bases relevant to the most common haplotype observed are shown with haplotype frequency (*f*).

Haplotype																									Location								Total	*f*
																									LR UK	CW UK	Basque	Greece	Anza	Tifnit	Imourane	Cap Ghir		
Linhap 1		T	C	G	A	T		T		G	C	A	A	T	T		T	A		A	A	G	G		2	1	2	1	1	1	1	0	9	0.32
Linhap 2											G					C	C						C		0	0	0	0	1	0	0	0	1	0.035
Linhap 3	C																								0	0	0	0	0	1	0	0	1	0.035
Linhap 4	C													C					C				C		0	0	0	0	1	0	0	0	1	0.035
Linhap 5																									0	0	0	0	0	0	1	0	1	0.035
Linhap 6					G							G													0	0	0	0	0	1	0	0	1	0.035
Linhap 7		C																							0	0	0	0	0	0	1	0	1	0.035
Linhap 8													G												0	0	0	0	0	0	1	0	1	0.035
Linhap 9							C																		0	0	0	0	1	0	0	1	2	0.07
Linhap 10										A															0	0	1	0	0	0	0	0	1	0.035
Linhap 11	C																				G				0	0	0	0	0	1	0	0	1	0.035
Linhap 12	C																								0	0	0	0	0	0	0	1	1	0.035
Linhap 13																						A			0	0	0	0	0	1	0	0	1	0.035
Linhap 14			T															G							0	1	0	0	0	0	0	0	1	0.035
Linhap 15						C	C	C																	0	0	0	0	0	0	0	1	1	0.035
Linhap 16						C		C	C																0	0	0	0	0	0	0	1	1	0.035
Linhap 17	C					C		C																	0	0	0	0	0	0	0	1	1	0.035
Linhap 18	C					C																	C		0	0	0	0	0	0	1	0	1	0.035
Linhap 19	C			A											C					G					0	0	0	0	0	1	0	0	1	0.035
Linhap 20				A																			C		0	0	0	0	0	0	1	0	1	0.035

Figure 6.7 A

Haplotype	LR UK	CW UK	Basque	Morocco
Linhap 1	2	1	2	3
Linhap 14			1	
Linhap 9		1		
Linhap 2				2
Linhap 3				1
Linhap 4				1
Linhap 5				1
Linhap 6				1
Linhap 7				1
Linhap 8				1
Linhap 11				1
Linhap 12				1
Linhap 13				1
Linhap 15				1
Linhap 16				1
Linhap 17				1
Linhap 18				1
Linhap 19				1
Linhap 20				1
Linhap 10				1

Figure 6.7 B

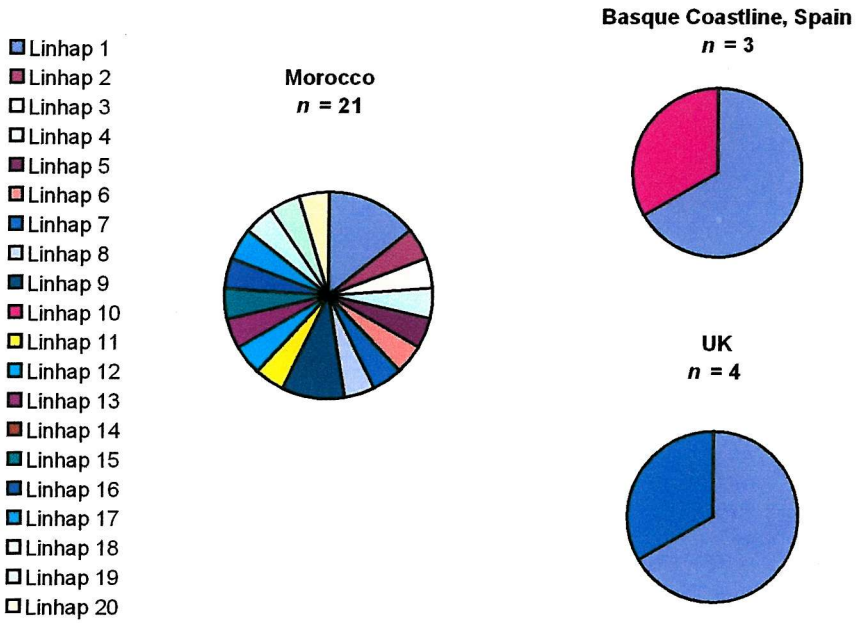


Figure 6.7. *Osilinus lineatus*: A. Haplotype distribution and abundance of the partial 16S mtDNA sequence. The number of samples representing each haplotype is listed according to geographical location (represented by different degrees of shading). B. Genetic diversity of each sampling location illustrated by pie charts of the 16S haplotypes.

6.3.8 Phylogenetic analysis using 16S sequence data

Neighbour-joining

The 16S NJ bootstrap analysis tree is given in Figure 6.8. The tree is rooted with *G. umbilicalis,* which forms a basal monophyletic sister group to the Atlantic /Mediterranean *Osilinus* ingroup with 84% bootstrap support. All species form monophyletic clades with 98 - 100% bootstrap support. Two distinct *Osilinus* clades are evident. An *O. lineatus, O. atratus* and *O. articulatus* clade (50%) and an *O. turbinatus, O. edulis* clade (86%). The former places *O. lineatus* as a sister species to a bifuricating clade (61% support) in which *O. articulatus* is basal to *O. atratus.* The latter places *O. turbinatus* as basal to *O. edulis* with 100% support.

Maximum-parsimony analysis

All 96 16S sequences were included in the initial maximum-parsimony analysis, each comprising 479 base pairs (characters). Gaps were treated as both a 'fifth base' and as missing characters to compare the effect on phylogeny. In all heuristic parsimony searches starting trees were obtained via stepwise addition, with 10 replicates of random addition sequence, one tree held at each step. The branch-swapping algorithm Nearest Neighbour Interchange (NNI) was enforced. Branches were collapsed if maximum length equalled zero and no topological constraints were enforced. For analysis performed with gaps treated as a fifth base, 273 of these characters were constant, 37 variable characters were parsimony-uninformative, and the remaining 169 variable characters parsimony-informative. When gaps were treated as missing data, 304 characters were constant, 28 were parsimony-uninformative and 147 characters parsimony-informative. The heuristic Fitch parsimony search performed with gaps treated as a fifth base tried 2242 rearrangements, and found one best tree of score 404 (Figure 6.9). The same heuristic search repeated with gaps treated as missing data tried 2322 rearrangements, and found two best trees of score 327. The two best trees shared the same interspecific tree topology, but differed in intraspecific relationships within the *O. lineatus, O. edulis* and *O. atratus* clades (Figure 6.10). Each analysis was repeated as a bootstrap analysis (1000 replicates). The 50% majority-rule consensus trees for both analyses again shared the same interspecific tree topologies with very similar support values. The tree produced under the bootstrap analysis with gaps treated as missing data is shown in Figure 6.12 with support values of the bootstrap tree produced with gaps treated as a fifth base in parentheses for compatible nodes.

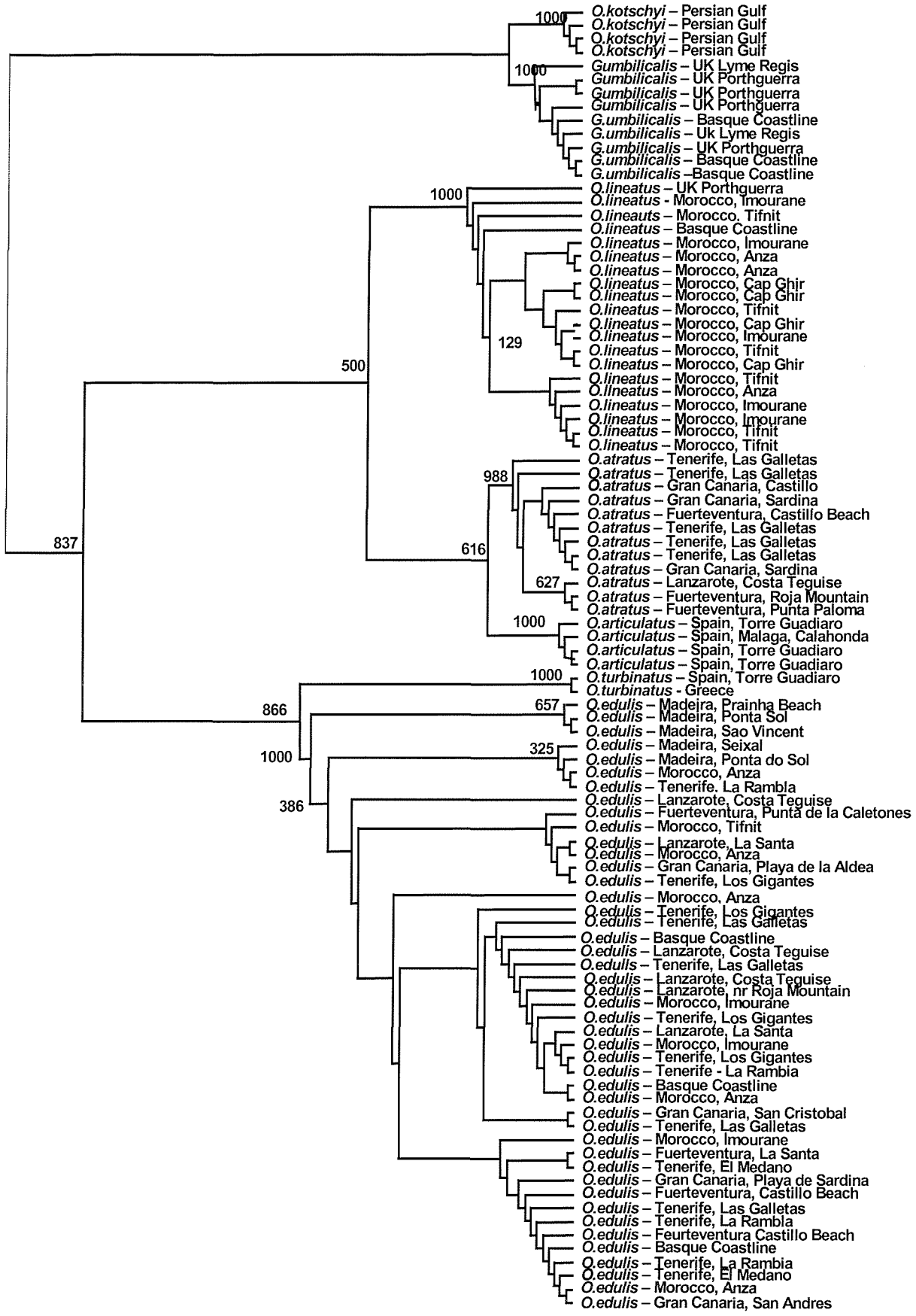


Figure 6.8. 16S mtDNA Neighbour-Joining tree with bootstrap support values (1000 bootstrap replications) given to the left of nodes.

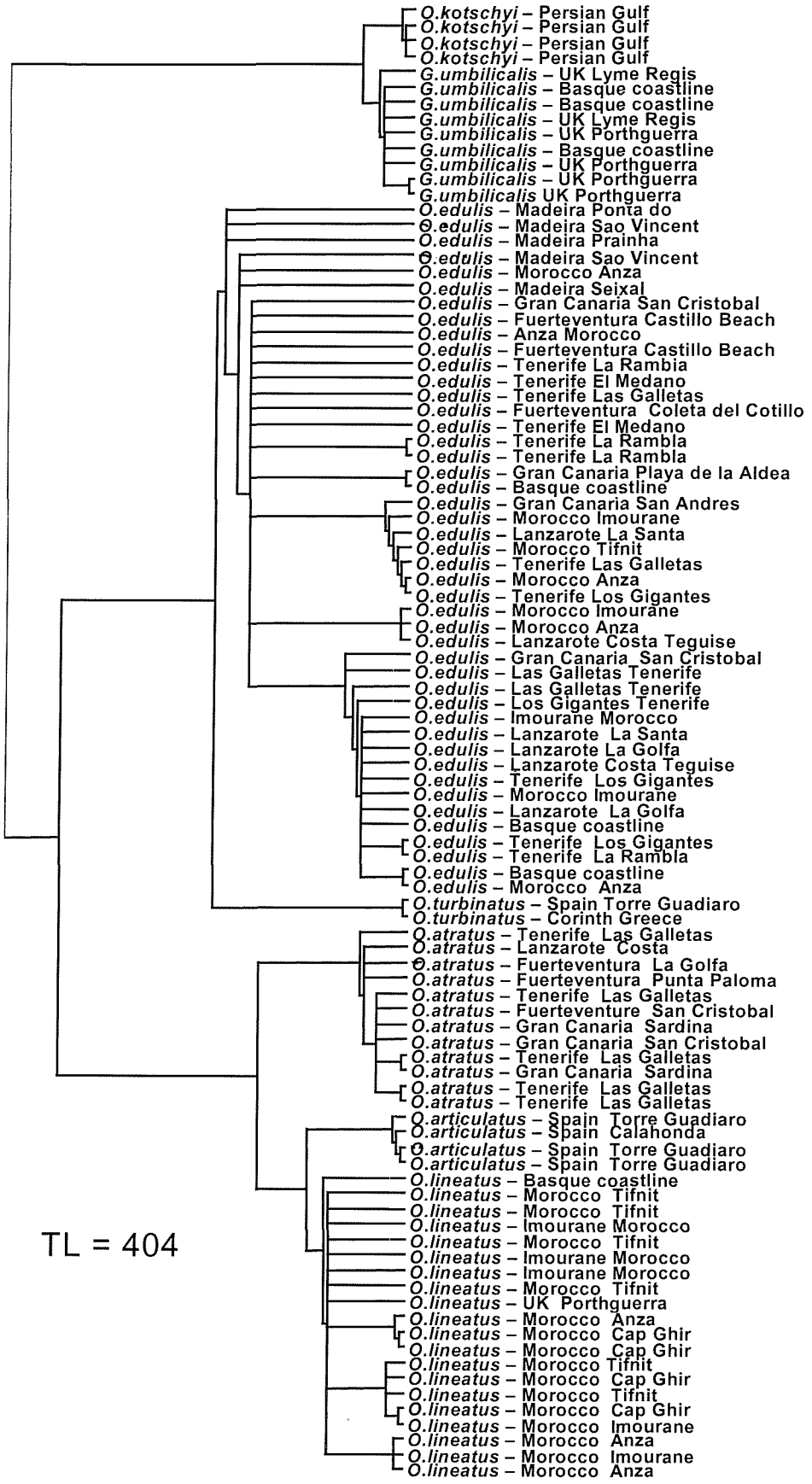


Figure 6.9. 16S mtDNA Maximum-parsimony tree. Heuristic search with characters unordered and equal weight with gaps treated as fifth base. TL = total length.

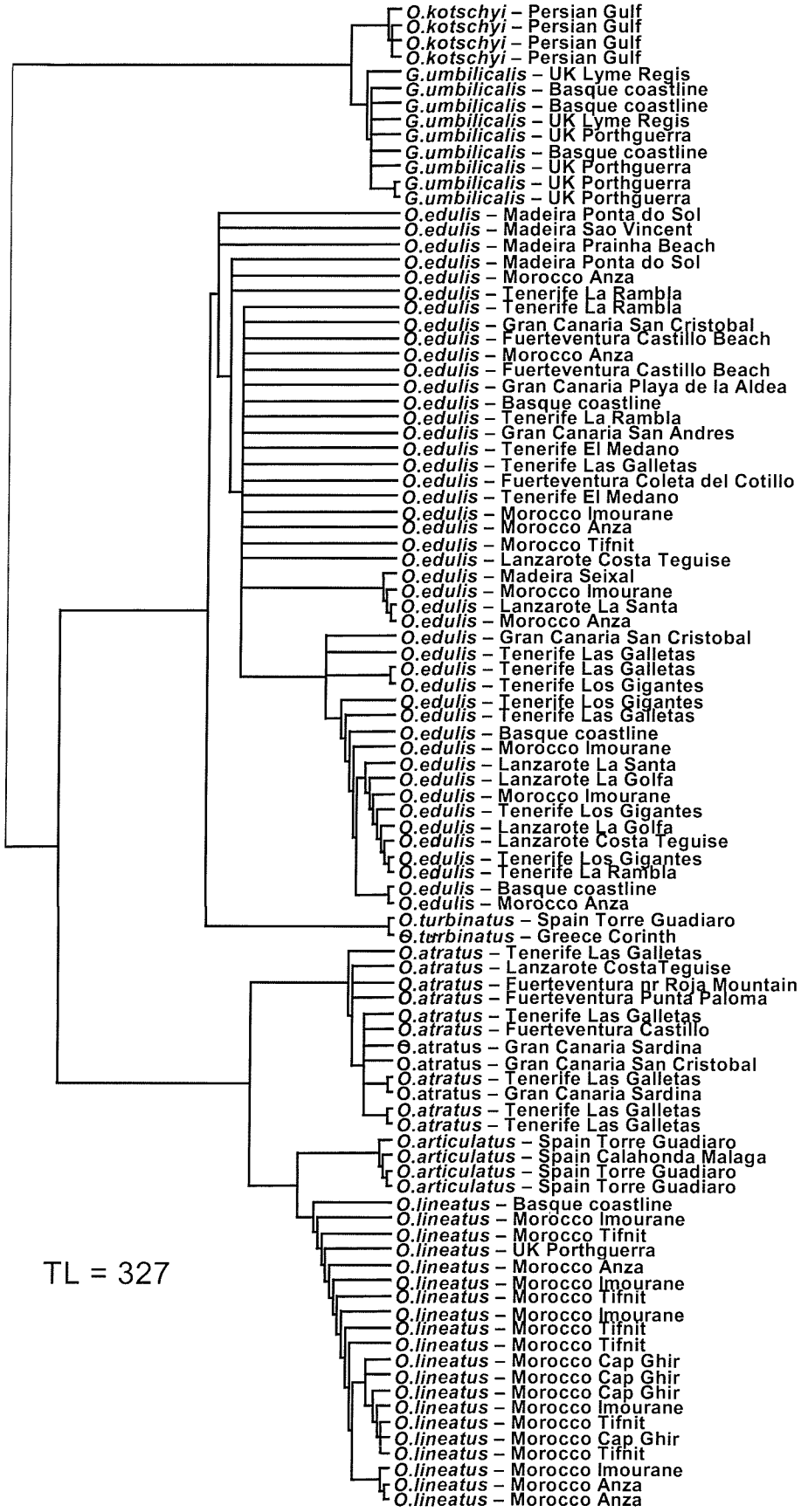


Figure 6.10. 16S mtDNA Maximum-parsimony tree. Heuristic search with characters unordered and of equal weight with gaps treated as missing. TL = total length.

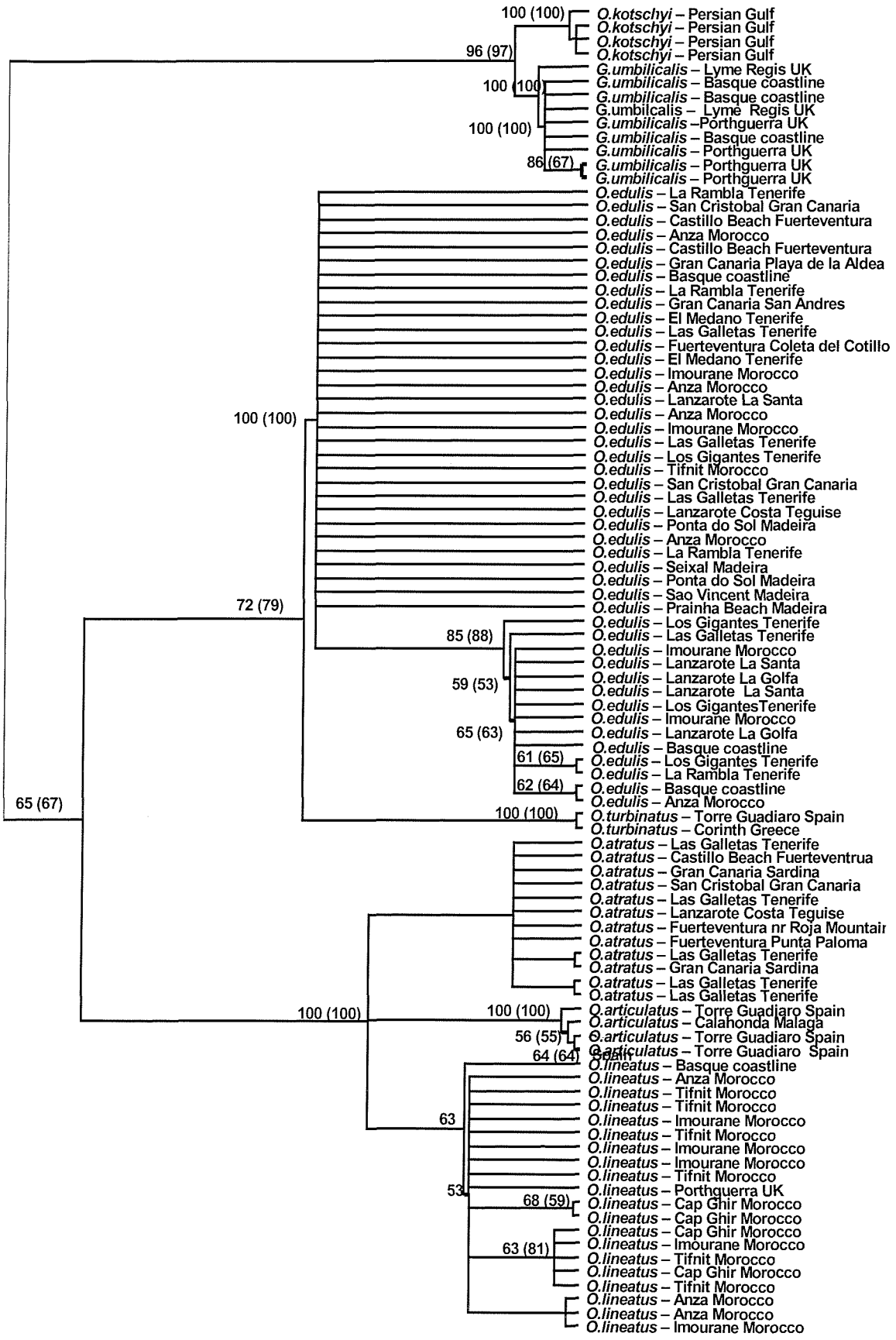


Figure 6.11. 16S mtDNA Maximum-parsimony Bootstrap (1000 replicates) 50% majority-rule consensus tree produced with gaps treated as missing data (bootstrap support values given at left of node) and gaps treated as a 5th based (bootstrap support values given at left of node in parenthesis).

The bootstrap analysis confers 96% support on *O.kotschyi* and *G. umbilicalis* as a monophyletic outgroup to the monophyletic Atlantic/Mediterranean *Osilinus* ingroup. There was also 100% bootstrap support for both outgroup species clades, reflected in the topology of both the maximum-parsimony trees in Figures 6.10 and 6.11. Considering the Atlantic/Mediterranean *Osilinus* clade, in both maximum-parsimony trees (Figure 6.10 and 6.11) and the bootstrap analysis, *O. edulis* and *O. turbinatus* form a bifurcating clade (72% support) sister to an *O. lineatus, O. atratus,* and *O. articulatus* clade (65% bootstrap support). In both the heuristic searches *O. atratus* is basal to *O. articulatus* and *O. lineatus.* In the bootstrap analysis, the phylogenetic relationships of these three species is unresolved. All species clades are given 100% bootstrap support.

A branch and bound parsimony search was attempted on the whole data set. After 200 hours of processing the search was aborted as no trees had been retained. Two smaller data sets were selected to allow computation of an exhaustive maximum-likelihood search and branch and bound maximum-parsimony search. The 12 16S sequences used for the subset analysis, selected on a basis of haplotype frequency and geographic distribution, are listed in Table 6.11.

Table 6.11. Species, ID number and haplotype name (where applicable) of the 16S mtDNA sequences included in the subset analysis. Location is given for samples representing unique haplotypes. Where a haplotype represents consensus sequences see Table 6.3.

Species	Sample	Haplotype	Location	Map Ref.
Gibbula umbilicalis	131	-	Cornwall, UK	2
Osilinus kotschyi	401	-	Khor Al Hamra, UAE	
O. turbinatus	96	-	Cadiz, Spain	4
	256	-	Greece	9
O. lineatus	038	Linhap1	Various (see Table 6.2)	
	139	Linhap14	Cornwall, UK	2
O. atratus	069	Atrahap1	Various (see Table 6.2)	
	385	Atrahap5	Gran Canaria, Sardina	34
O. articulatus	106	-	Cadiz, Spain	4
	108	-	Malaga, Spain	5
O. edulis	346	Eduhap1	Various (see Table 6.2)	
	197	Eduhap20	Madeira Island	36

A branch-and-bound search was performed on the 12-subset sequences. *Osilinus kotschyi* and *Gibbula umbilicalis* were designated as outgroup taxa. Constant (uninformative) characters were given a weight of zero to be effectively excluded from the analysis. All gaps were considered missing and the 95 parsimony informative characters assigned a weight of 1. Furthest addition sequence was in effect. One best tree was retained with a total length of 284 (Figure 6.12). The search was repeated with the same settings, except gaps were treated as a fifth base. Two best trees were found (total length 341). One tree was of identical tree topology to Figure 6.12, and the second differed within the (*O. lineatus, O. atratus, O. articulatus*) clade (Figure 6.13).

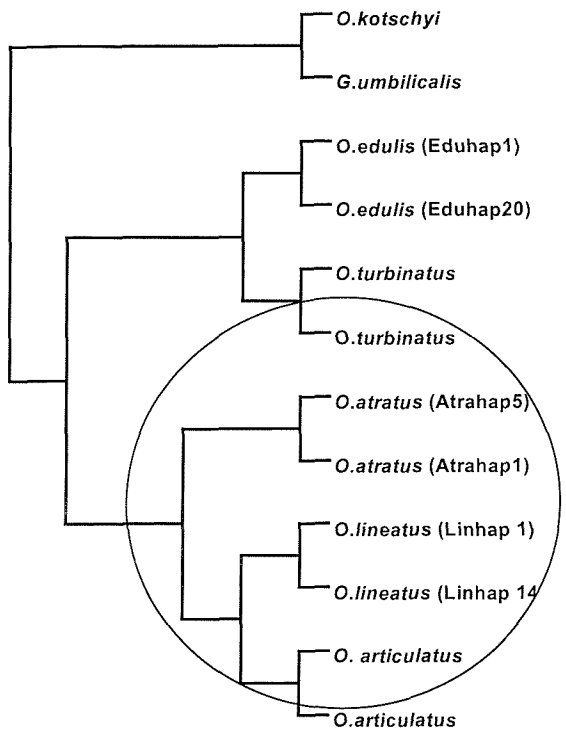


Figure 6.12. 16S mtDNA Maximum-parsimony branch and bound tree. Character weightings: Constant (uninformative) characters = 0. Gaps were considered missing. Parsimony informative characters = 1. The initial upper bound was computed heuristically, furthest addition sequence was in effect and branches coliapsed if the maximum branch iength equaled zero. Total length = 284

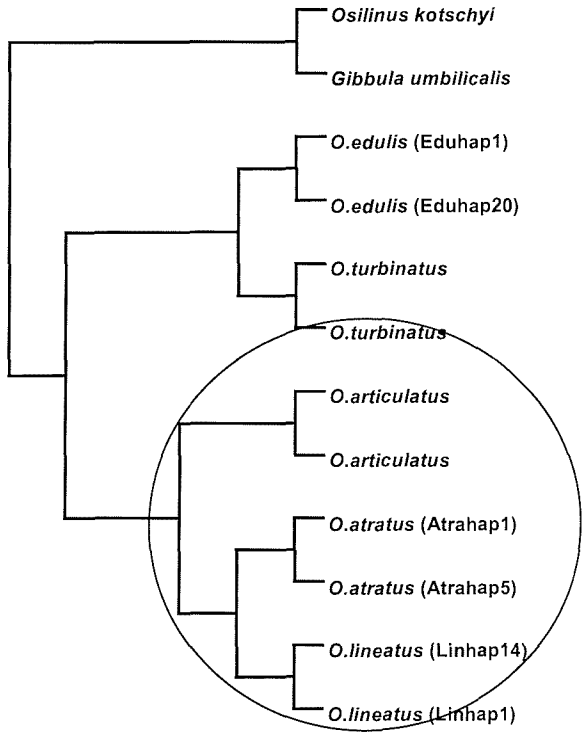


Figure 6.13. 16S mtDNA Maximum-parsimony branch and bound tree. Character weightings: Constant (uninformative) characters = 0. Gaps were treated as a fifth base. Parsimony informative characters = 1. The initial upper bound was computed heuristically, furthest addition sequence was in effect and branches collapsed if the maximum branch length equaled zero. Total length = 341

The phylogenies produced by the subset analysis retained the tree topology of two major sister clades within *Osilinus*, one containing *O. turbinatus* + *O. edulis*, the other *O. lineatus* + *O. articulatus* + *O. atratus.* Figure 6.12 placed *O. atratus* as basal to *O. lineatus* + *O. articulatus*; in Figure 6.13 the relative positions of *O. articulatus* and *O. atratus* to *O. lineatus* were reversed.

Maximum-likelihood analysis

The likelihood score of the 16S neighbour-joining tree (Figure 6.8) was estimated by 56 models within the GTR family to establish the most suitable model of DNA substitution for the 16S data set. The tree likelihood scores were subsequently compared for goodness of fit using the likelihood ratio test statistic (Huelsenbeck & Crandall, 1997; Huelsenbeck & Rannala, 1997), following the hierarchical hypothesis testing framework prescribed under the Modeltest software (Posada & Crandall, 1998). The GTR+G model of DNA substitution was chosen as conferring the best fit given the data, with a tree likelihood score of -*ln*L –2456.46. The base frequencies were estimated at A = 0.31, C = 0.13, G = 0.22, T = 0.34. The Gamma shape distribution was estimated at 0.33 and the rate matrix estimated as below (Table 6.12).

Table 6.12. DNA substitution rate matrix estimated by likelihood analysis of the neighbour-joining tree under the GTR+G model. The nucleotide bases are represented by A = adenine, G = Guanine, C = cytosine and T = thymine.

	A	C	G	T
A	-	1.072	5.646	1.647
C		-	3.788	6.655
G			-	1.000
T				-

The DNA substitution matrix and gamma shape distribution estimated under the GTR+G model of substitution were subsequently employed in a heuristic likelihood search. A molecular clock was not enforced. The heuristic search was conducted using the subtree-pruning-regrafting (SPR) branch-swapping algorithm, with initial swapping performed on the neighbour-joining tree. The heuristic search tried 84,751 rearrangements and found two trees with the best likelihood score (2443.72, Figure 6.14). The two best maximum-likelihood trees differ only in the relationship between two *Osilinus edulis* sequences from Coleta del Cotillo, Fuerteventura and El Medano, Tenerife. Tree 1 places these in two taxa clade; whilst tree 2 retains both sequences as tips of separate branches in the larger polytomy *O. edulis* clade (circled in Figure 6.14).

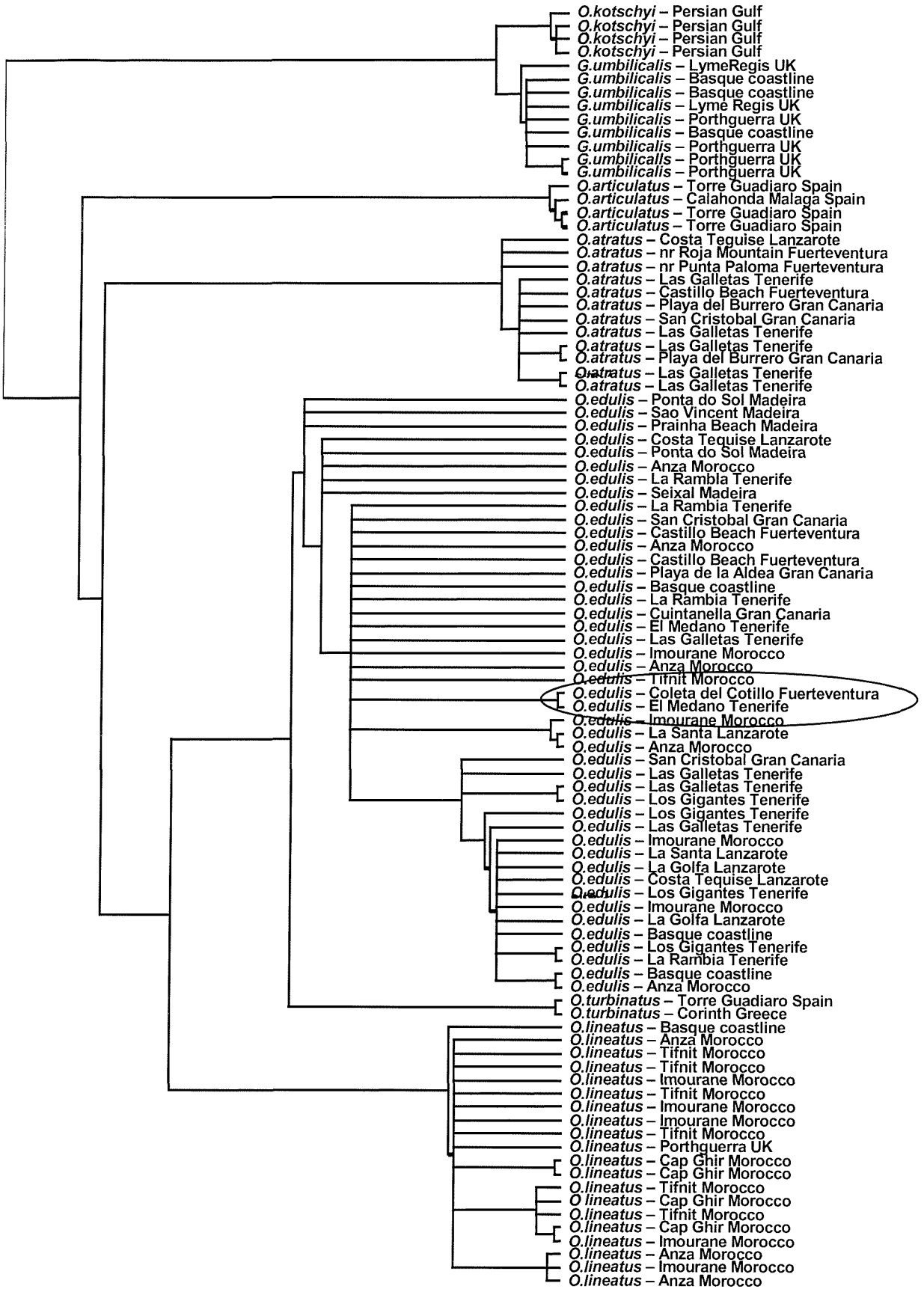


Figure 6.14. 16S mtDNA Maximum-likelihood tree. –lnL = 2443.72. This is representative of the two best ML trees produced by the heuristic search. The two trees differed in the circled clade, which was collapsed in tree 2.

The likelihood tree score of tree one was estimated under all the GTR models of DNA substitution computed for the neighbour-joining tree above, to establish if any simpler models could be accepted, further models rejected or the parameters improved (Table 6.13).

Table 6.13. Comparison of the likelihood tree scores of the maximum-likelihood tree 1 estimated by six models of DNA substitution using the Likelihood Ratio Test and X^2 distribution.

Model	Tree –*ln*L score	Likelihood ratio test			Degrees freedom	X^2 critical value for P > 0.001	Null model
		Null model	Alternative model	LRT*			
JC	2607.06						
F81	2582.49	JC	F81	49.14	3	16.27	Rejected
HKY 85	2535.36	F81	HKY	32.12	1	10.83	Rejected
GTR	2528.31	HKY	GTR	94.26	4	18.47	Rejected
GTR+Γ	2443.51	GTR	GTR+Γ	169.6	1	10.83	Rejected
GTR+Γ+I	2443.50	GTR+Γ	GTR+Γ+I	0.02	1	10.83	Accepted

*LRT = 2(-*ln*L*null* - -*ln*Lalternative)

The GTR+Γ model of DNA substitution was therefore retained as the most suitable model of DNA substitution for the 16S data set, with a tree likelihood score of -2443.51. The base frequencies were estimated at A = 0.31, C = 0.12, G = 0.22, T = 0.35, the Gamma shape distribution was estimated at 0.33 and the rate matrix estimated as tabulated below (Table 6.14). The parameters estimated under the GTR+ Γ model of DNA substitution showed little change from the likelihood analysis of the neighbour-joining tree and were therefore considered stabilised.

Table 6.14. DNA substitution rate matrix estimated by likelihood analysis of the maximum-likelihood tree one under the GTR+Γ model. The nucleotide bases are by A = adenine, G = guanine, C = cytosine and T = thymine.

	A	C	G	T
A	-	1.331	6.005	1.767
C		-	4.676	7.999
G			-	1.000
T				-

To establish whether the sequence data behaved in a clock-like manner, the likelihood score of both best maximum-likelihood trees was subsequently re-estimated under the GTR+Γ model with a molecular clock enforced. Enforcing a molecular clock requires a rooted tree, therefore the trees were rooted with the outgroup taxa *Osilinus kotschyi* and *Gibbula umbilicalis* as a paraphyletic group to the monophyletic ingroup taxa (all remaining *Osilinus* spp.). The tree likelihood score of tree 1 was -2726.2787, and tree 2 –2727.4044. The base frequencies of both trees were estimated at A = 0.30, C = 0.13, G = 0.24, T = 0.33, with the Gamma shape distribution 0.38 and the substitution rate matrix given below (Table 6.15).

Table 6.15. DNA substitution rate matrix estimated by likellhood analysis of the best maximum-likelihood trees under the GTR+G model with a molecular clock enforced. The nucleotide bases are represented by A = adenine, G = guanine, C = cytosine and T = thymine.

Tree 1				
	A	C	G	T
A	-	0.913	4.742	1.779
C		-	2.802	5.475
G			-	1.000
T				-

Tree 2				
	A	C	G	T
A	-	0.950	5.016	1.862
C		-	2.898	5.648
G			-	1.000
T				-

The tree likelihood score of maximum-likelihood tree one under the GTR+Γ with a molecular clock forced (-2726.28) was worse than the previously estimated GTR+Γ model with no molecular clock enforced (-2443.51). Therefore the null model was rejected showing the data does not conform to the molecular clock hypothesis.

The phylogeny in Figure 6.14 was therefore considered the maximum-likelihood estimate of the 16S phylogeny: *O. edulis* and *O. turbinatus* form a sister clade to *O. lineatus. O. atratus* is basal to *O. lineatus, O. edulis* + *O. turbinatus*. *O. articulatus* is basal in the Atlantic/Mediterranean *Osilinus* monophyletic group. The outgroup taxa *O.kotschyi* and *G. umbilicalis* are sister taxa to the monophyletic Atlantic/Mediterranean *Osilinus* clade.

The 16S subset data was also subjected to the Modeltest software v3.0 (Posada & Crandall, 1998). This used a neighbour-joining tree as the starting tree to estimate the likelihood score under the 56 models within the GTR family of DNA substitution models. Although the model of best fit was not expected to change, estimation of the substitution rate matrix and among-site variation may have been affected by the smaller data set. The GTR+G model was retained as the most suitable with an estimated rate matrix (Table 6.16), base frequencies estimated at A = 0.31, C = 0.11, G = 0.21, T = 0.37 and a gamma shape estimate of 0.15.

Table 6.16. Modeltestv3.0 derived DNA substitution rate matrix estimated by likelihood analysis of a neighbour-joining tree one under the GTR+Γ model. The nucleotide bases are represented by A = adenine, G = guanine, C = cytosine and T = thymine.

	A	C	G	T
A	-	0.038	6.675	1.259
C		-	1.183	4.855
G			-	1.000
T				-

These settings and estimates were employed in a likelihood heuristic search. The starting tree was obtained via stepwise addition with one tree held at each step. The NNI branch-swapping algorithm was enforced, branches were collapsed with branch lengths less than zero. Three best trees of likelihood score 1638.14 were found (Figure 6.15). In all three best trees the taxa *O.kotschyi* and *G. umbilicalis* form a monophyletic outgroup basal to the Mediterranean /Atlantic *Osilinus* clade. Sister species *O. turbinatus* and *O. edulis* form a clade with *O. lineatus* as basal. Trees (i) and (ii) retain the same topology but not leaf taxa; tree (i) places *O. atratus* as basal to the Mediterranean /Atlantic *Osilinus* clade and tree (ii) *O. articulatus* as basal. Tree (iii) is unresolved with respect to the relationship of *O. atratus* and *O. articulatus*.

The maximum-likelihood heuristic search was repeated as a bootstrap analysis with 100 replicates with all 474 characters re-sampled in each replicate. The bootstrap 50% majority-rule consensus tree is shown in Figure 6.16. The Atlantic/Mediterranean *Gibbula* and *Osilinus* species form a monophyletic group. *O. edulis* and *O. turbinatus* remain sister species but the phylogenetic relationship of remaining species clades is unresolved, reflected in the *O. lineatus, O. atratus, O. articulatus* polytomy.

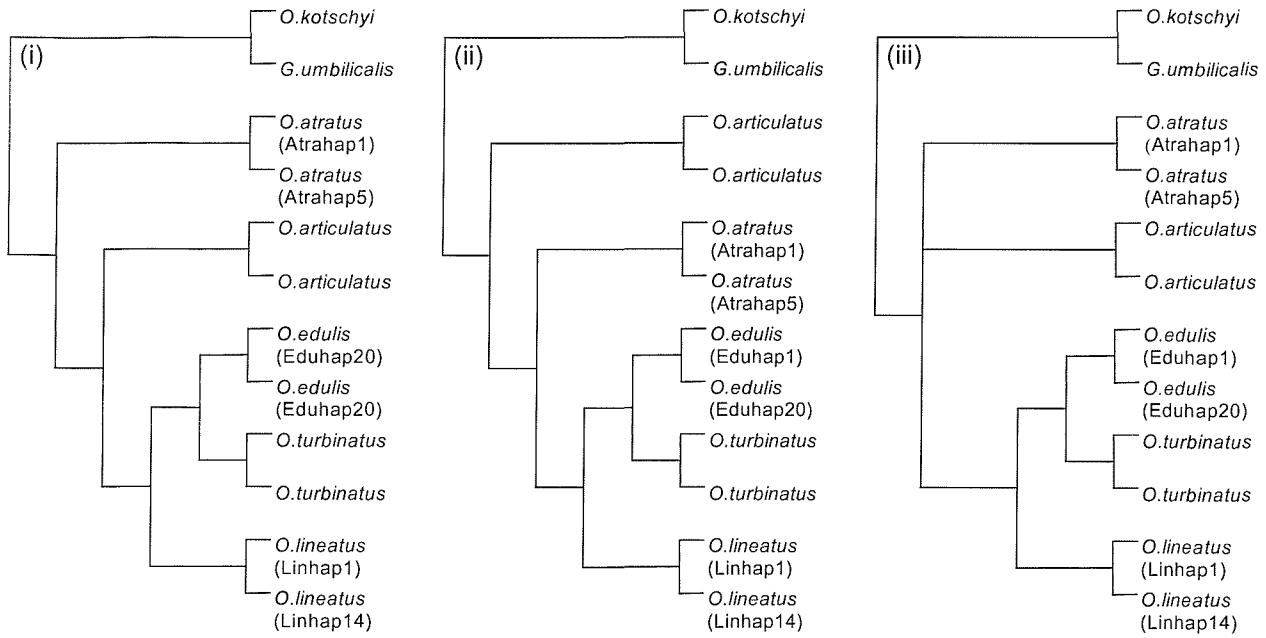


Figure 6.15. 16S mtDNA Maximum-likelihood heuristic search tree best trees with likelihood score –ln1638.14. Trees (i) and (iii) retain the same topology but tree (i) places *O. atratus* as basal to the Mediterranean /Atlantic *Osilinus* clade and tree (ii) *O. articulatus* as basal. Tree (iii) is unresolved with respect to the relationship of *O. atratus* and *O. articulatus.*

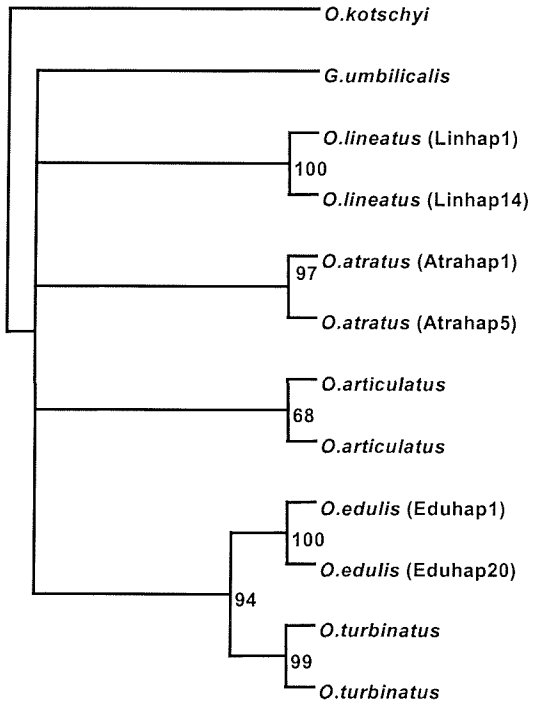


Figure 6.16. 16S mtDNA Maximum-likelihood heuristic search parametric bootstrap 50% majority-rule consensus tree. Bootstrap support values are shown at the right of nodes.

6.3.9 Phylogenetic analysis using COI sequence data

The COI neighbour-joining bootstrap tree is given in Figure 6.17 rooted with *G. umbilicalis*. All species form clades with 100% bootstrap support. The NJ tree supports an *O. lineatus* and *O. atratus* clade (73%), and an *O. articulatus, O. turbinatus* and *O. edulis* clade (37%). Within the latter, *O. articulatus* is basal to *O. turbinatus* and *O. edulis*, 44% bootstrap support is conferred on *O. turbinatus* and *O. edulis* as sister species, with the former basal. The COI *O. turbinatus* clade exhibits strong support for two separate clades (99.5% and 98.6%) with clear geographic patterning. Samples from Calahonda, Malaga, Spain from a distinct sister clade to samples collected from Puerto Duguesa in Spain, Cyprus and Corinth, Greece.

Maximum-parsimony analysis

All 74 COI sequences were included in a heuristic maximum-parsimony search. All characters were unordered and gaps were treated as a fifth base. Parsimony uninformative characters were weighted as zero, with all parsimony informative characters (168) being assigned a base weight of one. The starting tree was obtained via stepwise addition with ten random addition sequence replicates. One tree was held at each step and the tree-bisection-algorithm (TBR) branch-swapping algorithm was enforced. Branches were collapsed if maximum branch length equalled zero. No topological constraints were enforced. The search tried 1217068 rearrangements and found four best trees of total length 343 (Figure 6.18). Each tree was only found (hit) once, and all four best trees shared the same interspecific clade topology.

The maximum-parsimony phylogeny is rooted by the outgroup taxa *G. umbilicalis*. The *Osilinus* ingroup phylogeny is considerably divergent from the neighbour-joining phylogeny estimate (Figure 6.17). All species clades are monophyletic: *O. turbinatus* is basal to the group containing *O. articulatus, O. edulis, O. lineatus* and *O. atratus*. Within that group *O. articulatus* is basal. The two distinct clades present in the neighbour-joining tree including all *Osilinus* spp. are not apparent in the maximum-parsimony phylogeny (Figure 6.18). Two separate clades are estimated regarding *O. edulis, O. lineatus* and *O. atratus; O. edulis* is a monophyletic sister clade to the *O. lineatus* + *O. atratus* clade.

The intraspecific clades constant between the four best trees are circled (Figure 6.18). Within *O. turbinatus,* the Puerto Duguesa Spain, Cyprus and Corinth, Greece samples retain the same topology, with the Calahonda, Malaga Spain samples as basal. All four best trees also retain the *O. edulis* Madeiran samples exclusively present in a monophyletic clade along side the Tifnit and Cap Ghir Moroccan samples. The topology of the remaining *O. edulis* is variable between the four trees with no clear geographic distribution of the branches, or clear phylogenetic resolution. Within the *O. lineatus* intraspecific phylogeny, three clades are constant with the remaining four taxa differing in their basal

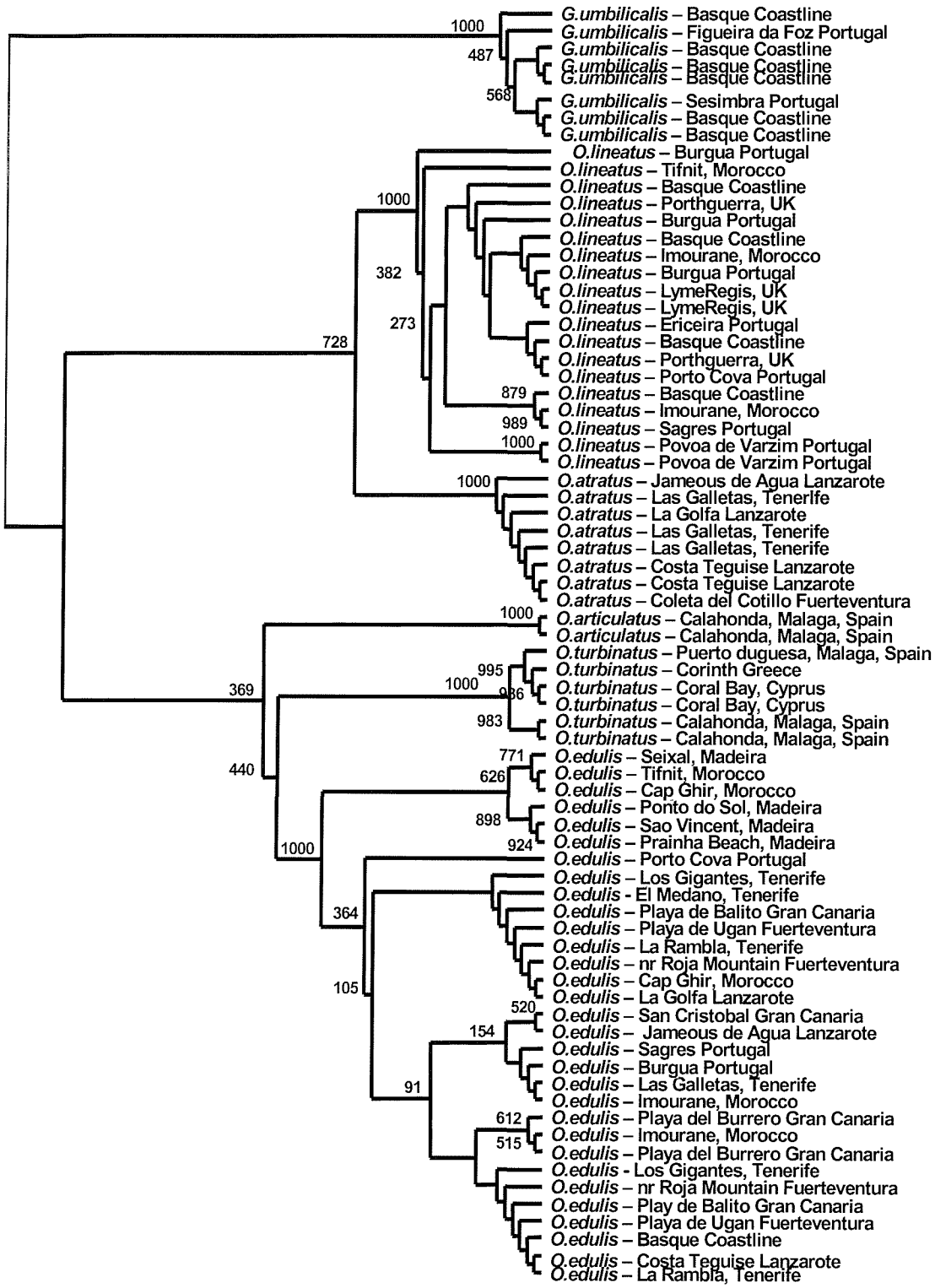


Figure 6.17. Neighbour-joining phylogeny of the 74 mtDNA COI partial gene sequences with bootstrap analysis (1000 replicates). Bootstrap support values are shown to the left of nodes.

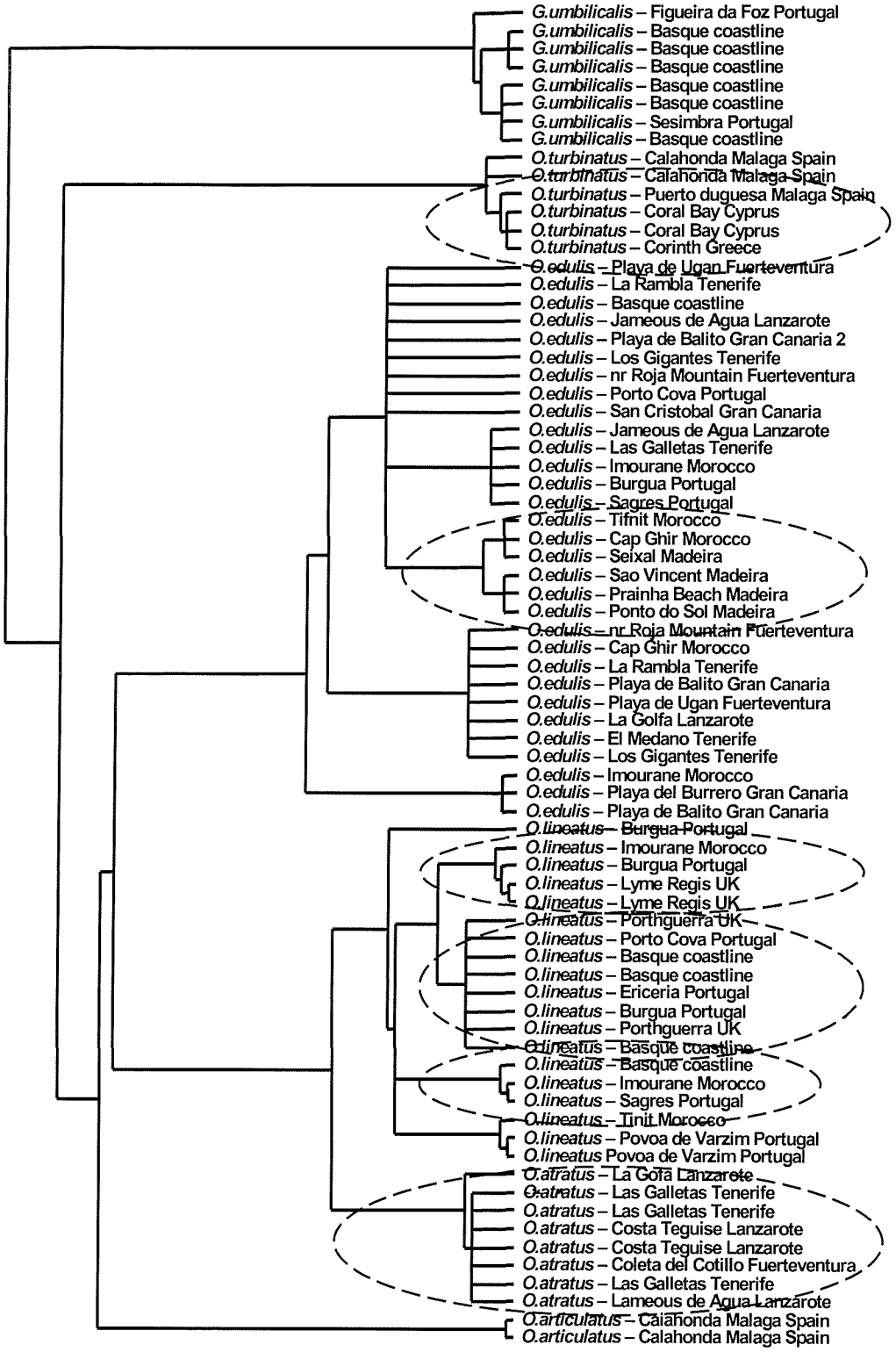


Figure 6.18. COI mtDNA maximum-parsimony tree TL 343. Representative of the four best trees found in the heuristic search. The interspecific tree topology was constant between the four trees. The intraspecific clade topologies shared by all four trees are circled.

position within the clade. The largest of the *O. lineatus* clades contains sequences sampled from the UK, Basque coastline and Portugal. This is potentially a reflection of a northern population albeit lacking any internal phylogenetic structure. This geographic bias is not shared by the remaining two consensus clades. One clade includes Moroccan, Portuguese and UK sequences; the second consists of Basque, Portuguese and Moroccan samples. This indicates, as in the neighbour-joining phylogeny, the gene tree is perhaps not reflective of the current biogeographical distribution at the intraspecific level within the given sample size. The topology of the entire *O. atratus* intraspecific clade is shared by all four best trees, but is also unresolved showing no internal phylogenetic or geographical structure.

The maximum-parsimony analysis was repeated as a bootstrap (Figure 6.19). The bootstrap analysis (100 replications) supports distinct species status for all taxa involved in the analysis: 100% bootstrap support is given to all species as monophyletic groups (clades). The interspecific phylogenetic relationships are, however, partially unresolved. *O. articulatus* is placed basal to the remaining *Osilinus* taxa by the 50% majority rule consensus tree. *O. lineatus* and *O. atratus* have 74% bootstrap support value as sister clades, as in the neighbour-joining and maximum-parsimony searches. The phylogenetic relationships of *O. edulis* and *O. turbinatus* within the genus remain unresolved, forming a trichotomy with the *O. lineatus* + *O. atratus* clade. This polytomy may also be interpreted as simultaneous divergence, although this seems unlikely given the diversity in interspecific phylogeny estimates produced under the analyses performed so far.

Some intraspecific geographic patterning is evident in the maximum-parsimony bootstrap analysis tree; 63% support is conferred on the Madeiran and Moroccan *O. edulis* clade, containing a three-sample Madeiran clade with 72% bootstrap support value. The *O. turbinatus* clade exhibits the strongest biogeographical pattern within the phylogeny. 97% bootstrap support is conferred on the Cyprus and Corinth, Greece samples as a monophyletic group relative to the Calahonda and Puerto Duguesa Spanish samples. The Puerto Duguesa, Spain sample is basal to the Cyprus and Corinth, Greece samples with 61% support. The Calahonda, Spain samples also have 61% bootstrap support as a monophyletic group basal to the remainder of the *O. turbinatus* clade. The *O. atratus* intraspecific phylogeny is an unresolved polytomy. This is also the case for the majority of the *O. lineatus* clade. Although high bootstrap support (98%, 97% and 95%) is given to three clades within the *O. lineatus* clade, as in the previous analyses, they do not contain geographically adjacent samples.

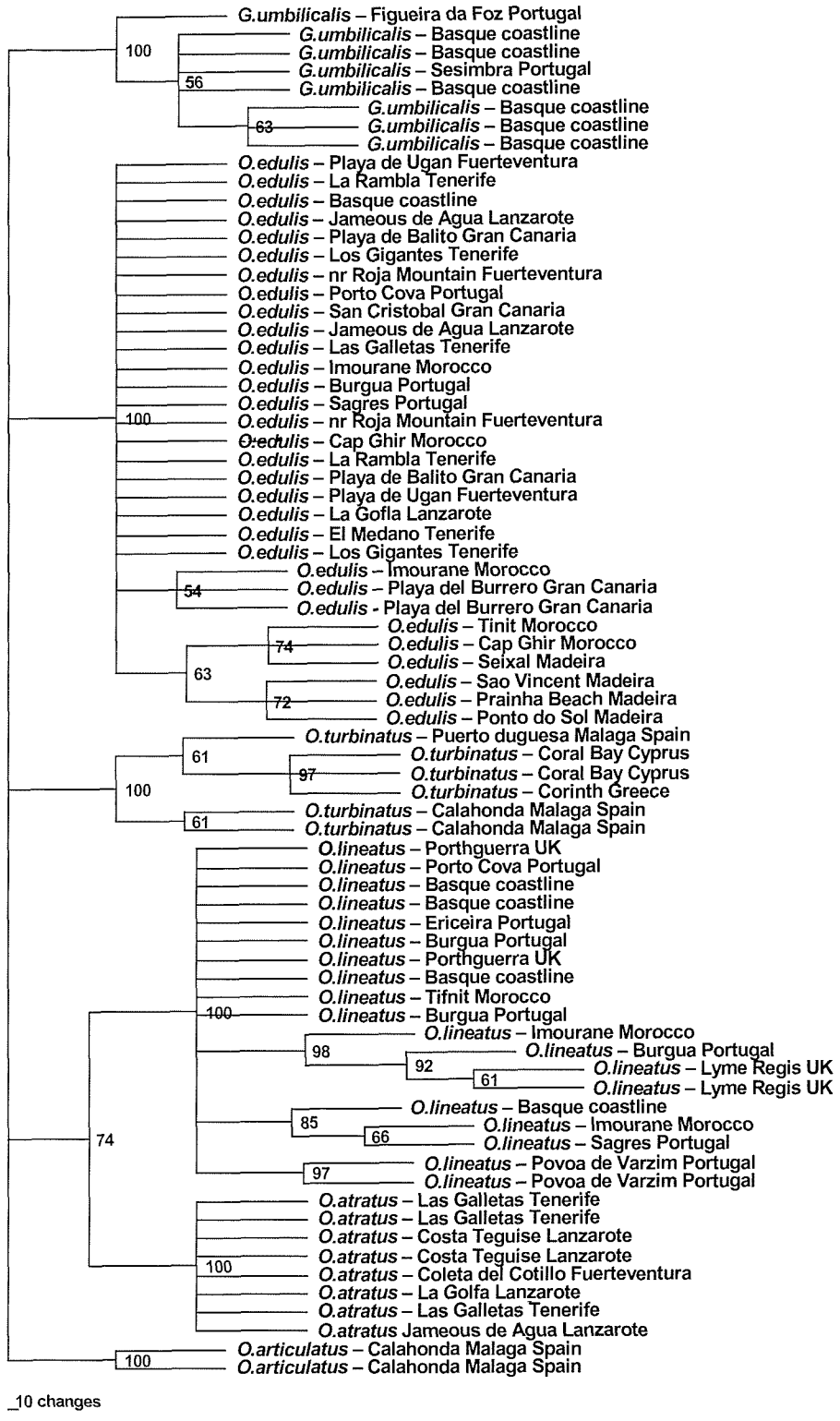


Figure 6.19. COI mtDNA Maximum-parsimony 50% majority-rule consensus phylogram. 100 bootstrap replicates were performed. Support values greater than 50% are shown to the right of relevant nodes. The scale at the left bottom corner of the tree indicates the branch length (number of evolutionary steps, i.e. substitutions).

A total of 30/74 sequences were chosen for the maximum-parsimony subset analysis (Table 6.17).

Table 6.17: List of species and samples included in the COI maximum-parsimony subset analysis with map reference (refers to Figures 3.2 and 3.3), species ID number, species name and sample location.

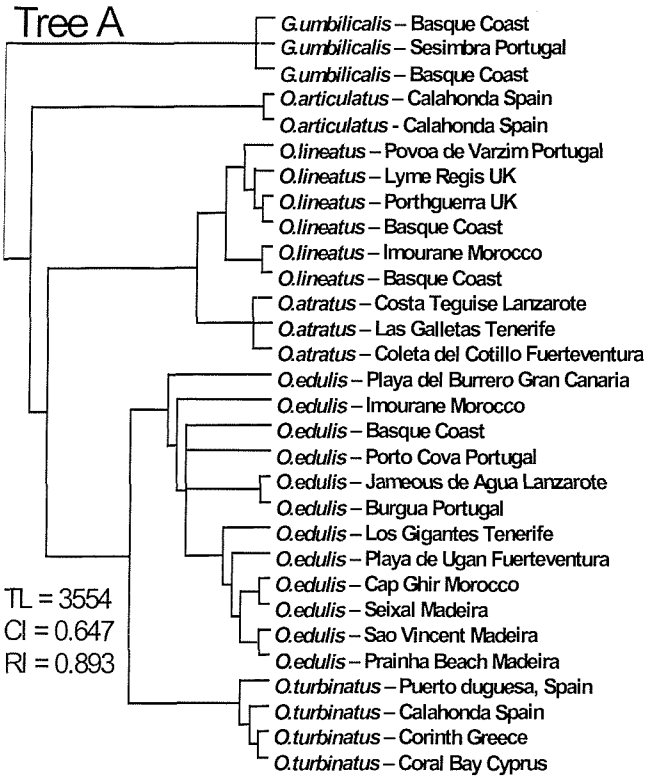
Map Ref	Species Id	Species	Location
3	246	*Gibbula umbilicalis*	Basque coastline
28	338	*Gibbula umbilicalis*	La Golfa, Lanzarote
44	436	*Gibbula umbilicalis*	Porto Cova, Portugal
12	029	*Osilinus lineatus*	Imourane, Morocco
1	133	*Osilinus lineatus*	Lyme Regis, UK
2	139	*Osilinus lineatus*	Cornwall, UK
3	235	*Osilinus lineatus*	Basque coastline
9	251	*Osilinus lineatus*	Corinth, Greece
41	452	*Osilinus lineatus*	Figueira da Foz, Portugal
5	091	*O. articulatus*	Calahonda, Spain
5	093	*O. articulatus*	Calahonda, Spain
14	069	*O. atratus*	Las Galletas, Tenerife
26	277	*O. atratus*	Costa Teguise, Lanzarote
19	321	*O. atratus*	Coleta del Cotillo, Fuerteventura
12	031	*O. edulis*	Imourane, Morocco
13	054	*O. edulis*	Cap Ghir, Morocco
15	076	*O. edulis*	Los Gigantes, Tenerife
39	182	*O. edulis*	Seixal, Madeira
38	183	*O. edulis*	Sao Vincent, Madeira
36	192	*O. edulis*	Prainha Beach, Madeira
3	226	*O. edulis*	Basque Coastline
27	263	*O. edulis*	Jameous de Agua, Lanzarote
24	339	*O. edulis*	Playa de Ugan, Fuerteventura
31	354	*O. edulis*	Playa del Burrero, Gran Canaria
45	464	*O. edulis*	Sagres, Portugal
48	493	*O. edulis*	Burgua, Portugal
7	102	*O. turbinatus*	Puerto duguesa, Spain
5	112	*O. turbinatus*	Calahonda, Spain
8	124	*O. turbinatus*	Coral Bay, Cyprus
9	256	*O. turbinatus*	Corinth, Greece

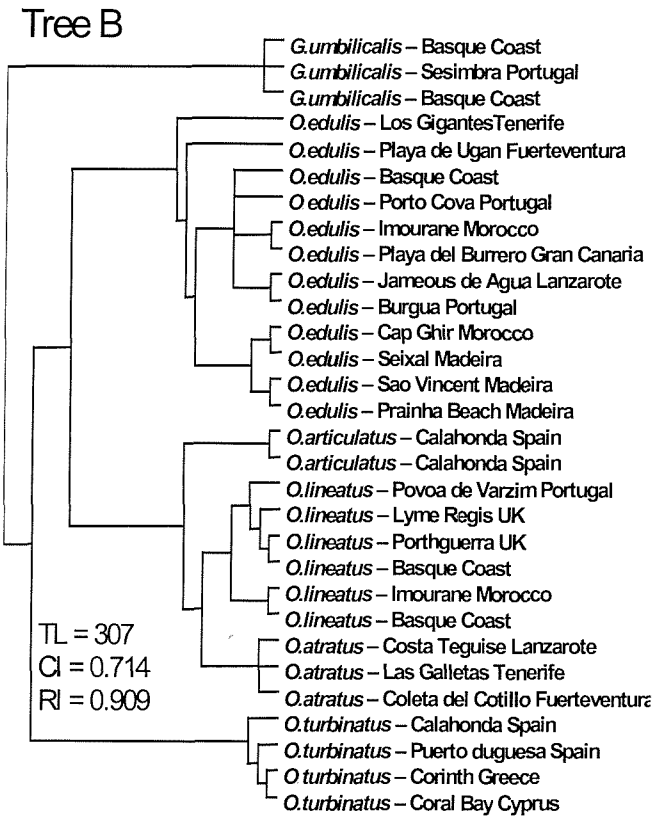
Maximum-parsimony analysis was performed under several character types and weighting schemes to produce a suite of starting trees. Unless stated, the heuristic search criteria were kept constant for all analysis. Starting trees were obtained via stepwise addition, with 100 random addition sequence replicates and 10 trees held at each step. The Tree-bisection-reconnection (TBR) branch- swapping algorithm was in effect, with branches collapsed if the maximum branch length equalled zero. The initial number of maximum trees was set at 100 with 100 increments if required. No topological constraints were enforced on the trees, which were unrooted.

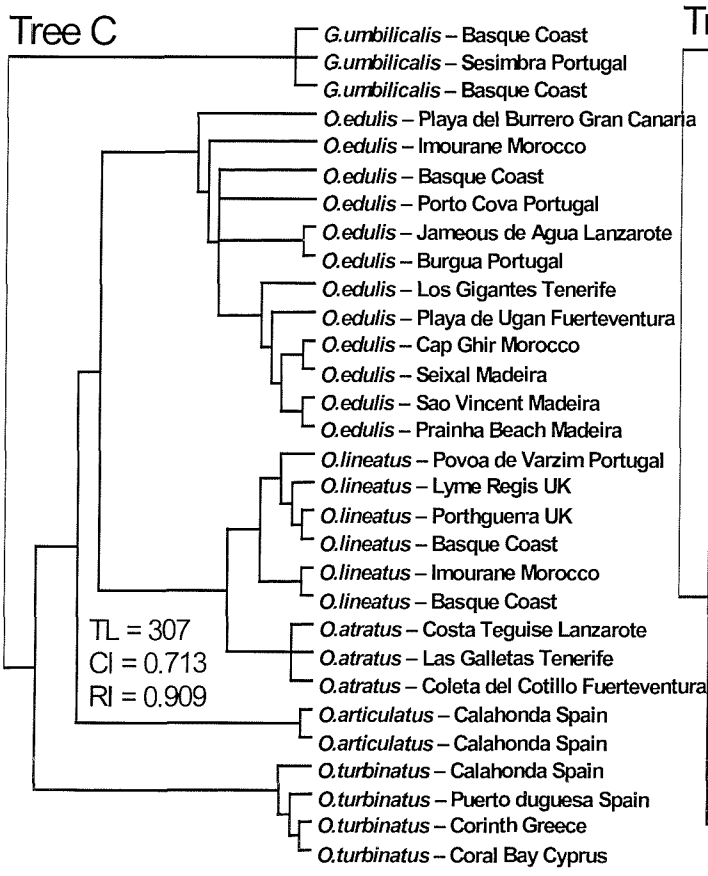
The heuristic parsimony searches were performed under the following criteria: all characters of equal weights for all positions and transformation types, characters unordered and gaps treated as a fifth base. All characters of equal weights for all positions transformation types, characters ordered and gaps treated as a fifth base. All characters were assigned equal weights for all positions, with transversion/transition weight ratio of 2:1, gaps treated as missing. All characters assigned equal weights for all positions, with transversion/transition weight ratio of 5:1, gaps treated as missing. The transformation weightings were enforced using a user defined step matrix (see Table 5.2, chapter 5). When gaps were treated as a fifth base, the 534 base pair sequence data set contained 356 constant characters, 26 variable parsimony uninformative characters and

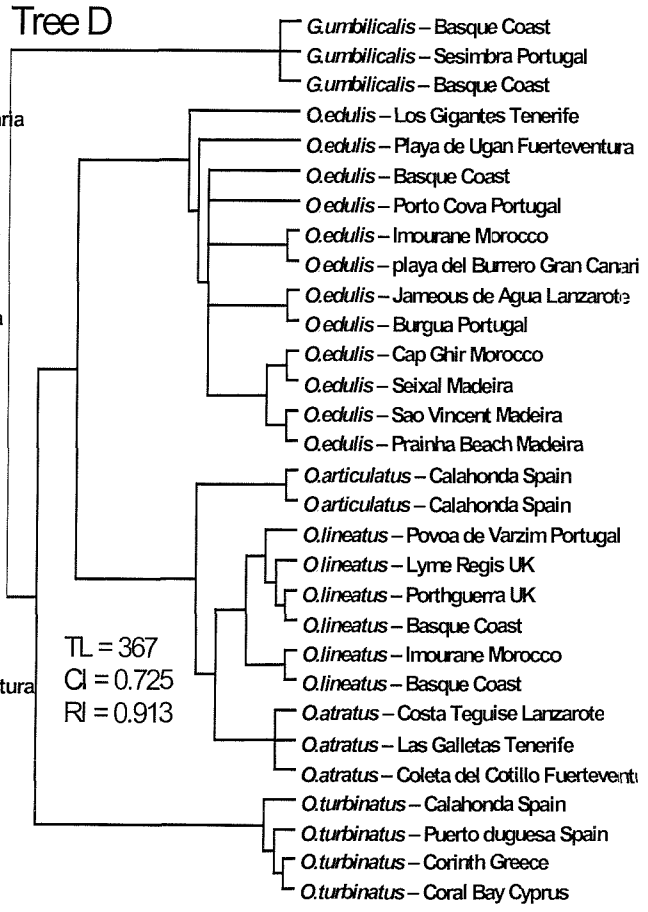
152 parsimony-informative characters. When gaps were treated as missing data the character status changed to 19 variable parsimony uninformative characters and 150 parsimony informative characters.

The ordered (Wagner) parsimony heuristic search tried 739,436 rearrangements and found two best trees of score 3554. The unordered parsimony heuristic search tried 587,719 rearrangements and found 12 best trees of score 307. The weighted transformation type parsimony search under the 2:1 tv/ts ratio performed 54,395,355 rearrangements and retained 36 trees of best score 367. When the 5:1 tv/ts ratio was imposed, 5,601,203 rearrangements produced 180 best trees of score 574. All 230 trees produced under the maximum-parsimony analyses above were examined for differences in tree topology and a representative suite of trees was subsequently chosen (Figure 6.20). The two trees produced by the Wagner parsimony analysis shared the same topological relationships between all species, but differed in topology within the *O. turbinatus* clade. Tree A was arbitrarily chosen from the two (Figure 6.20). The 12 trees produced under unordered parsimony contained 2 basic tree topologies with regard to interspecific relationships, with all 12 trees differing with in the *Osilinus edulis* clade (Trees B & C, Figure 6.20). All of the 36 best scoring trees produced by weighted parsimony (2:1) shared the same interspecific topological relationships, but varied within intraspecific relationships (Tree D, Figure 6.20). Again, all 180 trees found by 5:1 weighted parsimony shared the same interspecific topological relationships (Tree E, Figure 6.20). The CI and RI tree scores for all five trees were calculated under parsimony.


Tree A
G.umbilicalis – Basque Coast
G.umbilicalis – Sesimbra Portugal
G.umbilicalis – Basque Coast
O.articulatus – Calahonda Spain
O.articulatus - Calahonda Spain
O.lineatus – Povoa de Varzim Portugal
O.lineatus – Lyme Regis UK
O.lineatus – Porthguerra UK
O.lineatus – Basque Coast
O.lineatus – Imourane Morocco
O.lineatus – Basque Coast
O.atratus – Costa Teguise Lanzarote
O.atratus – Las Galletas Tenerife
O.atratus – Coleta del Cotillo Fuerteventura
O.edulis – Playa del Burrero Gran Canaria
O.edulis – Imourane Morocco
O.edulis – Basque Coast
O.edulis – Porto Cova Portugal
O.edulis – Jameous de Agua Lanzarote
O.edulis – Burgua Portugal
O.edulis – Los Gigantes Tenerife
O.edulis – Playa de Ugan Fuerteventura
O.edulis – Cap Ghir Morocco
O.edulis – Seixal Madeira
O.edulis – Sao Vincent Madeira
O.edulis – Prainha Beach Madeira
O.turbinatus – Puerto duguesa, Spain
O.turbinatus – Calahonda Spain
O.turbinatus – Corinth Greece
O.turbinatus – Coral Bay Cyprus
TL = 3554
CI = 0.647
RI = 0.893


Tree B
G.umbilicalis – Basque Coast
G.umbilicalis – Sesimbra Portugal
G.umbilicalis – Basque Coast
O.edulis – Los GigantesTenerife
O.edulis – Playa de Ugan Fuerteventura
O.edulis – Basque Coast
O.edulis – Porto Cova Portugal
O.edulis – Imourane Morocco
O.edulis – Playa del Burrero Gran Canaria
O.edulis – Jameous de Agua Lanzarote
O.edulis – Burgua Portugal
O.edulis – Cap Ghir Morocco
O.edulis – Seixal Madeira
O.edulis – Sao Vincent Madeira
O.edulis – Prainha Beach Madeira
O.articulatus – Calahonda Spain
O.articulatus – Calahonda Spain
O.lineatus – Povoa de Varzim Portugal
O.lineatus – Lyme Regis UK
O.lineatus – Porthguerra UK
O.lineatus – Basque Coast
O.lineatus – Imourane Morocco
O.lineatus – Basque Coast
O.atratus – Costa Teguise Lanzarote
O.atratus – Las Galletas Tenerife
O.atratus – Coleta del Cotillo Fuerteventura
O.turbinatus – Calahonda Spain
O.turbinatus – Puerto duguesa Spain
O turbinatus – Corinth Greece
O.turbinatus – Coral Bay Cyprus
TL = 307
CI = 0.714
RI = 0.909


Tree C
G.umbilicalis – Basque Coast
G.umbilicalis – Sesimbra Portugal
G.umbilicalis – Basque Coast
O.edulis – Playa del Burrero Gran Canaria
O.edulis – Imourane Morocco
O.edulis – Basque Coast
O.edulis – Porto Cova Portugal
O.edulis – Jameous de Agua Lanzarote
O.edulis – Burgua Portugal
O.edulis – Los Gigantes Tenerife
O.edulis – Playa de Ugan Fuerteventura
O.edulis – Cap Ghir Morocco
O.edulis – Seixal Madeira
O.edulis – Sao Vincent Madeira
O.edulis – Prainha Beach Madeira
O.lineatus – Povoa de Varzim Portugal
O.lineatus – Lyme Regis UK
O.lineatus – Porthguerra UK
O.lineatus – Basque Coast
O.lineatus – Imourane Morocco
O.lineatus – Basque Coast
O.atratus – Costa Teguise Lanzarote
O.atratus – Las Galletas Tenerife
O.atratus – Coleta del Cotillo Fuerteventura
O.articulatus – Calahonda Spain
O.articulatus – Calahonda Spain
O.turbinatus – Calahonda Spain
O.turbinatus – Puerto duguesa Spain
O.turbinatus – Corinth Greece
O.turbinatus – Coral Bay Cyprus
TL = 307
CI = 0.713
RI = 0.909


Tree D
G.umbilicalis – Basque Coast
G.umbilicalis – Sesimbra Portugal
G.umbilicalis – Basque Coast
O.edulis – Los Gigantes Tenerife
O.edulis – Playa de Ugan Fuerteventura
O.edulis – Basque Coast
O edulis – Porto Cova Portugal
O.edulis – Imourane Morocco
O edulis – playa del Burrero Gran Canari
O.edulis – Jameous de Agua Lanzarote
O edulis – Burgua Portugal
O.edulis – Cap Ghir Morocco
O.edulis – Seixal Madeira
O.edulis – Sao Vincent Madeira
O.edulis – Prainha Beach Madeira
O.articulatus – Calahonda Spain
O articulatus – Calahonda Spain
O.lineatus – Povoa de Varzim Portugal
O lineatus – Lyme Regis UK
O.lineatus – Porthguerra UK
O.lineatus – Basque Coast
O.lineatus – Imourane Morocco
O.lineatus – Basque Coast
O.atratus – Costa Teguise Lanzarote
O.atratus – Las Galletas Tenerife
O.atratus – Coleta del Cotillo Fuerteventu
O.turbinatus – Calahonda Spain
O.turbinatus – Puerto duguesa Spain
O.turbinatus – Corinth Greece
O.turbinatus – Coral Bay Cyprus
TL = 367
CI = 0.725
RI = 0.913

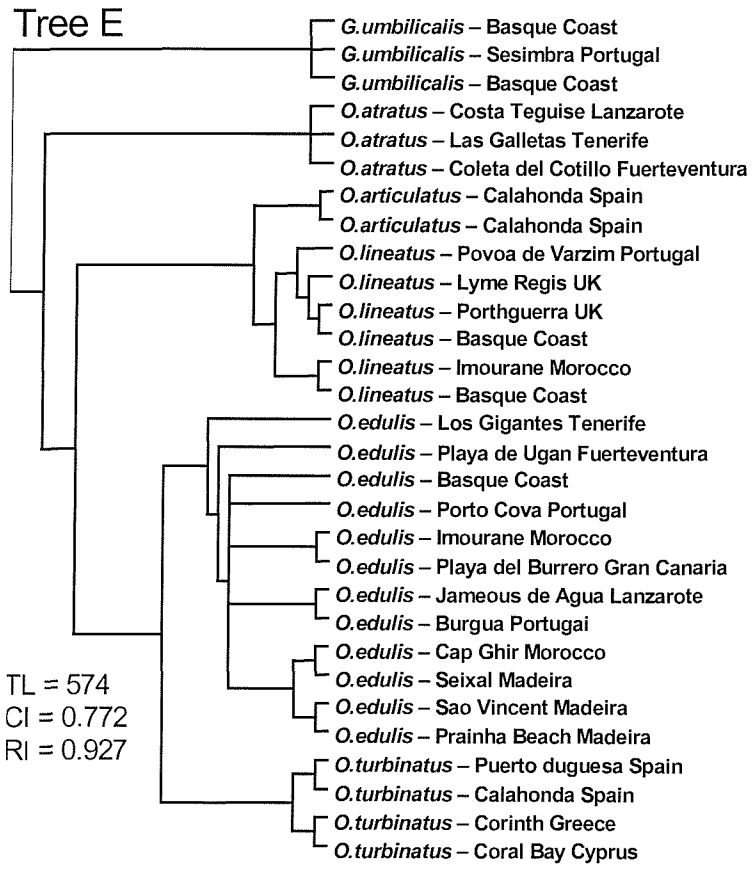


Figure 20: COI maximum parsimony trees produced by heuristic searches under the weighting schemes and character types described in the text. All trees are representative of the interspecific topologies produced under each parsimony analysis. Tree A is representative of the 12 tree topologies produced by the Wagner parsimony analysis. Trees B & C: representative of the 12 tree topologies produced by unordered parsimony analysis. Tree D: representative of the 36 tree topologies produced by the Fitch (2:1 TS/TV) parsimony analysis. Tree E: representative of the 36 tree topologies produced by the Fitch (5:1 TS/TV) parsimony analysis.

Two of the five tree topologies in Figure 6.20 shared the same interspecific phylogeny within *Osilinus*. Trees B and D estimated *O. turbinatus* as basal to the remaining ingroup speces. *O. edulis* formed a monophyletic sister clade to the *O. lineatus* + *O. atratus* + *O. articulatus* clade, in which the latter was basal. Tree A placed *O. articulatus* as basal to a phylogeny indicating two separate radiations: *O. lineatus* and *O. atratus* were sister species in one clade, and *O. turbinatus* and *O. edulis* sister species in the other. Tree E estimated a similar phylogeny but with *O. atratus* as basal to the *Osilinus* phylogeny and *O. articulatus* as the sister species to *O. lineatus*. Tree C exhibited the same interspecific phylogenetic relationships as the large maximum-parsimony phylogeny (Figure 6.19): *O. articulatus* was basal to the clade consisting of *O. edulis* as sister to the *O. lineatus* + *O. atratus* clade; in this tree *O. turbinatus* was basal within the ingroup *Osilinus* phylogeny.

As representative of the 230 trees produced under the various maximum-parsimony subset analyses, the four tree topologies represented in Figure 6.20 confirm the lack of complete resolution within the COI sequence data regarding the relative phylogenetic relationships of *O. turbinatus, O. articulatus* and *O. atratus* within the *Osilinus* genus. *O. edulis* and *O. lineatus,*

however, remain sister species in a dichotomous clade throughout the starting tree suite and previous maximum-parsimony and neighbour-joining analyses, supporting two separate radiations during the speciation of *Osilinus*. The five starting trees were subsequently used to estimate the tree likelihood scores under various models of DNA substitution to establish the most suitable model for maximum-likelihood analysis given the COI data set (Table 6.5).

Maximum-likelihood analysis

The likelihood score for all five COI maximum-parsimony trees (Figure 6.20) was estimated under various DNA substitution models within the GTR family to establish the most suitable model for the COI data set (Table 6.18). *G. umbilicalis* rooted all trees.

Table 6.18. Likelihood scores of the five maximum-parsimony COI mtDNA starting trees (Figure 6.20) under various nested models of DNA substitution

Model of DNA substitution	-*ln*L score Tree 1	Tree 2	Tree 3	Tree 4	Tree 5
JC	2345.30	2337.60	2339.49	2337.60	2355.62
F81	2331.68	2319.12	2321.98	2318.94	2338.47
K2P	2226.32	2226.51	2227.76	2226.51	2231.58
HKY85	2209.35	2206.97	2209.54	2206.97	2213.68
HKY85 + G	2114.09	2116.30	2118.60	2116.30	2113.53
HKY + G + I	2113.71	2116.13	2117.97	2116.13	2113.85
GTR	2204.66	2200.75	2203.37	2200.75	2209.85

The tree likelihood scores were subsequently compared for goodness of fit using the Likelihood ratio test statistic, following the hierarchical hypothesis testing framework prescribed under the Modeltest software (Posada & Crandall, 1998). All five trees subjected to the likelihood ratio test accepted the same model, and therefore the HKY85 + Γ model of DNA substitution was chosen as best fitting the data set (Table 6.19).

Table 6.19. Likelihood ratio test of the likelihood tree scores of the five COI mtDNA maximum-parsimony trees under various models of DNA substitution. X^2 critical value at *P*= 0.01

P=0.01	Tree 1	Tree 2	Tree 3	Tree 4	Tree 5
JC vs. F81 3 df, X^2 critical value = 11.34	27.24 Reject JC	36.96 Reject JC	35.02 Reject JC	37.32 Reject JC	34.30 Reject JC
F81 vs. HKY 1 df X^2 critical value = 6.63	244.66 Reject F81	224.30 Reject F81	224.88 Reject F81	223.94 Reject F81	249.58 Reject F81
HKY vs. GTR 4 df X^2 critical value = 13.28	9.38 Accept HKY	12.44 Accept HKY	12.34 Accept HKY	12.44 Accept HKY	7.66 Accept HKY
HKY vs. HKY+G 1 df X^2 critical value = 6.63	190.52 Reject HKY85	181.34 Reject HKY85	181.88 Reject HKY85	181.34 Reject HKY85	200.30 Reject HKY85
HKY+GvsHKY+G+I	0.76 Accept HKY+G	0.34 Accept HKY+G	1.26 Accept HKY+G	0.36 Accept HKY+G	-0.27 Accept HKY+G

A heuristic maximum-likelihood search was performed on all 74 sequences using the HKY85 + Γ model of DNA substitution. This model assumes two substitution types, transitions and transversions, unequal nucleotide frequencies and allows different mutation/variation rates at variable sites along the DNA sequence (gamma distribution of rates at variable sites). The shape of the gamma distribution (alpha) and the transition/transversion ratio was estimated by the search. Empirical base frequencies were assumed. The starting tree was obtained via stepwise addition with ten replicates of random sequence addition. One tree was held at each stepwise addition. The nearest-neighbour interchange (NNI) branch-swapping algorithm was in effect. Branches were collapsed if maximum branch length equalled zero. No topological constraints were enforced and the tree was unrooted. The search produced one best tree of likelihood score –lnL - 2576.85 (Figure 6.21). The transition/transversion ratio was estimated at 2.76, kappa at 5.63. Alpha was estimated at 0.18236.

The maximum-likelihood search was repeated as a bootstrap analysis (100 replicates) with replacement. All search criteria and likelihood settings were kept constant; the transversion/transition ratio and gamma distribution shape estimated in the heuristic search were assumed. The bootstrap 50% majority rule consensus phylogram is shown in Figure 6.22.

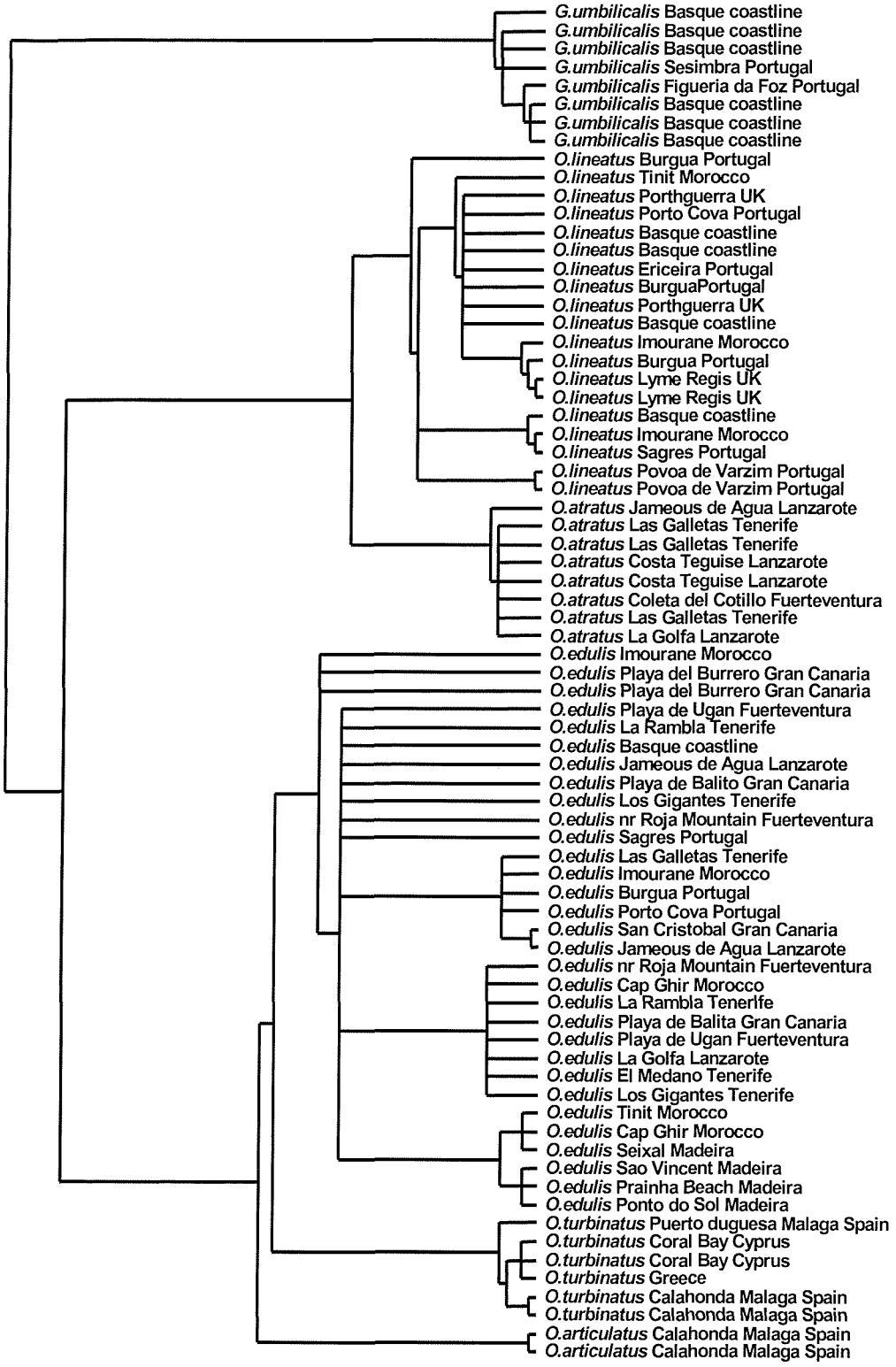


Figure 6.21. COI mtDNA Maximum-likelihood tree found in a heuristic search assuming the HKY85 + Γ model of DNA substituion (Tree likelihood score = –lnL 2576.85).

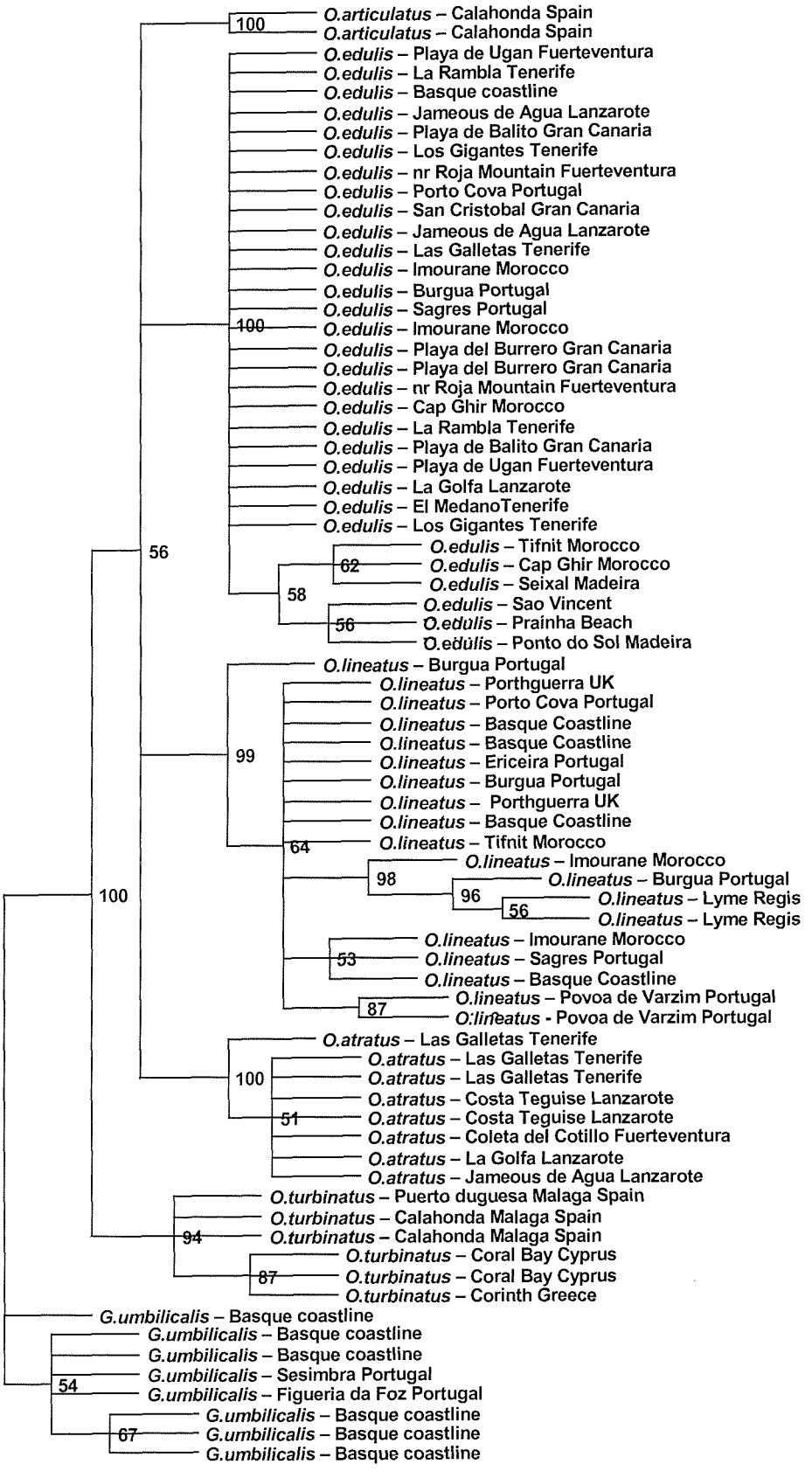


Figure 6.22. The COI mtDNA gene fragment maximum-likelihood bootstrap analysis 50% majority-rule consensus phylogram. Bootstrap support values are shown to the right of nodes.

The maximum-likelihood heuristic search produced a tree topology showing two radiations within the Atlantic/Mediterranean *Osilinus*. One clade contained *O. atratus* and *O. lineatus*. The second clade contained *O. edulis, O. turbinatus* and *O. articulatus*, with the latter basal to *O. edulis* and *O. turbinatus* as sister species. The bootstrap analysis did not reflect these interspecific relationships: 100% bootstrap support was given to *O. turbinatus* as the most recent common ancestor to the remaining *Osilinus* species. The 50% majority-rule consensus tree, however, placed *O. articulatus, O. atratus, O. lineatus* and *O. edulis* as a polytomy. This could reflect a simultaneous explosive radiation within *Osilinus.* The lack of phylogenetic resolution present in the sequence data for determining interspecific relationships is, however, the most likely explanation.

High bootstrap values (94% & 100%) were given to all species clades. The *O. turbinatus* clade exhibited geographic patterning. The Cyprus and Corinth, Greece samples were given 87% bootstrap support as a sister group to the Spanish samples. The *O. edulis* clade was largely unresolved except for the Madeiran and Moroccan samples which formed a bifurcating clade with 58% bootstrap support, containing an exclusively Madeiran clade (56%) and a clade containing a Madeiran and Moroccan sample. As in the previous analyses, this is notable because of the absence of Madeiran samples in the remainder of the *O. edulis* intraspecific phylogeny.

6.3.10 Phylogenetic analysis using combined 16S and COI sequence data

Neighbour-joining analysis

NJ searches were performed on the combined data set with *G. umbilicalis* designated as the outgroup taxa using the absolute distance (uncorrected 'p') and General time reversible distance measures. Both searches found the same tree topology (Figure 6.23).

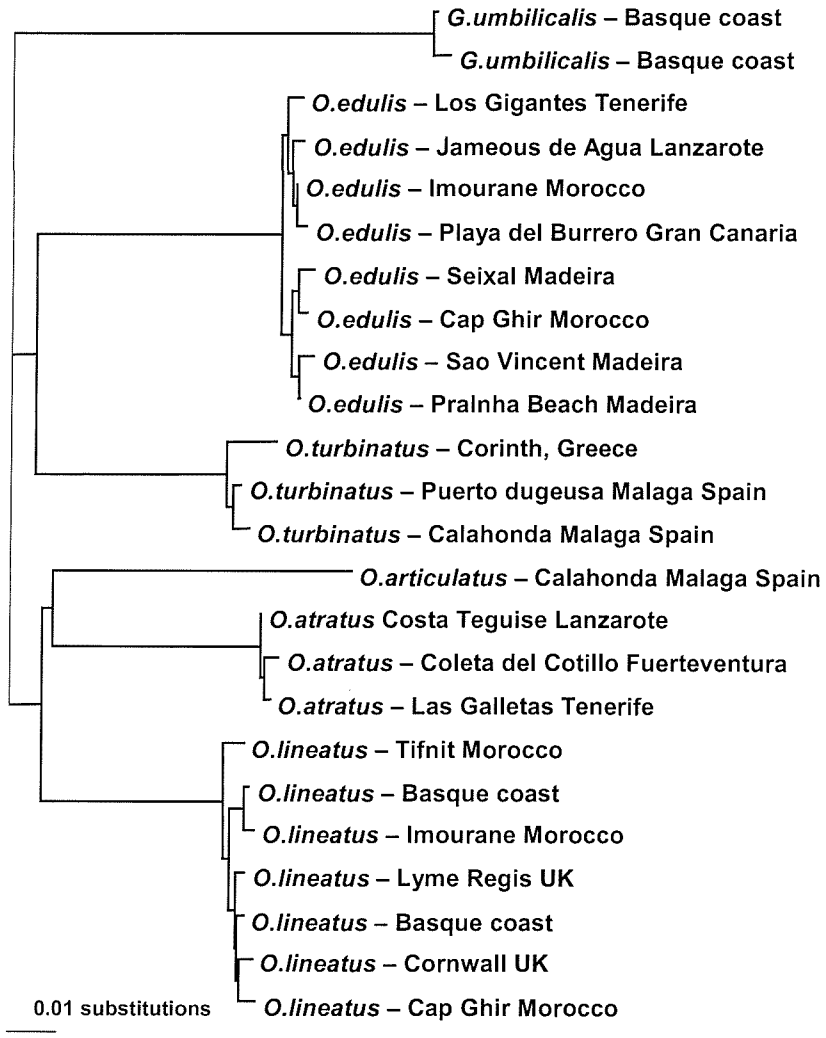


Figure 6.23. The 16S and COI combined mtDNA gene fragment sequence Neighbour-Joining phylogeny for both uncorrected and GTR distance measures.

The distance analysis (minimum evolution) was repeated as a bootstrap analysis with replacement (1000 replications), using the GTR distance measure. Starting trees were obtained by neighbour-joining and the TBR branch swapping algorithm was applied (Figure 6.24).

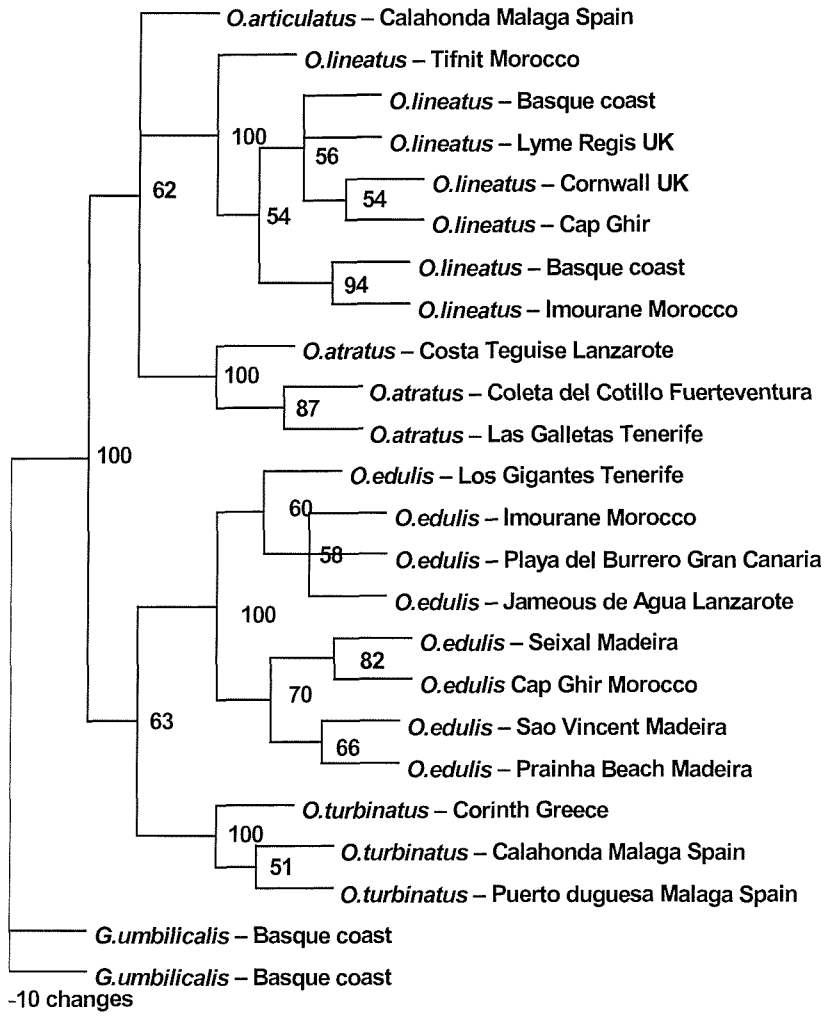


Figure 6.24. The 16S and COI combined mtDNA gene fragment Minimum Evolution Bootstrap 50% majority-rule consensus tree. The bootstrap support values are given to the right of nodes.

Both the neighbour-joining and minimum evolution phylogenies support all species as separate clades, with 100% bootstrap support. The phylogenies in Figures 6.23, 24 also reflect two major clades within the *Osilinus* Atlantic/ Mediterranean ingroup, as evident in the 16S and COI analyses above. *O. edulis* and *O. turbinatus* are sister species in a clade with 63% bootstrap support. Both trees placed *O. lineatus* + *O. articulatus* + *O. atratus* as a sister clade to the *O. edulis* + *O. turbinatus* clade. The neighbour-joining tree placed *O. articulatus* and *O. atratus* as a sister clade to *O. lineatus.* In the minimum evolution bootstrap analysis, the phylogenetic relationship of these three species lacks resolution. Within the *O. edulis* clade, 100% bootstrap support was conferred on two sister clades, defined geographically by the lack or presence of samples from Madeira. The Madeiran *O. edulis* clade has 70% support for 2 sister groups, one containing only Madeiran samples, the other a Madeiran and Moroccan sample. The *O. turbinatus* clade shows geographical patterning with 100% bootstrap support conferred on the Corinth, Greece sample as basal to the Spanish samples.

Maximum-parsimony analysis

A branch-and-bound maximum-parsimony search was performed on the combined data set. All characters were unordered and given equal weight. 735 of the 999 characters were constant, and therefore parsimony-uninformative. Of the variable characters, 43 were parsimony-uninformative and 221 parsimony-informative. The search retained 28 trees of total length (score) 440. All 28 best trees differed only in the intraspecific relationships of *O. edulis*. The three Madeiran and Cap Ghir Morocco sequences remained in a constant clade relative to the variable topologies of the remaining *O. edulis* sequences (representative tree given in Figure 6.25). The branch-and-bound search was repeated as a bootstrap analysis with replacement (1000 replicates), (see Figure 6.26).

The maximum-parsimony branch-and-bound phylogeny in Figure 6.25 places *O. articulatus* as basal to the Atlantic/ Mediterranean *Osilinus* clade. The remaining *Osilinus* species again formed two major clades: one containing *O. edulis* + *O. turbinatus*, the other *O. lineatus* + *O. atratus*. The bootstrap analysis confered 100% support for all the *Osilinus* species as separate clades (Figure 6.26). Again *O. articulatus* was basal to the remaining species: *O. edulis* and *O. turbinatus* have 65% support as sister species in the two taxa clade. The phylogenetic relationship of *O. atratus* and *O. lineatus* are unresolved. Within *O. edulis*, 67% bootstrap support was conferred on the clade exclusively containing Madeiran samples, which has the same topology, and similar bootstrap support values as the minimum evolution tree (Figure 6.26). The geographic patterning was also observed within the *O. turbinatus* clade between the eastern and western Mediterranean basin samples.

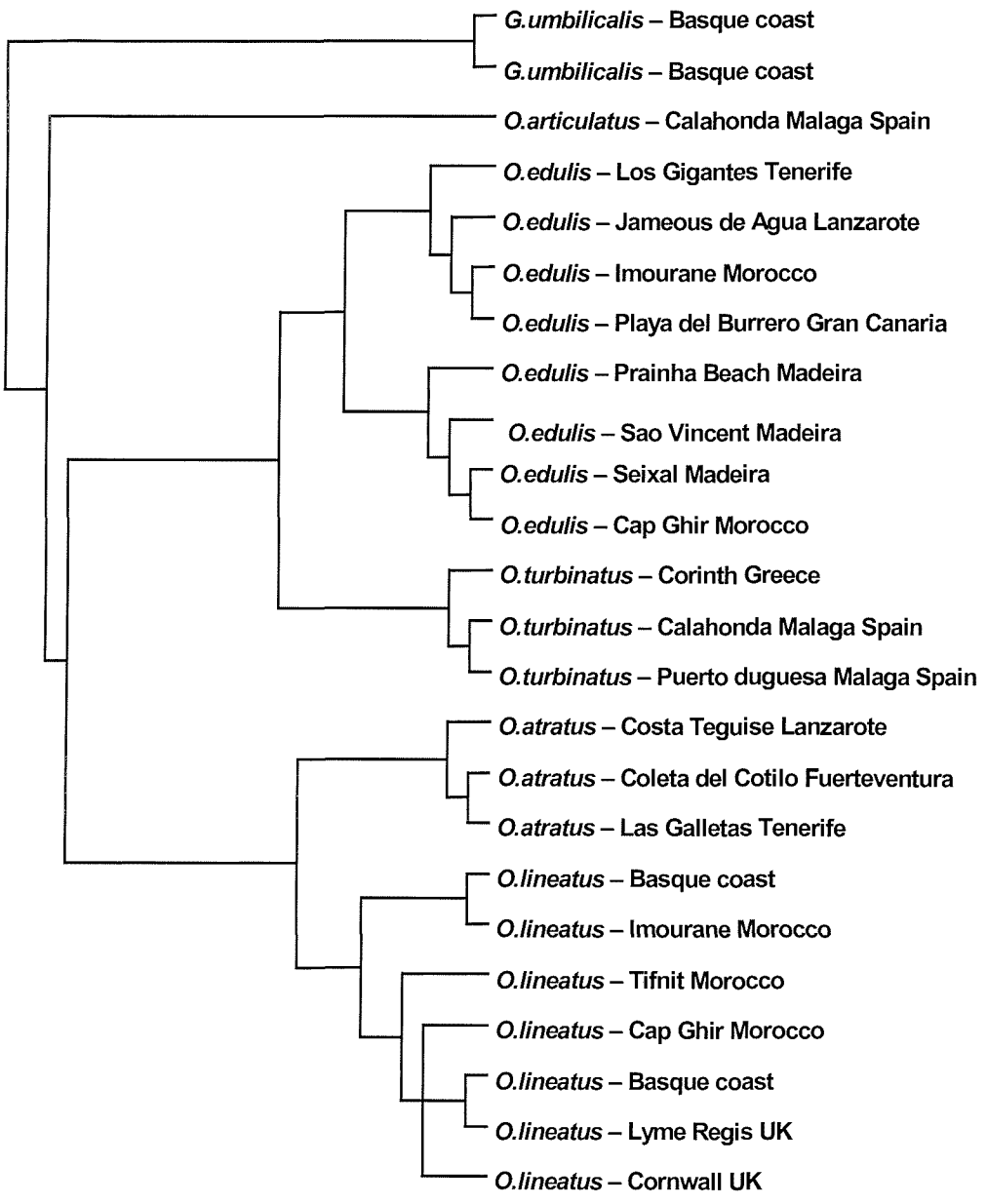


Figure 6.25. The 16S and COI combined mtDNA gene fragment maximum-parsimony branch-and-bound phylogeny. Total length = 440.

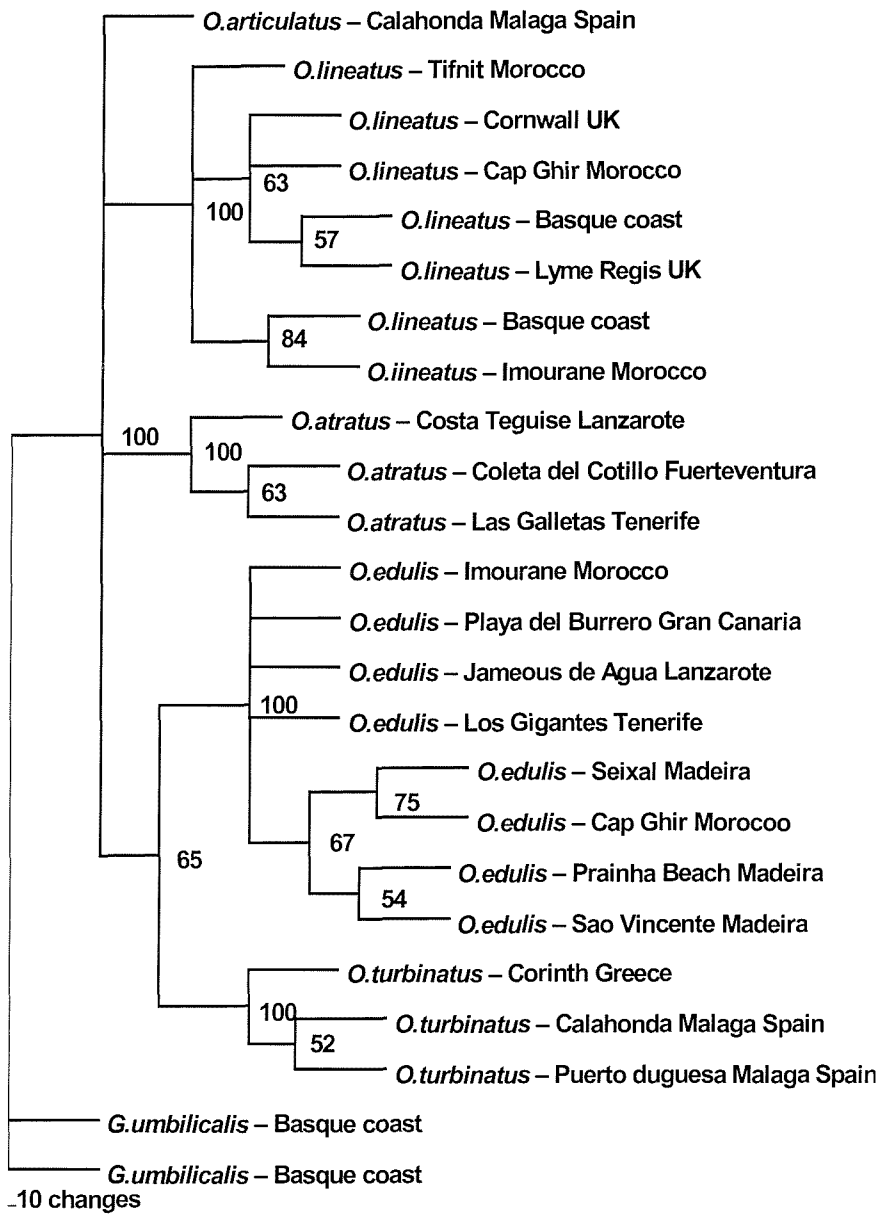


Figure 6.26. The 16S and COI combined mtDNA gene fragment maximum-parsimony branch-and-bound bootstrap 50% majority-rule consensus tree. The bootstrap analysis was performed with replacement and 100 replicates. Bootstrap support values are given to the right of nodes.

Maximum-likelihood analysis

The log likelihood scores of the 56 models of DNA substitution calculated using Modeltest v3.06 (©1998-2000 David Posada) are given in Table 6.20. These were subsequently compared using the hierarchical likelihood ratio test (hLRTs) (Table 6.21).

Table 6.20. The Log likelihood scores of the 16S and COI mtDNA neighbour-joining tree calculated by 56 hierarchical DNA substitution models.

	Log likelihood scores			
Model		+I	+G	+I+G
JC	3676.48	3676.47	3676.52	3675.43
F81	3637.37	3637.37	3637.58	3636.52
K80	3502.58	3502.58	3500.67	3499.20
HKY	3425.50	3426.50	3424.48	3423.85
TrNef	3502.58	3502.58	3500.66	3499.20
TrN	3426.49	3426.48	3424.47	3423.80
K81	3499.94	3499.94	3498.30	3496.78
K81uf	3426.50	3426.50	3424.45	3423.84
TIMef	3499.93	3499.93	3498.30	3496.78
TIM	3426.49	3426.49	3424.44	3423.80
TVMef	3494.56	3494.56	3492.35	3490.79
TVM	3425.03	3425.03	3422.78	3422.53
SYM	3494.55	3494.55	3492.34	3490.78
GTR	3425.02	3425.02	3422.70	3422.53

I = proportion of invariable sites. G = shape of rate variation

Table 6.21. The hierarchical Likelihood ratio test (hLRT) of the likelihood tree scores of the 16S and COI mtDNA neighbour-joining trees under various models of DNA substitution.

hLRT Null vs alternate model	-lnL0	-lnL1	LRT*	° freedom	P-value	Null model
Equal base freqs JC vs F81	3793.89	3762.33	63.12	3	<0.000001	Rejected
TS = TV F81 vs HKY	3762.33	3591.76	49.14	1	<0.000001	Rejected
Equal TS rates HKY vs Trn	3591.76	3591.53	32.12	1	0.4928	Accepted
Equal TV rates HKY vs K81uf	3591.53	3589.39	94.26	1	0.29599	Accepted
Equal rates among sites HKY vs HKY+Γ	3591.76	3424.47	334.58	1	<0.000001	Rejected
No invariable sites HKY+Gvs HKY+I+G	3424.47	3423.85	1.25	1	10.83	Accepted

The HKY+G was selected as the best fitting model with base frequencies A = 0.2919 C = 0.1584 G = 0.1804 and T = 0.3693. The TS/TV ratio was estimated at 5.5608 and the gamma shape parameter at 0.1379. The HKY+G model was therefore assumed in a maximum-likelihood heuristic search (Figure 6.27) with random sequence addition (10 replicates) and the TBR branch-swapping algorithm in effect. The search tried 6857 rearrangements and found one best tree of log likelihood score 3418.43. The search was then repeated with a molecular clock enforced. Seven best trees were found with a likelihood score –lnL = 3441.04087. Given that the molecular clock is the null

hypothesis, it can be rejected under the hLRT and concluded that the combined 16S and COI sequence data set does not behave in a clock-like manner. The maximum-likelihood heuristic search assuming the HKY+G model was repeated as a bootstrap analysis with replacement (100 replicates), Figure 6.28.

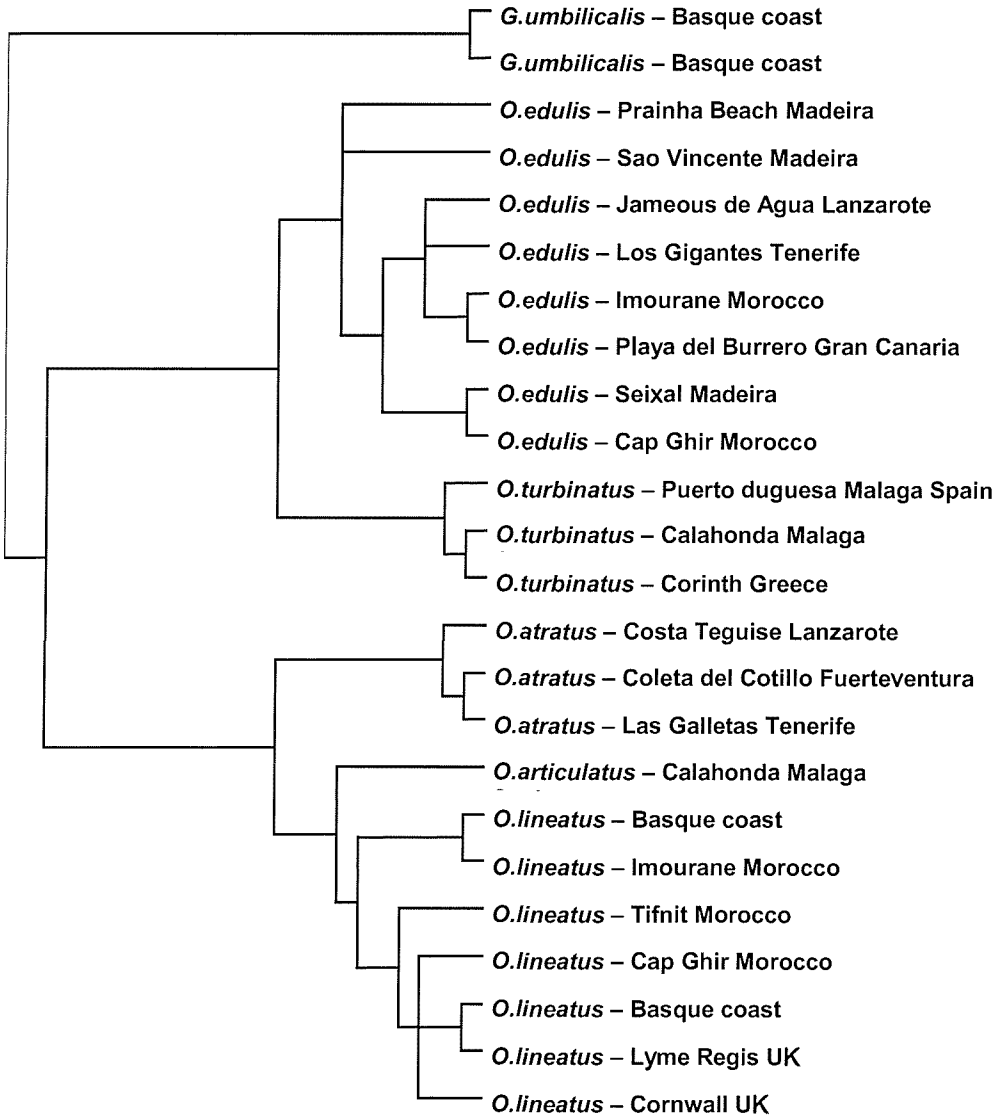


Figure 6.27. The 16S and COI combined mtDNA gene fragment Maximum-likelihood phylogeny assuming the HKY+G model of DNA substitution. Tree likelihood score = -lnL 3418.4363

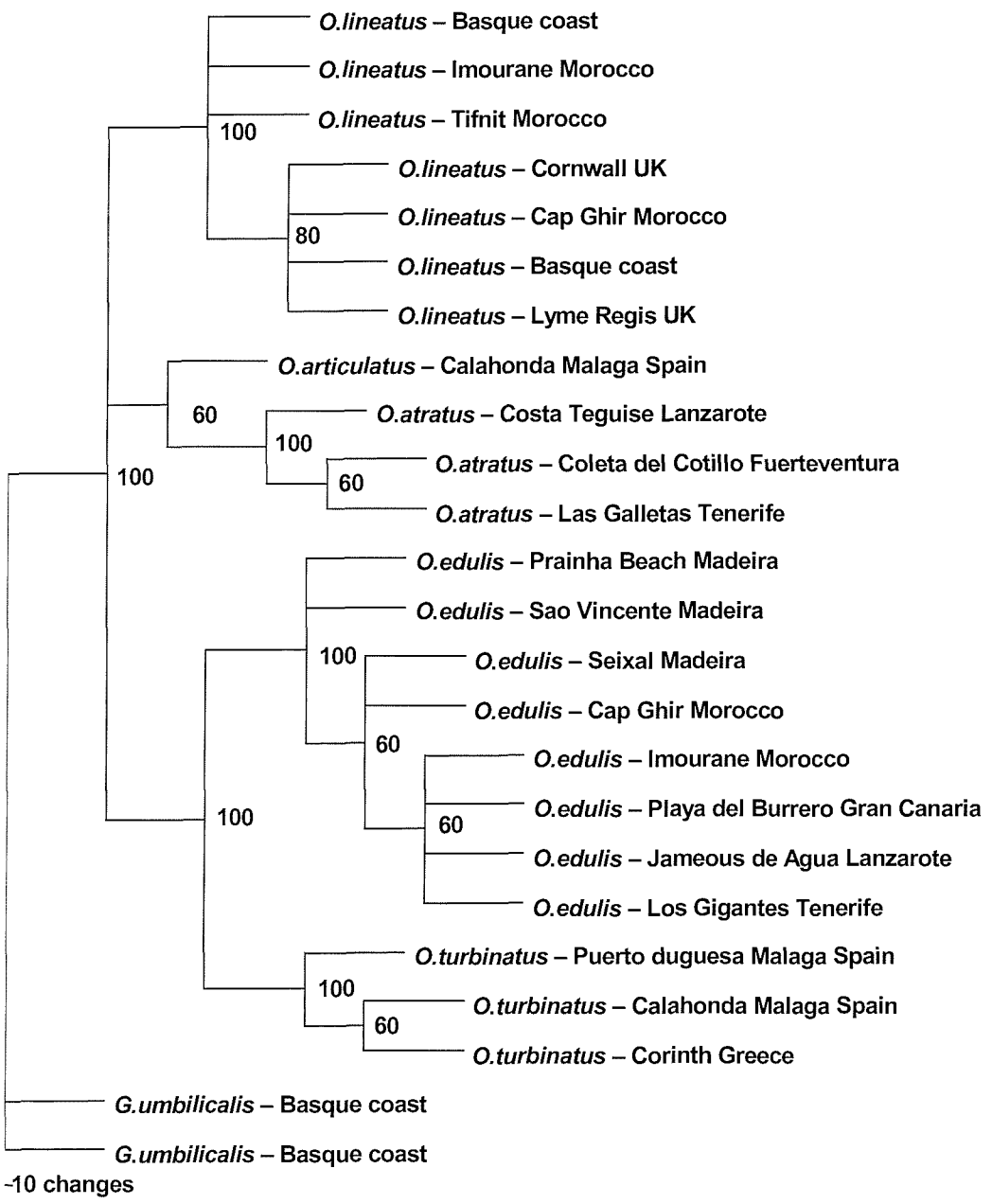


Figure 6.28. The 16S and COI combined mtDNA gene fragment HKY+G maximum-likelihood bootstrap with replacement 50% majority-rule consensus tree. Bootstrap values are given to the right of nodes.

The combined 16S and COI maximum-likelihood phylogeny in Figure 6.27 supports two radiations within the Atlantic/Mediterranean *Osilinus* group: there are two distinct sister clades, one containing *O. turbinatus* as the sister species to *O. edulis.* The other clade contains *O. atratus, O. articulatus* and *O. lineatus* with *O. articulatus* sister to *O. lineatus* and *O. atratus* basal to *O. lineatus* and *O. articulatus*. All species are monophyletic. This is reflected in the bootstrap analysis (Figure 6.28) that confers 100% support on all species clades. *O. edulis* and *O. turbinatus* are given 100% bootstrap support as sister species in a clade with *O. turbinatus* as basal. *O. lineatus* forms a separate clade, splitting from the *O. edulis* and *O. turbinatus* clade at the base of the Atlantic/Mediterranean phylogeny, further supporting the hypothesis of a separate radiation from that which gave rise to the *O. turbinatus* + *O. edulis* clade. It would seem that *O. articulatus* and *O. atratus* are sister species, with 60% bootstrap support. Their phylogenetic relationship to the remaining *Osilinus* species is, however, unresolved.

6.4 Discussion

The first aim of this chapter was to establish whether the species designations assumed in the analysis reflect biological species. The phylogenetic analyses clearly support *O. edulis, O. turbinatus, O. atratus, O. articulatus* and *O. lineatus* as monophyletic groups. This fulfils the species criteria of the Phylogenetic Species Concept (Rosen, 1979; Eldredge & Cracraft, 1980). Without exception, each species formed a single clade in the neighbour-joining, maximum-parsimony and maximum-likelihood phylogenetic analyses of the 16S, COI and combined mtDNA gene fragments. The bootstrap analysis furthermore conferred 94-100% bootstrap support on the monophyly of all species.

The classically accepted test for species as evolutionary independent units under the Biological Species Concept (Dobzhansky, 1937) is that populations retain separate identities in sympatry (Avise, 1994). *O. edulis* occurs in sympatry with *O. lineatus* along the Basque coastline, Portuguese and Moroccan Atlantic coasts. *O. atratus* occurs alongside *O. edulis* on the Canary Islands (Tenerife, Gran Canaria, Fuerteventura and Lanzarote) and apparently Madeira and the N. African coast (Fischer-Piette, 1958). *O. turbinatus* and *O. articulatus* are sympatric within the Mediterranean (Spanish coast, Greece and Cyprus) with a few incursions of the Atlantic species *O. edulis* and *O. lineatus* east of the Gibraltar straits to Fuengirola (Gofas *pers. comm.,* 2000). That each *Osilinus* species occurs in sympatry with another species represented in the phylogeny at at least one sample location, is support for reproductive isolation at the genetic level, thus supporting species status for the *Osilinus* species assumed *a priori* as a working hypotheses.

Allopatric species formation is closely associated with the accumulation of genetic differentiation between isolated or semi-isolated demes (Mayr, 1940). Genetic divergence leading to the reproductive isolation and therefore species status of all taxa included in the analyses is given further weight by the saturation curve and transition/ transversion ratio analyses. The saturation

curve and clustering in the transition/ transversion data for both the 16S and COI mtDNA gene fragments are an indication of the discrete and non-contiguous levels of genetic variation occurring at the micro (population) and macro (systematic) evolutionary levels. The divergence between interspecific *Osilinus* sp. and *Gibbula* sequences is greater than all intraspecific *Osilinus* comparisons for all 14,692 16S and COI sequence pairwise comparisons with no contiguity between the two levels of variation. This does not suggest that the current classification of *Osilinus* obscures sibling species or species complexes (contrary to Gosling 1994). The transversion /transition plot clustering also demonstrates that *Gibbula umbilicalis* and *O.kotschyi* are not included within the Atlantic /Mediterranean *Osilinus* group, exhibiting a well-defined greater genetic divergence and transition/ transversion ratios expected of an outgroup (Avise, 1994).

A second aim of this chapter was to investigate the evolutionary relationships of *Osilinus*, and to propose hypotheses of speciation modes and mechanisms. Complete interspecific phylogenies, with all living members of a clade present, can provide a powerful tool for answering questions on the evolutionary relationships of the taxa. The precision of the answers provided, however, depends on the size of the data set (Purvis, 1996). The evolutionary relationships of the Atlantic/Mediterranean *Osilinus* species have not been fully resolved by the 16S, COI and combined mtDNA gene phylogenies. There is a lack of consistency in the estimated phylogenetic relationships of *Osilinus* between the optimality criteria chosen and the gene fragment used. The *Osilinus* phylogeny is complete in its inclusion of all living taxa, but the lack of resolution may be attributed to the limitations of the sequence data.

A gene tree represents only part of the story of the history of genetic descent (Maddison, 1995) and may not accurately reflect the species tree. Rates of evolution among mtDNA genes are also known to vary within a lineage (Brown *et al.*, 1982). This is particularly true of COI that has been shown to exhibit five-fold differences in rate between taxa (Brown & Simpson, 1981; Crozier *et al.*, 1989). Although the 16S and COI genes are effectively from the same locus, their different rates of evolution may be partly responsible for the differing phylogenetic estimates produced by the two different gene fragments, this is particularly evident within *O. articulatus*.

Although it is advisable to use multiple phylogenetic estimates (Avise, 1994), different phylogenetic methods can differ dramatically in the likelihood of finding the correct tree, and in their efficiency with an increase in data (effectively sequence length). Some phylogenetic methods are inconsistent, that is they converge on the wrong tree as more data are collected (Huelsenbeck, 1995). A comparison of different methods ability to resolve the correct phylogeny found that maximum-likelihood (K2P) and maximum-parsimony weighted analyses showed the greatest accuracy for both compared to UPGMA and NJ uncorrected methods. NJ Kimura distance and Parsimony uncorrected varied in accuracy dramatically under different starting conditions (Huelsenbeck & Hillis, 1993).

There are consistent patterns in my phylogenetic analysis of *Osilinus* that provide insight into the evolutionary relationships and speciation of this gibbuline group in the North-eastern Atlantic. The phylogenetic analysis seems to suggest that two radiations took place at similar times during the speciation of the Atlantic /Mediterranean *Osilinus*. A dual radiation within *Osilinus* is supported by the analyses of 16S, COI and combined data sets where two clades are found. In the 16S and combined neighbour-joining, the 16S maximum-parsimony and the combined maximum-likelihood phylogenies these sister clades are: *O. edulis* + *O. turbinatus*, and *O. articulatus* + *O. atratus* + *O. lineatus,* with bootstrap support values between 37-100%. The neighbour-joining and maximum-likelihood heuristic COI phylogeny supports an *O. lineatus* + *O. atratus* clade, and an *O. articulatus* + *O. turbinatus* + *O. edulis* clade.

Not all the analyses support two clades among the Atlantic/Mediterranean *Osilinus* species. In all the resolved phylogenies, however, *O. lineatus* and *O. edulis* are constant members of contrasting sister clades, and are never exclusively sister species. In the combined data maximum-parsimony branch-and-bound bootstrap phylogeny the *O. edulis* and *O. turbinatus* clade has 65% support, forming a sister group to an *O. lineatus* and *O. atratus* clade. *O. articulatus* is basal to the entire *Osilinus* clade. *O. articulatus* is also basal in the 16S maximum-likelihood phylogeny, followed by *O. atratus*; then *O. edulis* and *O. turbinatus* as a sister group to *O. lineatus.* In the COI large data set maximum-parsimony heuristic search, *O. turbinatus* was placed basal to the rest of the *Osilinus* species, within which *O. articulatus* was basal to two sister clades, one containing *O. edulis,* the other *O. lineatus* and *O. atratus.* The COI maximum-parsimony subset analysis produced several trees exhibiting various interspecific topologies, that were constant in the presence of two distinct sister clades containing *O. lineatus* and *O. edulis.*

The lack of resolution within the Atlantic/Mediterranean *Osilinus* was evident in the bootstrap analyses. In the large COI sequence data set maximum-parsimony bootstrap, *O. lineatus* and *O. atratus* are sister species in a clade with 74% support, but the relationship of the remaining *Osilinus* species is unresolved. In the COI maximum-likelihood bootstrap phylogeny, *O. turbinatus* is basal but the remaining *Osilinus* species form an unresolved polytomy. Again in the 16S maximum-likelihood bootstrap analysis the *O. turbinatus* and *O. edulis* clade has 94% support, but the phylogenetic relationships of *O. lineatus, O. articulatus* and *O. atratus* remain unresolved. The combined maximum-likelihood analysis confers 100% bootstrap support on *O. edulis* and *O. turbinatus* as sister species. The *O. lineatus* radiation and the branch leading to *O. articulatus* and *O. atratus* as sister species, however, form a polytomy at the base of the *Osilinus* phylogeny. Since bootstrapping indicates how well groupings of operational taxonomic units (OTU's) are supported by the existing data, this indicates that the data set was not extensive enough to provide significant support for the relationships among *O. atratus, O. articulatus* and *O. lineatus.* Further sampling, sequencing of the partial 16S and COI mitochondrial genes and phylogenetic analysis is therefore required to resolve fully the phylogeny of this group.

In summary, there is strong support for *O. edulis* and *O. turbinatus* as sister species. The relationship of the remaining Atlantic/Mediterranean *Osilinus* is less clear. It seems that the *O. edulis* and *O. turbinatus* radiation was mirrored by a separate radiation, giving rise to *O. lineatus*. The phylogenetic analysis suggests that *O. articulatus* and *O. atratus* may have speciated from the same radiation that produced *O. lineatus,* and were possibly basal to that species, but this is not consistently supported and therefore remains a working hypothesis.

As a working hypothesis, however, the combined 16S and COI maximum-likelihood HKY+G (Figure 6.27) and the maximum-parsimony branch-and-bound (Figure 6.25) phylogenies will be used as the best estimates of the evolutionary relationships within *Osilinus,* because of the robust nature of the methods, and the greater sequence length increasing the likelihood of finding the correct tree. According to the COI pairwise distance matrix, interspecific comparisons with *O. articulatus* were greater than any other Atlantic/Mediterranean *Osilinus* species, suggesting basal status on *O. articulatus.* This was not born out in the 16S mtDNA pairwise distance matrix in which *O. edulis* exhibited the greatest interspecific pairwise differences.

Speciation mode can be inferred from the distribution of sister taxa, if added evidence supports the speciation mode (Losos & Glor, 2003). This approach does, however, assume that current distribution is a reliable indicator of historical ranges, which is not always true (Lynch, 1989; Barraclough & Vogler, 2000). Under a vicariance model of historical biogeography (Avise, 1994; Cunningham & Collins, 1994) the phylogenetic relationships among taxa, should reflect the historical relationships among the disjunct geographic regions occupied by each taxon, since under this model the continuous ranges of ancestral taxa would have been split by geographic (vicariant) events. The alternative hypothesis is that active or passive dispersal occurred across pre-existing geographic or ecological barriers (Avise 1994).

The interspecific phylogeny of the Atlantic/ Mediterranean *Osilinus* supports apparent allopatric speciation, since allopatry is greatest in the most recent sister lineages. The ranges of the sister species *O. edulis* and *O. turbinatus* do not overlap geographically (except for a few rare incursions of *O. edulis* east of the Gibraltar Straits) the former occurring in the Atlantic, the latter the Mediterranean. Therefore sympatic speciation is rejected and allopatric speciation by vicariance is inferred. A physical barrier does not presently separate the two taxa. The Mediterranean and Atlantic have, however, been historically isolated from each other.

Further support for allopatric speciation of *O. edulis* and *O. turbinatus* is given by the fact their geographic pattern is shared by other sets of sister taxa (Losos & Glor, 2003). Notably, the sister species *O. lineatus* and *O. articulatus* (Figure 6.27). A very similar geographic distribution is true for these sister taxa as for *O. edulis* and *O. turbinatus. O. articulatus* is endemic to the Mediterranean and *O. lineatus* is found on the Atlantic coast from the UK south to N. Africa. It is therefore suggested that these sister taxa also arose by vicariant allopatric speciation, most likely

as a result of the historical physical barriers separating the Atlantic and Mediterranean splitting the ancestral population and causing divergence of the two species. The relationship of *O. articulatus* has, however, proved the most topologically variable taxon within the phylogenetic trees produced throughout the analyses, and is the branch that is most disparate when comparing Figures 6.25 and 6.27. *O. articulatus* is also the taxa with the least number of samples and most limited geographic sampling. More *O. articulatus* samples are required to fully resolve its relationship within *Osilinus* and therefore add support to this hypothesis on its mode of speciation.

Peripatric speciation of sister taxa can be inferred when their ranges do not overlap, and one is smaller than the range of another (Losos & Glor, 2003). This is true for *O. lineatus* and *O. atratus.* Peripatric speciation occurs when a small founder population is geographically isolated from a large parent population and diverges rapidly under the effects of genetic drift and inbreeding (Hartl & Clark, 1989). The range of *O. lineatus* is greater than that of *O. atratus*, which only occurs on the Atlantic Islands (Madeira, Salvegens, Canary Islands and Cape Verde Islands), although it may be found on the coast of Senegal, N. Africa (Hickman & McLean, 1990). The phylogeny infers that *O. atratus* may therefore have arisen by a rare dispersal event; that colonisation of the Atlantic Islands by a founder population led to divergence from the geographically isolated mainland population. The Atlantic Islands are oceanic, and have never been connected to the mainland (Nunn, 1994). The occupying fauna must therefore originate by dispersal of a source population (Paulay, 1994). There are no western Atlantic sister-lineages for *Osilinus* (Hickman & McLean, 1990), further supporting speciation by founder event for *O. atratus* on the Atlantic Islands from a European/ N. African mainland source population.

An alternative explanation for the geographical distribution of the sister taxa *O. lineatus* and *O. atratus* is that low sea stands during the Pleistocene Ice Ages and the consequent exposure of seamounts (Epp & Smoot, 1989) facilitated the range expansion of the ancestral population from the mainland to the Atlantic Islands via a stepping stone model (Kimura & Weiss, 1964). Subsequent rises in sea level would have created a physical barrier to gene flow if the distance between the continent and remaining Atlantic Islands was greater than the larval dispersal ability. The island and mainland populations would subsequently have diverged and therefore speciated by vicariant allopatry.

Hansen (1983) describes a link between developmental mode and rates of speciation and extinction in marine invertebrates. Gastropods with low dispersal potential tend to speciate more often than species with high dispersal potential; although this is not always true (Palumbi, 1992, 1994). A powerful tool in biogeographic reconstruction is the mapping the mode of reproduction or larval dispersal potential characters on phylogenetic trees. The life history strategies of *Osilinus* are relatively unknown. *O. lineatus* has leicithotrophic larvae with a planktonic stage of 5 days to 2 weeks (Fretter & Graham, 1994). The lack of geographic structure within the *O. lineatus*

phylogeny indicates that gene flow occurs along its Atlantic range, perhaps facilitated by a stepping stone-like model along the coastline between suitable rocky shores.

The absence of *O. atratus* on the European coastline suggests that this species may have a limited larval dispersal compared to *O. edulis*, with which is is sympatric on the Atlantic Islands. The current range of *O. edulis* reflects the combined range of *O. lineatus* and *O. atratus* (although *O. edulis* is not found as far north as *O. lineatus*). It may therefore be inferred that the distance between the Atlantic Islands and mainland is not an effective barrier to dispersal of *O. edulis* larvae. Conversely, the same distance appears to be an effective dispersal filter for *O. lineatus* and *O. atratus.* This may be an example of isolation-by-distance, or exclusion of *O. atratus* on the mainland by competition for limited available ecological niches. Since *O. edulis* and *O. atratus* are sympatric on the Atlantic Islands, and *O. edulis* and *O. lineatus* are sympatric on the Portuguese and Moroccan coastline, competition from *O. lineatus* may maintain the current distribution of *O. atratus.* An investigation into the mode of reproduction and larval dispersal potential of all the *Osilinus* species is key to understanding the current biogeography and will offer further insight into mechanisms of speciation within these northeast Atlantic and Mediterranean trochids.

The colonisation of the Atlantic Islands by larvae from mainland populations is most likely to have occurred via the Canary Current, assuming historical surface currents in the Northeast Atlantic were similar to those of present day. The intraspecific phylogeny *O. edulis* infers that two colonisation events of the Atlantic Islands may have occurred historically. Within the *O. edulis* intraspecific phylogeny, Madeira and Canary Island population samples do not occur in the same clade, but in separate clades including only N. African and Iberian population haplotypes. The intraspecific relationships of the *O. edulis* and *O. atratus* morphotypes cited as endemic to the Selvagens to the rest of the Atlantic island populations is of interest; the Selvagen population may be ancestral to the more common morphotypes radiated throughout the younger Atlantic Islands. These questions cannot be answered, however, until sequences are obtained from Madeiran *O. atratus* samples, and Salvagens *O. atratus* and *O. edulis* samples.

In contrast to the known planktonic larval dispersal potential of *O. lineatus* and inferred high dispersal of *O. edulis,* the *O. turbinatus* clade exhibited strong geographic patterning. The relatively high intraspecific diversity of *O. turbinatus* is perhaps surprising considering the sample size (7) and geographic area represented (Spain, Greece and Cyprus) when compared to the lower genetic variation within *O. edulis* (0.009) from a considerably larger sample size (40) and geographic distribution (Basque, Portuguese, Spanish and Moroccan Atlantic coastlines, the Madeiran and Canary Islands). Samples from Cyprus and Greece in the eastern Mediterranean basin have high phylogenetic support as a separate clade to the Spanish samples from the western Mediterranean basin. This may reflect a much shorter planktonic larval stage, or even a non-planktonic life history strategy in this species.

The potential geographic range of a species is limited by suitability of environmental conditions, its ecological tolerance (Connell & Slatyer, 1977). Within this ecological tolerance zone actual geographic distribution is also influenced by historical demographic factors, dispersal patterns and larval dispersal potential (Avise, 1994; Avise, 2000) and interspecific competition (Connell, 1961). A species range is also affected by the availability of ecological niches (Grinnell, 1918). In all of the sympatric ecological pairings, each of the species has evolved from separate lineages. When found in sympatry, *Osilinus* species tend to occupy different heights on the rocky shore, or different substrates. They are very rarely seen in abundance on the same rocks or same shore height. The present disruption of *Osilinus* is therefore consistent with niche specialisation driven by interspecific competition (Gause, 1934).

The biogeography and interspecific phylogeny of *Osilinus* have been used to test modes of speciation. Allopatric speciation appears to account for the evolutionary divergence exhibited by this genus. The vicariant mechanisms driving this speciation, however, can only be hypothesised based on knowledge of palaeoceanographic events within reasonable timescales. Time since divergence cannot be confidently estimated from pairwise distances since the data does not behave in a strict clock-like manner, however a general mitochondrial clock of 2-4 Myr-1 can be used to infer approximate times of lineage splitting (Table 6.22). The dates inferred are to be considered with further caution because of the absence of fossil records to calibrate any clock assumed.

Table 6.22. Estimates of taxa divergence times (Million years) based on mtDNA COI (above diagonal) and mtDNA 16S (below diagonal) pairwise distances and assuming a general molecular clock of 2-4% Myr^{-1}.

	O.lin	*O. atratus*	*O.artic*	*O.ed*	*O.turb*	*O.kots*	*G.umb*
O. lineatus (20)	--	2.73 - 5.45	3.58 - 7.15	3.08 - 6.15	3.03 - 6.05	-	4.08 - 8.15
O. atratus (12)	1.93 - 3.85	--	3.6 - 7.25	3.2 - 6.4	3.33 - 6.65	-	4.4 - 8.8
O. articulatus (4)	2.33 - 4.65	1.6 - 3.2	--	3.35 - 6.7	3.45 - 6.9	-	4.53 - 9.05
O. edulis (45)	2.98 - 5.95	2.53 - 5.05	3.13 - 6.25	--	2.73 - 5.45	-	4.28 - 8.55
O. turbinatus (2)	2.78 - 5.55	1.75 - 3.5	2.33 - 4.65	2.13 - 4.25	--	-	3.85 - 7.7
O.kotschyi (4)	4.83 - 9.65	4 - 8	4.03 - 8.05	4.5 - 9	4.58 - 9.15	--	-
G. umbilicalis (9)	3.93 - 7.85	3.30 - 6.6	3.33 - 6.65	3.9 - 7.8	3.75 - 7.5	4.45 - 8.90	--

Using the working hypotheses based on the phylogenies represented in the, maximum-parsimony and maximum-likelihood trees (Figures 6.25 and 6.27 respectively) there are two key points in the evolutionary history of the Atlantic/ Mediterranean *Osilinus*. The first is the split between the *O. edulis* + *O. turbinatus* clade, and the *O. lineatus* + *O. atratus* clade. The second event (or events) is that which drove speciation within each of these two clades producing the four extant *Osilinus* species. Two practically simultaneous and similarly speciose radiations appear to have occurred within the Atlantic/ Mediterranean *Osilinus*. One radiation resulted in the Atlantic *O. edulis* and Mediterranean *O. turbinatus*, the second in the Atlantic *O. lineatus* and *O. atratus*. These two

radiations suggest that either the same historical event may have caused both radiations, or that two different events occurred at similar times in the evolutionary history of *Osilinus.* For both mt DNA genes, the divergence times infer that the radiation of *Osilinus* took place over approximately 3 Million years.

Based on the inference of allopatric speciation in the two main radiations evident in the *Osilinus* phylogeny, discussed above, the range of the taxa ancestral to the *O. edulis* + *O. turbinatus* and *O. lineatus* + *O.atratus* + *O. articulatus* clades must have extending throughout the Northeast Atlantic and Mediterranean. Since the geographical range of the ancestor of these two ancestral lineages cannot be inferred, hypotheses on the palaeoceanographic events during the speciation of *Osilinus* will be limited to the events that drove speciation in the two radiations.

The sympatric Atlantic species *O. edulis* + *O. lineatus/ O. atratus* and sympatric Mediterranean species *O. turbinatus* + *O.articulatus* have evolved from two separate radiations. It is likely that the Messinian Salinity Crisis (MSC, see chapter 2), the onset of which is estimated at 5.96 ± 0.02 Ma, and consequent isolation of the Mediterranean from the Atlantic was the vicariant event that initiated divergence in two separate lineages. The molecular clock estimates support this (Table 6.22). The sister species *O. edulis* and *O. turbinatus* appear to have diverged between 4.25-5.45 Ma, *O. lineatus* and *O. articulatus* between 4.65 – 7.15 Ma (16S and COI estimates respectively). This was a synchronous event over the entire Mediterranean basin (Krijgsman *et al.,* 1999) that resulted in the isolation of the Mediterranean from the open Atlantic Ocean between 5.59-5.33 Ma (Krijgsman *et al.,* 1999). Prior to this, two pre-Messinian Atlantic gateways connected the Mediterranean basins to the Atlantic, the Betic corridor through the Iberian peninsular and the Rifian corridor through Morocco.

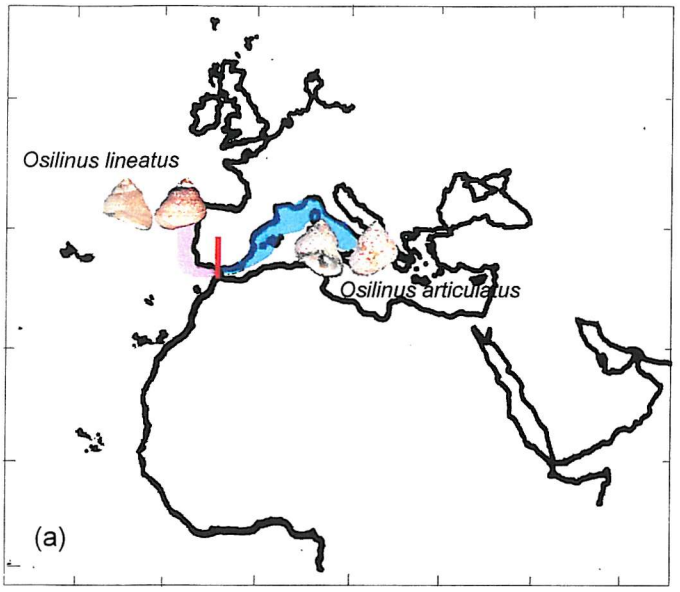
The Betic corridor closed during the latest Tortonian/earliest Messinian, and the Rifian corridor was severely restricted by earliest Messinian time, 6.1Ma (Garces *et al.,* 2001). The closing of the Betic corrider prior to the Rifian corridor may have served to isolate what became *O. lineatus* along the northern Atlantic coasts of the Iberian Peninsula before the onset of the MSC, and initated the divergence of the ancestor of *O. articulatus* within the Mediterranean. The later closing of the Rifian corridor and the continued access between the Mediterranean and Moroccan Atlantic coastline could have concurrently provided the equivalent speciation event for the *O. turbinatus/O. edulis* ancestor. The isolated populations that gave rise to *O. edulis* and *O. lineatus* speciated as they took refuge either side of what became the Gibraltar Straits along the Atlantic coast. The loss of Palaeo-Mediterranean Overflow Water from the Mediterranean to the Atlantic reduced the heat transfer to the north Atlantic and likely caused cooling of the European climate (Benson *et al.*, 1991). Molluscs may have shifted south to warmer climes at this point, down the African coast, or driven the adaptation of *O. lineatus* to a more temperate ecological niche, as reflected in its northern distribution.

The subsequent desiccation and sterilisation provided a hostile environment in the Mediterranean. The basal position of *O. turbinatus* in the *O. edulis* clade and *O. articulatus* in the *O. lineatus* clade may indicate that the ancestors of the present Mediterranean species may have survived in the very eastern parts of the Mediterranean where the desiccation was less extreme. The opening of the Gibraltar Straits reconnected the palaeo-Mediterranean and Atlantic at the beginning of the Pliocene epoch, 5 Ma (Por, 1989). The Mediterranean Sea was then colonised by taxa from the Atlantic (Sabelli & Taviani, 1984), and colonisation of the Atlantic was possible for relict Mediterranean taxa. The consequent flooding of the Mediterranean would have facilitated range expansion of *O. turbinatus* and *O. articulatus* throughout the eastern and western basins.

The formation of the Atlantic Islands provided an opportunity for speciation for the *O. atratus* and the range expansion of *O. edulis*. A 72 Ma hotspot is responsible for the Madeiran Archipelago, Desertas Islands and several seamounts between Madeira and the alkaline Serra do Monchique complex of SW mainland Portugal (Geldmacher & Heornle, 2000). Porto Santo is aged 11-14 Ma, Madeira is around 5 Ma and Desertas Island between 3.2-3.6 Ma. The Canary Islands also attributed to hotspot volcanism, exhibit a similar northeast to southwest age gradient, represented at its extremes by El Hierro aged 1 Ma, and Fuerteventura aged at 20 Ma (Juan *et al.*, 2000). The Selvagen Islands are aged at 20-24 Ma and are thus older than the Madeiran Archipelago.

The Atlantic Islands existed before the *O. lineatus/O. articulatus* ancestor was isolated from the Paleo-Mediterranean upon closure of the Betic corridor. In the maximum-likelihood phylogeny (Figure 6.27) *O. atratus* is basal within the *O. lineatus* + *O. articulatus* + *O. atratus* clade. This suggests that the colonisation, and subsequent isolation of populations of *Osilnus* on the mainland and Atlantic Islands (which became *O. lineatus* and *O. atratus* respectively), may have preceeded the speciation events that occurred as a result of the MSC. Contrary, in the maximum-parsimony phylogeny (figure 6.25) O. *articulatus* is basal in the *Osilinus* monophyletic clade, and *O. atratus* + *O. lineatus* diverged later in a radiation mirroring that of *O. edulis* and *O. turbinatus*. The divergence times (Table 6.22) of *O. lineatus* and *O. atratus* (3.85-5.45 Ma; 16S and COI estimates respectively) support a later divergence time, similar to that of *O. edulis* and *O. turbinatus*. The later divergence estimate of *O. atratus* + *O. lineatus* supports a model of peripatric speciation via a rare dispersal event over the vicariant model, since the low sea stands during the Messinian Salinity Crisis that may have facilitated the range expansion of *O. edulis* (and possibly *O. atratus*) by the exposure of sea mounts occurred previously, 5.75 and 5.55 Ma. However since these estimates are a rough guide, neither theory can be excluded. The hypotheses of the evolutionary history of *Osilinus* is summarised, Figure 6.29.

The genus *Osilinus* offers an interesting insight into allopatric speciation in the paleo-oceanographically complex region of the Northeast Atlantic and Mediterranean. The interspecific relationships have been partially resolved by this work and provide some hypotheses as a basis for further work. The analysis has also provided a robust molecular basis for current taxonomic



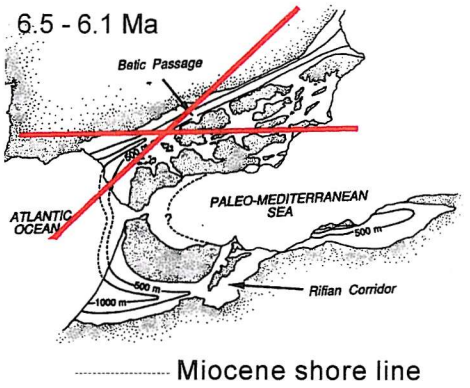
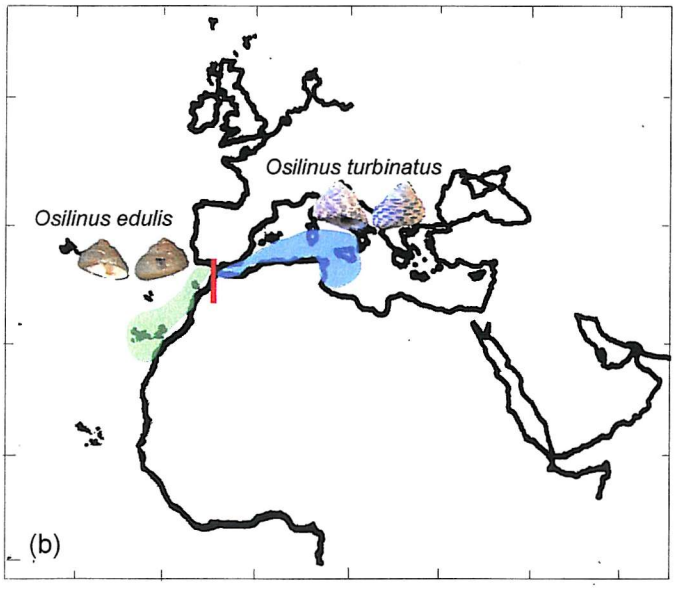


Figure 6.29(a) vicariant speciation model of *O. lineatus* and *O. articulatus*. The Betic Corridor closed between 6.5-6.1 Ma signalling the onset of the Messinian Salinity Crisis (MSC), and separating the previously continuous population of ancestral *Osilinus* taxa either side of the Iberian peninsula.



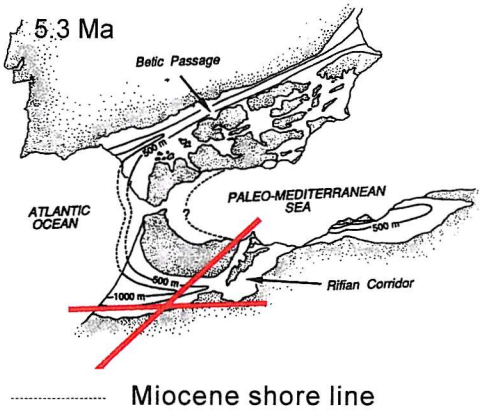
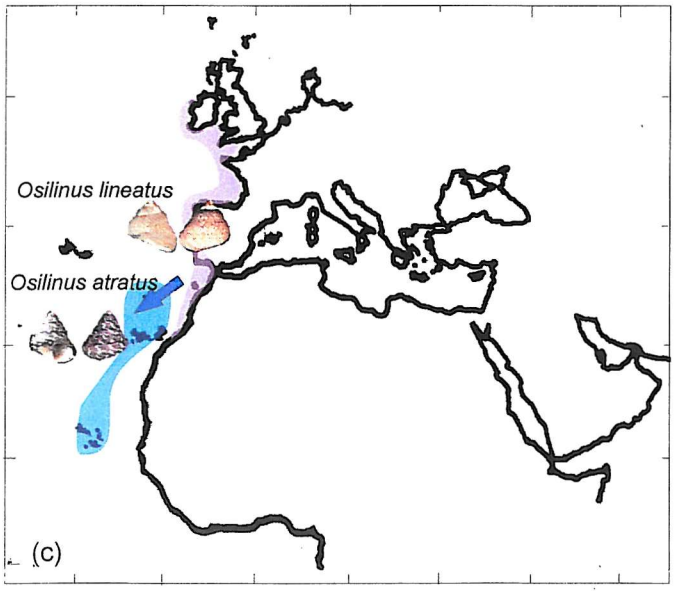


Figure 6.29(b) vicariant speciation model of *O. edulis* and *O. turbinatus*. The Rifian Corridor closed 5.3 Ma isolating the Mediterranean from the Atlantic Ocean, and dividing the previously continuous population of ancestral *Osilinus* taxa either side of what became the Gibraltar Straits.



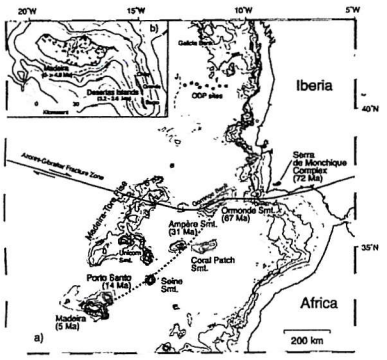


Figure 6.29(c) vicariant or dispersal speciation model of *O. lineatus* and *O. atratus*. The low level sea stands during the MSC facilitated colonisation, by ancestral *Osilinus* taxa, of the Atlantic Islands (Madeira, Canary Islands and Cape Verde Is) via exposed sea mounts. Consequent submerison of the seamounts created an isolation-by-distance barrier and the populations diverged. Alternatively the Atlantic Islands were colonised by a rare dispersal event after the MSC.

Figure 6.29. Radiation hypotheses of the evolutionary history of *Osilinus* in the Northeast Atlantic and Mediterranean

classifications. Further sampling of *O. atratus* and *O. edulis* from the Salvegens Is, and *O. atratus* from Madeira would serve to complete the unanswered questions posed by this study. It is likely that the small sample sizes of *O. articulatus,* and possibly *O. atratus,* are responsible for the lack of phylogenetic resolution. More *O. articulatus* and *O. turbinatus* sequences are also required from a greater geographic range of samples, representing the eastern and western Mediterranean more extensively.

Chapter 7

Cladistic analysis of morphological characters and evolution of the radula in *Osilinus* (Gibbulini: Trochidae)

7.1 Introduction

Morphological data are currently used less than DNA sequence data for phylogenetic reconstruction, even though most of our knowledge of phylogeny is based on classifications founded on morphological data (Platnick, 1979; Scotland *et al.*, 2003). DNA sequence data can provide increased accuracy in phylogenetic reconstruction because of the increased number of characters available (Hillis, 1987; Cummings *et al.,* 1995). There is no ambiguity in assigning character states in aligned sequence data. Identifying a suitable number of discrete morphological characters for accurate phylogenetic reconstruction, however, is often problematic (Hillis, 1998). The justification and discussion of character selection creates ambiguity in using morphological data for phylogenetic construction (Patterson & Johnson, 1997; Hawkins *et al.*, 2000). Furthermore, current understanding of the processes underlying morphological evolution is less understood than the process of DNA sequence evolution (e.g. DNA substitution models) (Scotland, 2003). Morphology does play a role in phylogeny reconstruction, however, by providing reciprocal illumination and congruence in light of molecular phylogenies (Patterson, 1982; Scotland *et al.*, 2003). Since, in morphological cladistics, the problem of character coding increases with the number of characters, Scotland *et al.,* (2003) advise that effort is most productively focused on exploring anatomy and morphology for fewer characters to be used in the context of molecular phylogenies.

This chapter aims firstly to present a hypothesis of the phylogenetic relationships of *Osilinus* based on maximum parsimony analysis of morphological characters. Secondly, the characters and their states are mapped onto the 16S mtDNA maximum likelihood 50% majority-rule consensus phylogeny (Chapter 5) to investigate the patterns of evolution of the radula within *Osilinus.* This process provides insight into the adaptive evolution of the radula within Gibbulini and identifies phylogenetically informative radular characters. This chapter builds on the photographs of shell plates in chapter 3 by presenting Scanning Electron Micrographs (SEM) of radulae for all the species included in the analysis (except the radula of *A.concamerata*). Photographs of plates of the outroup species shells are also presented here. The shell and radula plates illustrate diagnostic morphological characters and provide examples of intraspecific variation in radula morphology. These are used to discuss the morphological character coding and referred to in the character state descriptions.

7.2 Materials and methods

Table 7.1 lists the species used in the cladistic analysis. The ingroup consists of all the inclusive *Osilinus* species and seven outgroup taxa from three genera of the Gibbulini tribe. These were chosen as appropriate outgroup taxa based on *a priori* knowledge as members of the monophyletic Gibbulini tribe based on the systematic revision of Trochacean gastropods (Hickman & McLean, 1990).

Table 7.1. List of species of Gibbulini used in the cladistic phylogeny.

Genus	Species	Distribution	Localities
Austrocochlea	*A. porcata*	Endemic to Australia; Queensland, to Houtman Abrolhos, Western Australia (WA), inc. Tasmania.	29, 30,
	A. concamerata	Endemic to Australia; Port Stephens, NSW, to Houtman Abrolhos, WA, inc. Tasmania.	27, 28,
	A. constricta	Endemic to Australia; northern NSW to WA.	26, 32, 29
Monodonta	*M.labio*	Indo-West Pacific	25, 31,
Oxystele	*O. variegata*	South Africa – endemic	24,
	O. sinensis	South Africa – endemic	24,
Gibbula	*G.umbilicalis*	N.E. Atlantic.	5, 6,
Osilinus	*O.kotschyi*	Persian Gulf, Gulf of Oman, Arabian Sea and Red Sea.	1, 2, 3, 4
	O.lineatus	N.E. Atlantic	5, 6,
	O.turbinatus	Mediterranean	7, 8, 9, 10, 11,
	O.articulatus	Mediterranean	7, 9, 11, 12, 13,
	O.edulis	N.E. Atlantic, Macronesian Is (ex. Azores)	14, 15, 16, 17, 18,
	O.atratus	Macronesian Is (ex. Azores)	19, 20, 21, 22, 23,

Localities from which anatomical material was examined in the study: 1. Al Mihd, Abu Dhabi, UAE. 2. Khor Al-Hamra, Ras Al-Khamah, UAE. 3. Delma Is. Abu Dhabi, UAE. 4. Rasal Qila, Abu Dhabi. Acc no 2393 NHM collection. 5. Lyme Regis, UK. 6. Lamorna Cove, UK. 7. Fethia Bay,S.Turkey. 8. La Rapiuta, Majorca, NHM collection. 9. Houmt Souk, Tunisia. NHM collection. 10. Mallorca. 11. Southern Turkey. 12. Cyprus. 13. S.France. 14. Salvagen Is. Petit Salvage. 15. Southwest Portugal. 16. Tifnit, Morocco. 17. Imourane, Morocco. 18. Ponta do Sol, Madeira. 19. Madiera Is. 20. Toston Reef, Fuerteventrua. 21. St vincente Is. Cape Verde Is. NHM Acc no 2324. 22. Madeira Bay, Cape Verde Is. 23. Sao Vincente, Cape Verde Is. 24. Hermanus, S. Africa. 25. Cape D'aguilar, Hong Kong . 26. Abrolhos Is. W.Australia. 27. NSW, Australia. Acc No. 2388. 28. Flinders Bay, Nr Augusta, W. Australia. Acc No. 2388. 29. Gerring, NSW, Australia. 30. Mollymook, NSW, Australia. Acc No. 2384. 31. Starfish Bay, Hong Kong. 32. Shoal Bay, W. Australia.

Anatomical material was examined from 13 gibbuline species obtained from the localities listed in Table 7.1. The sources of identifications for outgroup species are as follows: *Austrocochlea concamerata* (Wood, 1828), *A. constricta* (Lamarck, 1822) and *Monodonta labio* (Linnaeus, 1758) from Wilson (1993). For *Austrocochlea porcata* (Adams, A., 1853) identification was based on Parsons & Ward (1994). Identification of the *Oxystele* species was from Branch *et al.,* (1994). Identification for *Gibbula umbilicalis* was from Hayward & Ryland (1995). Hard part (shell and radula) characters were obtained from all species listed; soft part anatomical observations were taken from *Osilinus atratus, Osilinus kotschyi, Monodonta labio* and *Austrocochlea constricta*. All descriptions refer to character states found in adult specimens. All voucher material is deposited in the Natural History Museum, London (BMNH). Live collected animals were relaxed in 8% (w/v) $MgCl_2$ for 1-6hrs, fixed in 10% seawater-formalin, rinsed in fresh water and preserved in 80% ethanol. Each specimen was sexed by examination of a section of the gonads mounted on slides under a light microscope, and the shell dimensions recorded (height and width). Characters and observations of the headfoot, ctendium, kidney openings and alimentary system (stomach and rectum) were examined by dissection.

All species examined here possess a rhipidoglossate type radula (Cox 1960) following the formula ∞+5+R+5+∞ unless otherwise stated. The radula were prepared according to the methodology of Ridgeway *et al.* (1998): the first 10 rows of worn teeth were discarded and the remainder cleaned by soaking in dilute hypochlorite bleach solution at room temperature. The cleaned radula was rinsed in distilled water, mounted on a thin layer of polyvinyl acetate glue on glass cover slips attached to aluminium stubs, and coated with gold/palladium, before examination by scanning electron microscope. Six radulae were examined per species, three male and female from three locations. For comparison between species and individuals, each radula was photographed in the same orientations. In the plates, all radulae are in flat view unless otherwise stated.

7.3 Morphological descriptions of shell and radula

Descriptions and shell plates (Figures 3.5 – 3.10) of the species *Osilinus kotschyi, O. articulatus, O. atratus, O. edulis, O. lineatus,* and *O. turbinatus* are given in chapter 3 and are considered in conjunction with the plates showing radulae (Figures 7.6 to 7.13 respectively). The shell morphology of *Austrocochlea, concamerata, A. constricta, Monodonta labio, Oxystele variegata, O. sinensis* and *Gibbula umbilicalis* is shown in Figure 7.1. The radula morphology of *Austrocochlea* species, *Monodonta labio, Oxystele* species and *Gibbula umbilicalis* are shown in Figures 7.2 to 7.5 respectively.

7.4 Selection, description and coding of characters

The choice and coding of discrete morphological characters is a fundamentally subjective process that aims to determine phylogenetically informative characters from observation. Genuinely homologous characters are required for the cladistic analysis to be phylogenetically useful, since only homologous derived characters can provide synapomorphies to determine clades (Reid, 1999a). To achieve this, a broad sampling of characters is necessary based on observations from a number of individuals of both sexes from a variety of locations (Table 7.1). Initially characters were scored from shell, soft part and radula morphology.

The shell has been used extensively as a source of valid taxonomic characters in cladistic analyses of gastropods (e.g. Sasaki, 1998; Takada, 1992). Additionally, the large degree of variation in shell morphology exhibited by the species included in this analysis initially seemed a robust and extensive source of characters for cladistic analysis. After extensive examination of the shells from the NHM London dry collection plus specimens collected during fieldwork, it was decided not to include shell characters because of problems in defining discrete states. For example, the basal columella denticle proved difficult to score. Although observed as present (e.g. *Monodonta labio,* Figure 7.1) or absent (*Oxystele variegata,* Figure 7.1) in some species, there were also various intermediate states of tooth size (e.g. *Austrocochlea comamerata,* Figure 7.1 and *Osilinus lineatus,* Figure 3.9). This was also true of patterning on the shell base, shell shape and size of spiral cords. Defining discrete states in shell patterning was also problematic. Three main pattern types were identified: zig zag (e.g. *O. lineatus*, Figure 3.9), stepped blocks (*O. turbinatus*, Figure 3.10), and spiral stripes (e.g. *A. constricta,* Figure 7.1). Intraspecific variation in

shell patterning, however, and the presence of two or more pattern types in the shells of some species (e.g. *Osilinus articulatus*, Figure 3.6) excluded this as a scorable character.

Soft parts (e.g. the head-foot, ctenidium, kidney openings and rectum) have also proved useful for providing taxonomic resolution at the species level in several gastropods (e.g. Sasaki, 1998). The following anatomical features were examined for potential as characters in cladistic analysis: the head-foot, eye stalk and tentacles, neck lobe, stomach, ctenidium, the anus, rectum, left kidney opening and right kidney and genital opening, on the basis of their use as taxonomic characters in previous cladistic analysis of gastropods. Within the Gibbulini, however, the variation in soft parts was not sufficient between genera or species to provide useful characters. Morphological features such as the spiral ceacum of the stomach were similar between geographically distant species such as *A.constricta* and *O.lineatus*.

Radulae have been used extensively in systematic studies of gastropods at all levels of taxonomic resolution and provide a valuable source of phylogenetic information (Reid, 2000). Radular characters provide robust and significant sources of morphological characters for phylogenetic inference and higher-level taxonomy (e.g. Ponder & Lindberg, 1997) and are sometimes diagnostic at the species level. Their systematic value at intermediate levels (families and genera) has been recently questioned, however, due to striking examples of parallelism and convergence. Recent studies also show that radula can exhibit intraspecific variation due to several factors (variation in radula length due to rate of wear, teratology of radulae, asymmetry, ontogenetic variation, sexual dimorphism, ecophenotypic plasticity). For a review of the use of the radula in the taxonomy and phylogeny of Gastropoda see Reid (2000). Reid maintains that when characters are correctly coded and a wider set of morphological characters are used, radulae can still provide a powerful tool, albeit one to be applied with caution. When superimposed on molecular phylogenetic trees, morphological characters can be used to study the evolution of adaptation and convergence. As a taxonomically informative character, the radula is second only to the shell for the discrimination of species (Reid, 2000).

Based on the systematic revision of Hickman & McLean (1990) the radula features of the superfamily Trochoidea are as follows: a radula size comparable to other archaeogastropods (i.e. larger than caenogastropods), bilaterally symmetrical with more than 100 teeth per row, distinct central and marginal tooth fields in each row (a rhipidoglossate type radula, Cox, 1960), five lateral teeth per half row, rachidian and lateral teeth possess expanded bases that overlap and interlock, common presence of a food groove beneath the cusps of marginal teeth (a hallmark and synapomorphy of the Trochoidea), an expanded base of the innermost marginal tooth shaft resulting in the protolateromarginal plate. The derived character states of the family Trochidae include: a well differentiated rachidian, within-column interactions between bases and cusps of the rachidian tooth, interactions of rachidian tooth with laterals in other rows (diagonally) and rachidian height is greater than its width. In the subfamily Trochinae the M-shaped rachidian cusp top is said to be diagnostic (Hickman & McLean, 1990). The outer marginal tooth possesses a heavy basal

plate and there is no serration of the food-collecting groove in the undersides of the marginals. Within the tribe Gibbulini, regularly arcuate rows are typical of the family but with large variation in cusp morphology and basal expansion. The five lateral teeth per half row are similar to the rachidian in size and shape.

The characters of radula morphology chosen for the analysis were based on observed variation between species from SEM analysis and characters used in previous gastropod cladistic analysis and taxonomic studies that have been shown to be phylogenetically informative (Beck, 1986, 1995; Hazprunar, 1988; Herbert, 1994; Hickman, 1996). Table 7.2 lists the character states and descriptions used to score the radula for cladistic analysis. The descriptions of radula characters are discussed below with reference to the plates showing radulae of the species *A. constricta* and *A.porcata* (Figure 7.2), *Monodonta labio* (Figure 7.3), *Oxystele sinensis* and *O.variegata* (Figure 7.4), *Gibbula umbilicalis* (Figure 7.5), *Osilinus kotschyi* (Figure 7.6), *O.articulatus* (Figures 7.7 and 7.8), *O.atratus* (Figure 7.9), *O.edulis* (Figure 7.10), *O.lineatus* (Figures 7.11 and 7.12) and *O.turbinatus* (Figure 7.13).

Table 7.2. List of radular characters and character states used in the cladistic analysis of Gibbulini.

Character number	Character	Character state	Description of character state
1	Rachidian cusp length	0	small - rachidian cusp length less than lateral teeth cusp length
		1	large - rachidian cusp length less than lateral teeth cusp length
2	Rachidian shaft width	0	narrow - neck-shaft width less than half tooth length.
		1	broad - neck-shaft width equal or greater than half tooth length
3	Rachidian shaft wings	0	absent
		1	small
		2	large
4	Rachidian head	0	bifid
		1	not bifid
5	Rachidian base plate	0	straight
		1	convex
		2	concave
6	Number of lateral teeth	0	5 pairs
		1	6 pairs
7	Differentiation of outermost lateral	0	basal lobe present on all laterals
		1	basal lobe present only on outermost lateral
8	Denticles on lateral tooth cusp	0	denticles at cusp base
		1	denticles along entire length
9	Lateral tooth cusp shape	0	blunt or chisel-shaped
		1	pointed
10	Marginal tooth rachis	0	absent
		1	present
11	Denticles on marginal tooth cusp	0	absent
		1	denticles at cusp base
		2	denticles along entire length
12	Shape of marginal tooth denticles	0	long
		1	short

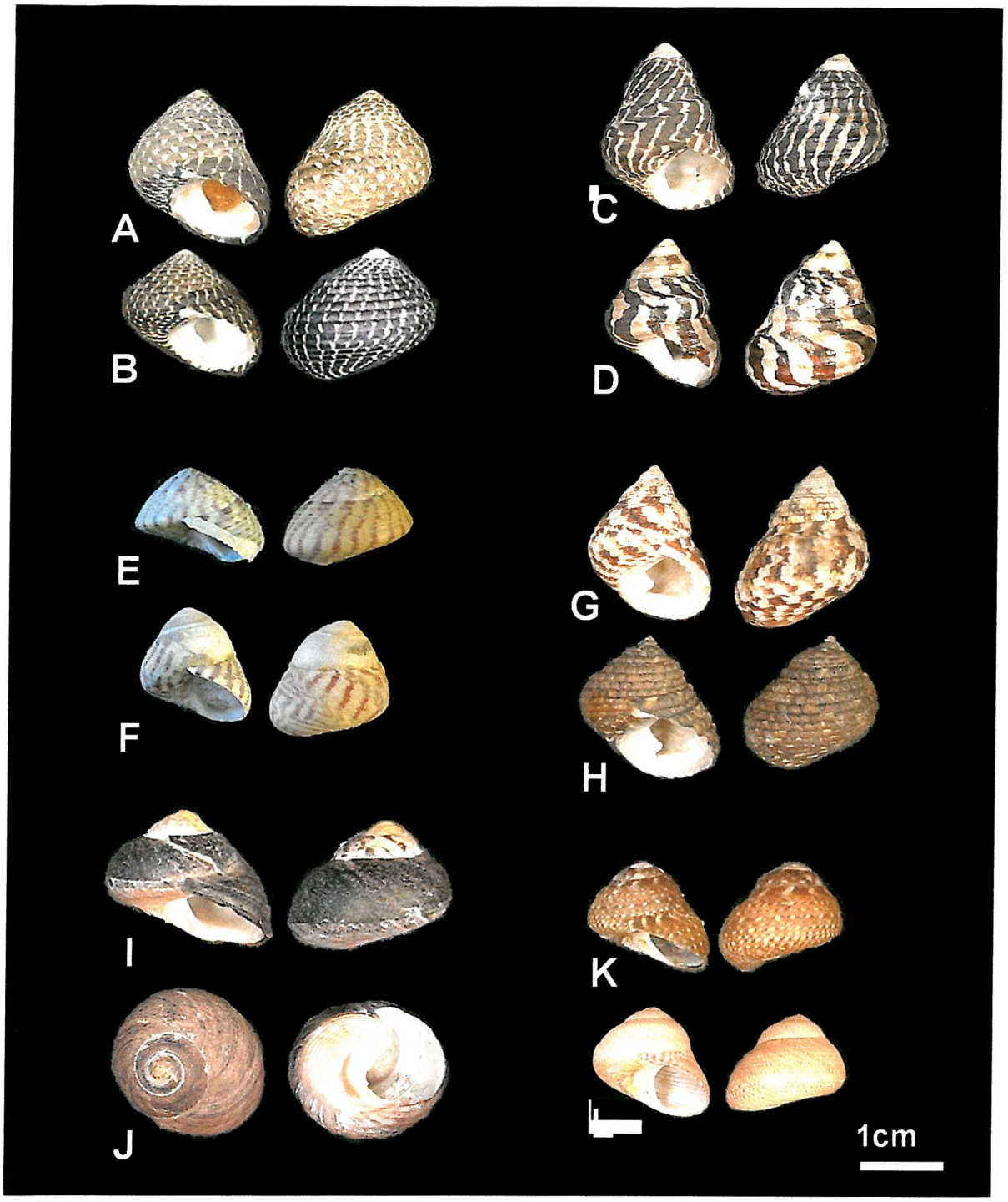


Figure 7.1. Plate of outgroup taxa shells. Species and specimen localities: **A**, **B:** *Austrocochlea concamerata* Sydney, Australia (BMNH acc. no. 2242) **C**, **D**: *Austrocochlea constricta* NSW Australia (BMHM acc no: 2036). **E, F**: *Gibbula umbilicalis* BMNH **E**: Northern France. **F**: British Coast. **G**, **H**: *Monodonta labio* Indian Ocean (BMNH acc. no. 1563). **I**, **J**: *Oxystele sinensis* South Africa (BMNH acc. no: 1761). **K**, **L**: *Oxystele variegata* **K**: Cape of Good Hope, South Africa (BMNH acc. no. 2341). **L**: Cape Colony (BMNH acc. no. 2351).

7.4.1 Rachidian (characters 1-5)

Character 1 (rachidian cusp length)

The rachidian cusp length showed wide variability within the gibbuline taxa, particularly in cusp size and morphology. The rachidian cusp was either *large*; analogous to the lateral cusps in size and shape (e.g. *Austrocochlea constricta,* Figure 7.2B) or *small*; it had a reduced cusp (of length half or less than the lateral cusp length) as in *Monodonta labio* (Figure 7.3B). In the *small* state, cusp shape was often entirely different in shape and denticulation to the lateral tooth cusps (e.g. *Monodonta labio* - Figure 7.3D), or exhibited a comparable but diminished cusp shape and denticulation (e.g. *Osilinus edulis* - Figure 7.10C). The rachidian of *Austrocochlea constricta* (Figure 7.2B) and *A. porcata* (Figure 7.2G) had a well-developed cusp 50-75μm long with 5-7 pronounced denticles. The *Monodonta labio* rachidian had a short broad cusp relative to those of the adjacent lateral teeth (Figure 7.3A, B). Cusp morphology in this species exhibited intraspecific variation possessing a broad, M shaped cusp (Figure 7.3A) or a distinct bifid cusp with fine serrations along the cusp edge (Figure 7.3B). The rachidian of *Oxystele variegata* was similar to that of *A.constricta,* possessing a large blunt-ended denticulate cusp 55-70μm in length similar in morphology to that of the lateral teeth with three to four large denticles extending along three quarters of the cusp edge (Figure 7.4C). In constrast, the rachidian of *Oxystele sinensis* had a smooth rounded cusp 50-70μm long with 2-3 small serrations at the cusp base. In *Gibbula umbilicalis* the rachidian has a small cusp 20-30μm long with a distinct pointed tip and 5-11 small serrations along its edge. The cusp is often bilaterally asymmetrical in this species. The *large* rachidian cusp of *Osilinus kotschyi* (Figure 7.6C) contrasted with the small cusp state observed in the European *Osilinus* species. *Osilinus articulatus,* however, showed unique intraspecific variation in rachidian cusp length. Both large lateral-like cusp and small residual like cusp shape was observed within this species (Figure 7.7B, 7.8C, D). Variation in cusp shape was also observed within *Osilinus atratus* and *Monodonta labio.*

Character 2 (rachidian neck-shaft width)

Neck-shaft width varied between species and appeared to be independent of rachidian cusp length. The rachidian exhibited either the *broad* or *narrow* state. The *broad* state was defined as the neck-shaft width being equal or greater than the half-tooth length, as in *Osilinus kotschyi* (large cusp length) (Figure 7.6C), and *Osilinus lineatus* (small cusp length) (Figure 7.11B). The *narrow* state was defined as the neck-shaft width being less than the half-tooth length, as in *Monodonta labio* (Figure 7.3B). The species *A. constricta, Oxystele variegata, O. sinensis* and *Gibbula umbilicalis* have a well-defined, narrow neck-shaft (Figures 7.2B, 7.4D, 7.4A, 7.5B respectively). In contrast, *A. porcata* exhibits the broad neck-shaft state (Figure 7.2G), as does *Monodonta labio,* in which the rachidian has a long thick neck-shaft (Figure 7.3B). Among the *Osilinus* species, the *narrow* state was found in *O. kotschyi, O. articulatus* and *O.lineatus* (Figures 7.6C, 7.7B and 7.11B respectively). The broad state was found in *O. atratus* (Figure 7.9D, E), *O. edulis* (Figure 7.10B) and *O. turbinatus* (Figure 7.13A).

Character 3 (neck-shaft wings)

The neck-shaft wings are the lateral protuberances either side of the neck-shaft extending from the outer cusp edge to the neck-shaft base. Three states were observed between the taxa: *absent*, *small*, defined as the maximum protuberance of each wing less than half neck-shaft width; or *large*, defined as the maximum protuberance of each wing, equal to or greater than the neck-shaft width. Neck-shaft wings were *absent* in *Osilinus edulis* and *O. atratus* (Figure 7.10B and 7.9D, E). *Small* neck-shaft wings were exhibited in the species *Mondonta labio* (Figure 7.3B), *Oxystele variegata* (Figure 7.4D), *O. lineatus* (Figure 7.11B) and *O. turbinatus* (Figure 7.13A). *Large* wings were found in *Gibbula umbilicalis* (Figure 7.5B) *A. constricta* and *A. porcata* (Figures 7.2B and 7.2G) extending from the cusp top edge to the base of the neck-shaft. The *large* neck-shaft wing state was also observed in *Oxystele sinensis* (Figure 7.4A) and in some *O. articulatus* individuals (Figure 7.8C, E).

Character 4 (rachidian cusp head shape)

The rachidian cusp head shape was either *bifid* (cusp head is two pronged, concave or possessed an indentation) – as in *Monodonta labio* (Figure 7.3B) and *O.atratus* (Figure 7.9D) or *non-bifid* as in *A. constricta*, (Figure 7.2B), *A. porcata* (Figure 7.2G), *Oxystele variegata* (Figure 7.4D) *Oxystele sinensis* (Figure 7.4A), *Gibbula umbilicalis* (Figure 7.5B), *Osilinus kotschyi* (Figure 7.6C), *O. articulatus* Figure 7.8C, B), *O. edulis* (Figure 7.10B) and *O. lineatus* (Figure 7.11B).

Character 5 (rachidian base plate shape)

Throughout the species examined the rachidian basal plate is very similar to that of the lateral teeth in size and shape, and observations upon this reflect observations of the laterals. The rachidian basal plate shape was determined by the plate edge contour from the base of the neck-shaft to the point of maximal basal plate width. Three states were observed: *straight, concave* or *convex*. The *straight* character state was exhibited in *A. constricta* (Figure 7.2B), *A. porcata* (Figure 7.2G), *O. sinensis* (Figure 7.4A), *O.articulatus* (Figure 7.7B), *O. edulis* (Figure 7.10B) and *O. atratus* (Figure 7.9D,E). The basal plate shape of the rachidian of *Monodonta labio* (Figure 7.3B) and *O. turbinatus* (Figure 7.13A) was *concave*. The *convex* character state was observed in *O.kotschyi* (Figure 7.6C).

7.4.2 Lateral teeth (characters 6 – 9)

Character 6 (number of lateral teeth pairs per row)

Five lateral teeth per half row is a feature of the trochacean radula (Hickman & McLean, 1990). In the re-description of *Osilinus kotschyi* by Herbert (1994), however, the presence of *six* lateral teeth per half row (Figure 7.6B, E, F) was observed, but considered to '*be a specific peculiarity and thus of little significance beyond this*'. Herbert proposed this as a further example of ontogenetic increase in lateral tooth number. The remaining taxa examined here all exhibited *five* lateral teeth pairs with the exception of *O.articulatus,* in which both *five* and *six* pairs of teeth occur (Figure 7.8). Only one *O.articulatus* specimen was observed with six pairs, supporting the hypothesis that lateral tooth number could be determined by ontogenetic factors as proposed by Waren (1990).

Character 7 (differentiation of outermost lateral tooth)

Differentiation of the outermost lateral tooth was characterized by the presence of a lobe-like structure on the outer cups base protruding below the cusp denticles. This was observed to differing degrees: either as a small bud-like protuberance as exhibited by *A.constricta* (Figure 7.2F, G) or a larger non-featured basal lobe as in *Monodonta labio* (Figure 7.3E, F). The basal lobe was observed to interact with the cusp denticles to differing degrees (*cf O.atratus* - Figure 7.9F and *O.articulatus* - Figure 7.8E). The *differentiated* outer tooth of *Gibbula umbilicalis* has a more pronounced basal lobe than *A.constricta* or *Monodonta labio*, which interacts with the cusp denticles (Figure 7.5C). In *Oxystele sinensis* the outer lateral tooth is differentiated by the presence of a simple basal lobe that interacts with the cusp denticles (Figure 7.4B). In contrast *no differentiation* was exhibited in *Oxystele variegata;* all five pairs of lateral teeth possessed lobes (Figure 7.4E).

Character 8 (lateral tooth cusp denticulation)

Lateral tooth cusp denticulation (character 8) was either basal, characterized by a few denticles extending less then half the cusp length as in all the *Osilinus* spp. (e.g. *O.articulatus* - Figure 7.7E) or many, extending more than half way or the entire cusp length as in *A.constricta* (Figure 7.2D), *Monodonta labio* (Figure 7.3C, E) and *Oxystele variegata* (Figure 7.4C, E). Lateral tooth denticulation extended less than half way in *Oxystele sinensis* (Figure 7.4B). In *Gibbula umbilicalis*, athough the denticulation is basal (Figure 7.5C), the 3-4 lateral tooth denticles extend further along the cusp edge than those of *O.lineatus* and *O.kotschyi.*

Character 9 (lateral tooth cusp shape)

The lateral tooth cusp shape was scored as *pointed*, as in *Osilinus kotschyi* (Figure 7.6C) and *O. articulatus* (Figure 7.8A, B) or either *blunt* or *chisel-shaped* as in *Oxystele variegata* (Figure 7.4C) and the remainder of species.

7.4.3 Marginal teeth (Characters 10-11)

Character 10 (marginal tooth rachis)

The longitudinal dorsal rachis (character 10) was either; *absent* giving a smooth appearance to the marginal teeth, as in *Oxystele sinensis* and *Osilinus kotschyi* (Figure 7.6G, H, I) or *present*, as in all the other species (e.g. *Osilinus atratus,* Figure 7.9G - I). In *O. lineatus,* as in all species with a rachis, pronouncement of the dorsal ridge progressively diminishes with tooth size (Figures 7.12A-C).

Character 11 (marginal tooth denticulation)

The marginal cusp denticles, as in the lateral teeth (albeit exaggerated), were classified as either *present along the entire cusp edge* to the tip, as in all the *Austrocochlea* species (Figure 7.2E and F), *Monodonta labio* (Figure 7.3E, F) and *Oxystele variegata* or, only *present at the tooth base*, as in all the other species (e.g. *Gibbula umbilicalis,* Figure 7.5D).

Character 12 (shape of marginal tooth denticles)

The denticles were described as either short and serrated, as in *Monodonta labio* (Figure 7.3E and F) or, long and feathery like *Osilinus lineatus* (Figure 7.11A-C), *Oxystele variegata* (Figure 7.4F), and the remaining species.

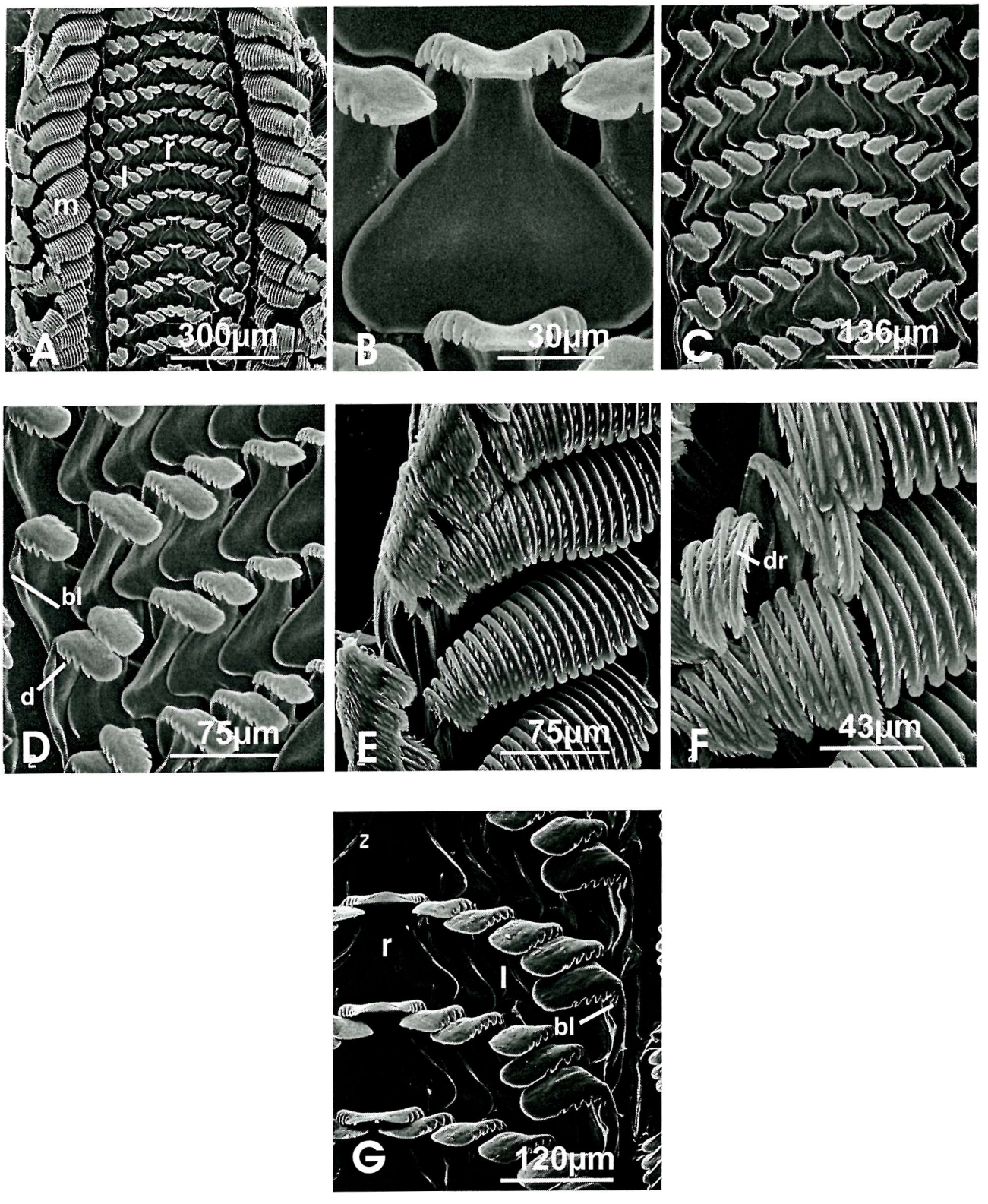


Figure 7.2. Scanning electron micrographs of radulae of *Austrocochlea* species. **A - F** *A. constricta* (Abrolhos I., W.Australia;(BMNH), male, SL= 12.0mm, RL = 6.6mm, 70+ rows) **A** Full width of radula, showing rachidian (r), lateral (l) and marginal (m) teeth. **B** Rachidian. **C** Rachidian and lateral teeth. **D** Lateral teeth with denticulation(d) and basal lobe (bl). **E** Marginal teeth. **F** Inner marginal teeth with dorsal rachis (dr). **G** *A.porcata* Mollybrook, NSW, Australia.(NHM London Acc.no. 2384), female, SL = 13.7mm, RL = 10.2mm, 65+ rows showing rachidian (r), lateral (l) teeth and basal lobe (bl).

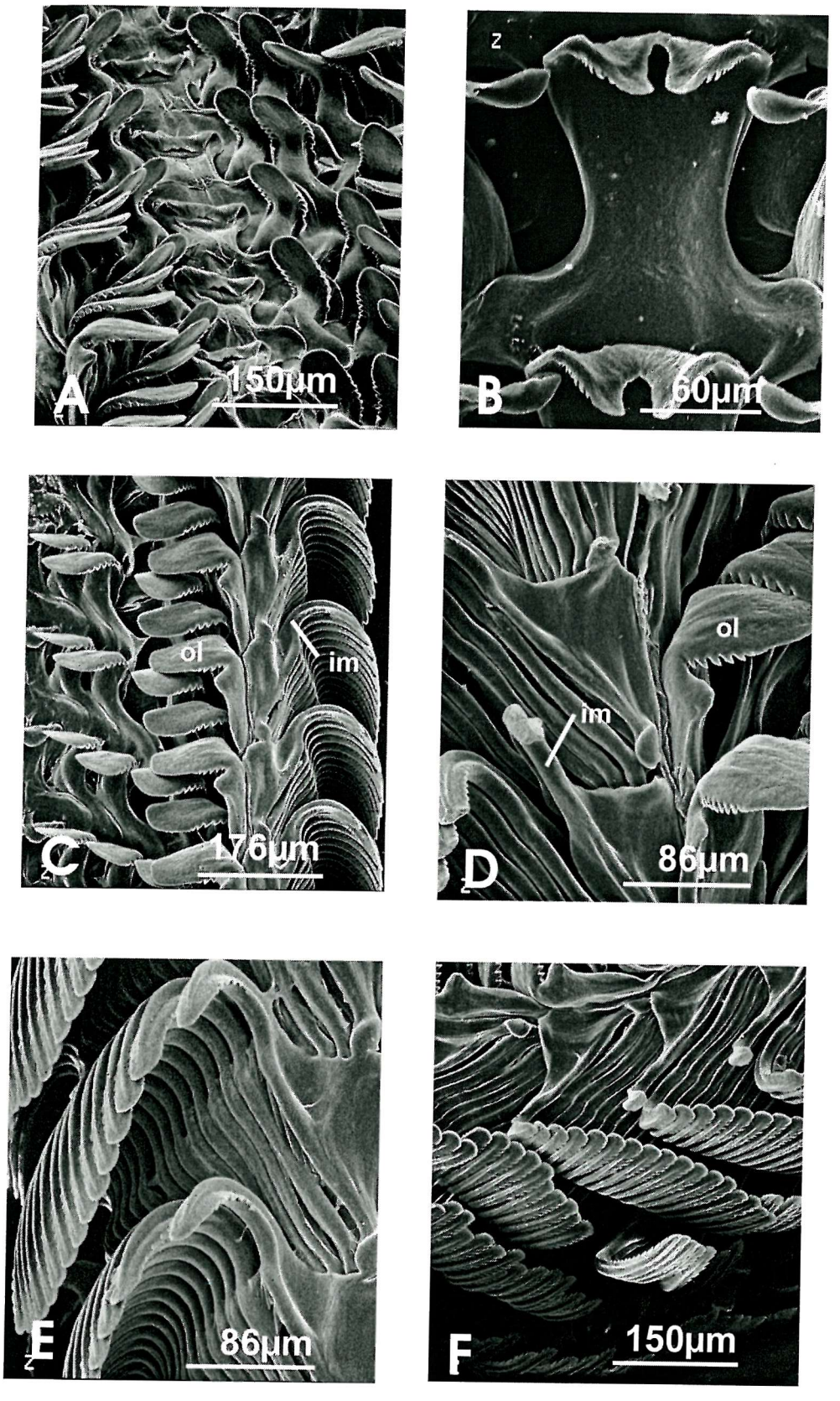


Figure 7.3. Scanning electron micrographs of radulae of *Mondonta labio* (Cape D'Aguilar, Hong Kong; female, SL = 18mm, RL = 16mm, 75+ rows) **A** view at 45' showing rachidian and lateral cusps. **B** Rachidian with bifid cusp. **C** and **D** Outer lateral tooth (ol), showing basal lobe and underside of inner marginal teeth (im). **E** and **F** view at 45' showing marginal cusps with serrated denticulation.

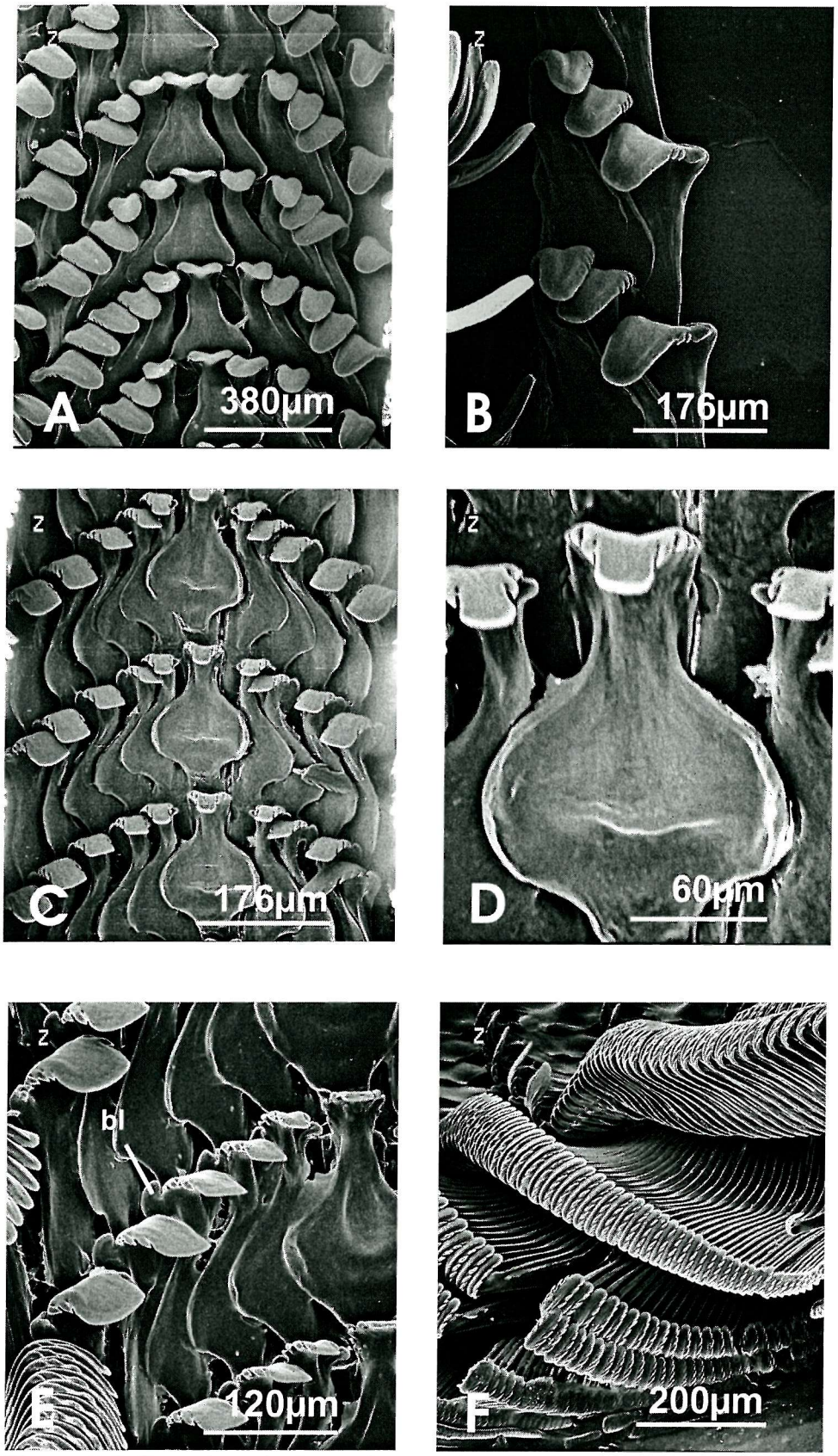


Figure 7.4. Scanning electron micrographs of radulae of *Oxystele* species. **A** and **B** *O.sinensis* (Hermanus, South Africa; female, SL = 22.8mm,RL = 16.1mm). **C - F** *O.varigeata* (Hermanus, South Africa; female, SL = 14.7mm, RL = 11.6mm, 62 rows) showing **C** rachidian and five pairs lateral teeth. **D** rachidian. **E** Basal lobes (bl) present on all five lateral teeth per half row. **F** Marginal teeth.

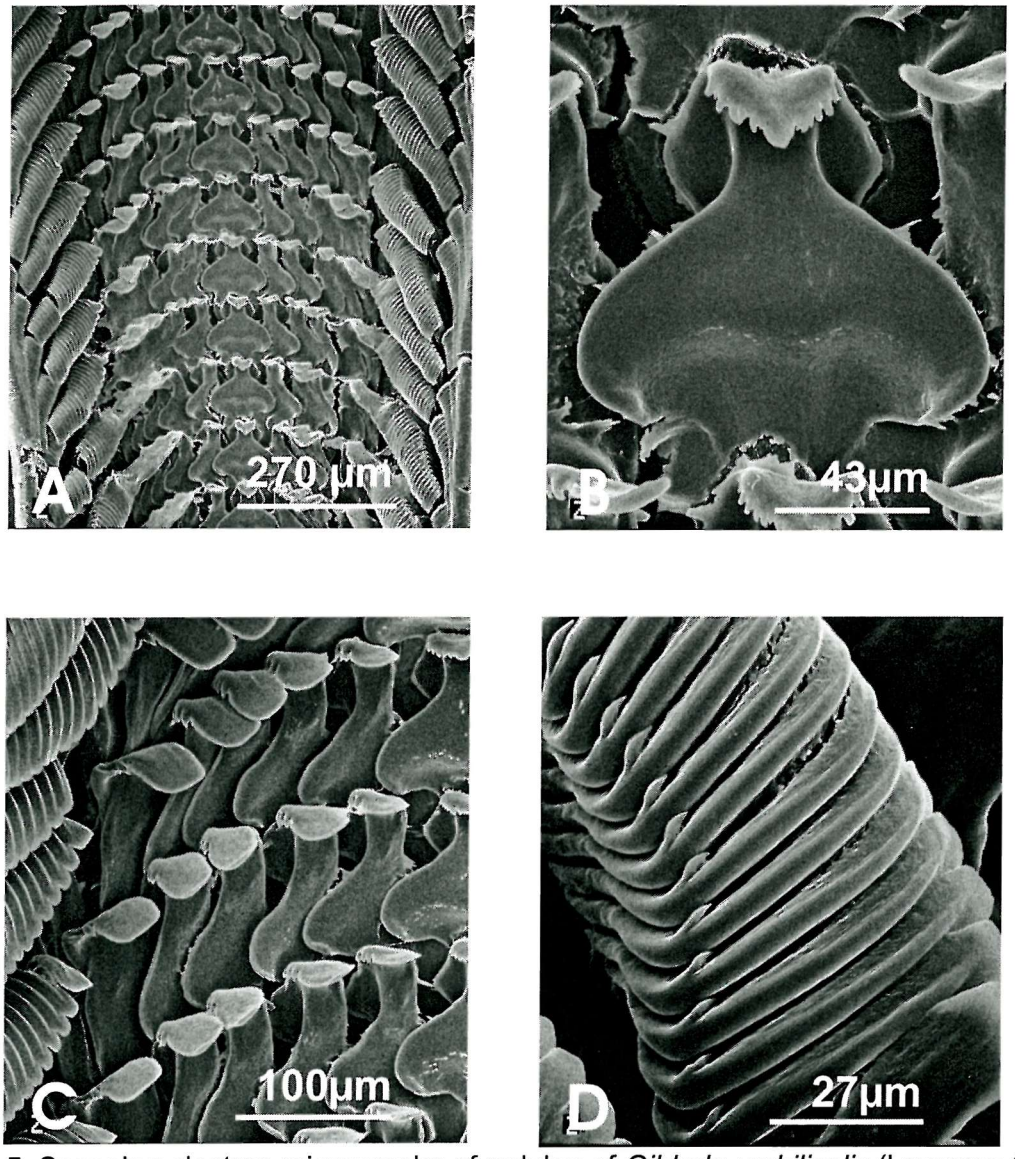


Figure 7.5. Scanning electron micrographs of radulae of *Gibbula umbilicalis* (Lamorna Cove, Cornwall, UK; male, SL = 11.5mm) showing **A** Radula width. **B** Rachidian. **C** Half row of lateral teeth with differentiated outer lateral. **D** Inner marginal teeth with basal denticles and dorsal rachis.

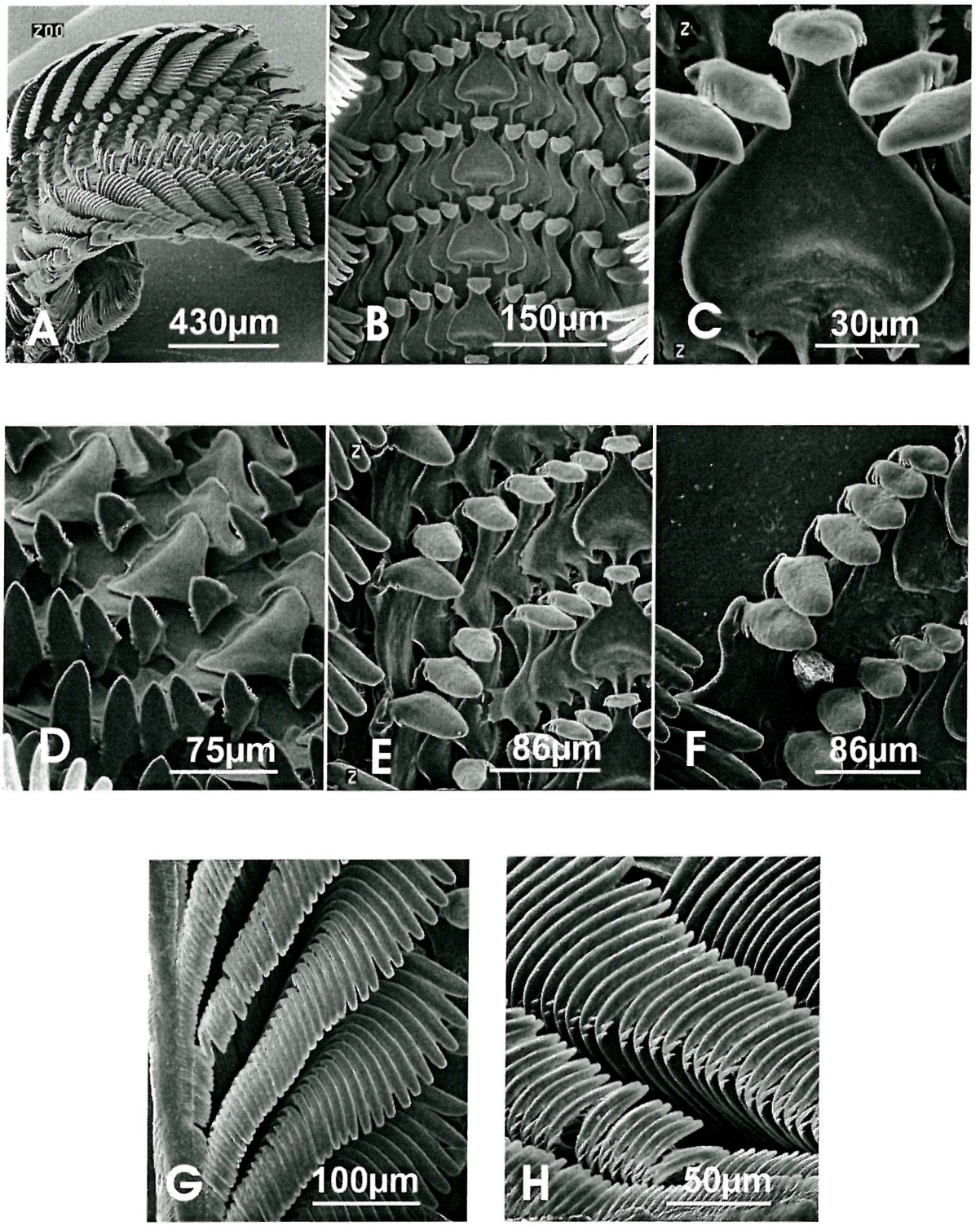


Figure 7.6. Scanning electron micrographs of radulae of *Osilinus kotschyi* (Sandy substrate, Khor Al-Hamra, Ras Al-Khamah, United Arab Emirates; female, SL = 14.3mm, RL = 6.6mm, 60+ rows). **A** View at 45° radula curled to expose cusps. **B** Rachidian and lateral teeth rows. **C** Rachidian. **D** View at 45° showing rachidian and lateral tooth cusps. **E** and **F** Half lateral rows with differentiated outer lateral tooth and basal lobe. **G** and **H** Showing marginal teeth lacking dorsal rachis.

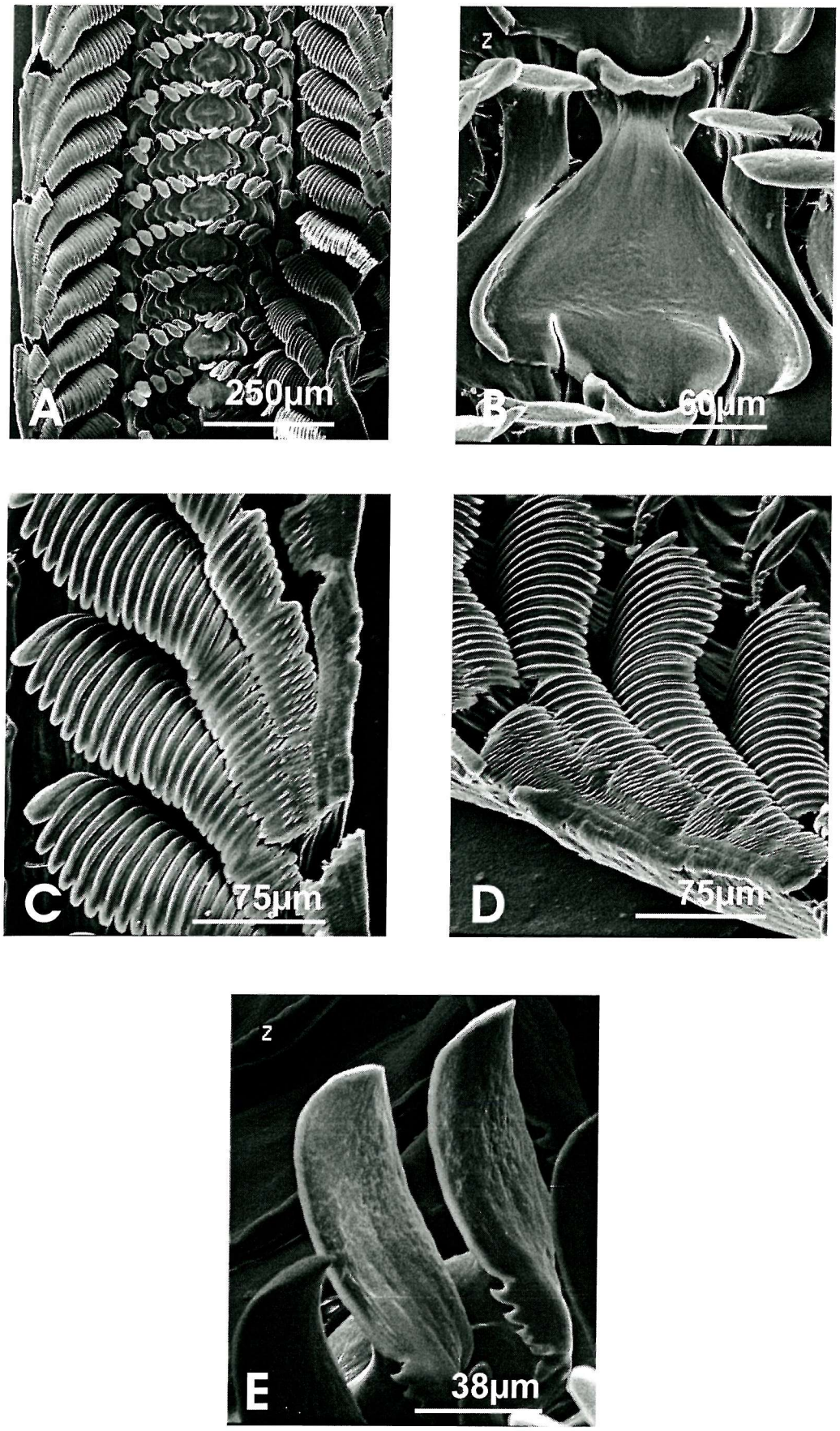


Figure 7.7. Scanning electron micrographs of radulae of *Osilinus articulatus*. **A** (Southern France; male, SL = 18.6mm, RL = 9.2mm, 60+ rows) showing radula width. **B** (Tunisia, N.Africa; female, SL = 28.3mm, RL = 12.6mm, 70+ rows) showing rachidian, **C** and **D** Marginal teeth. **E** View at 45° showing lateral teeth cusps with basal denticles

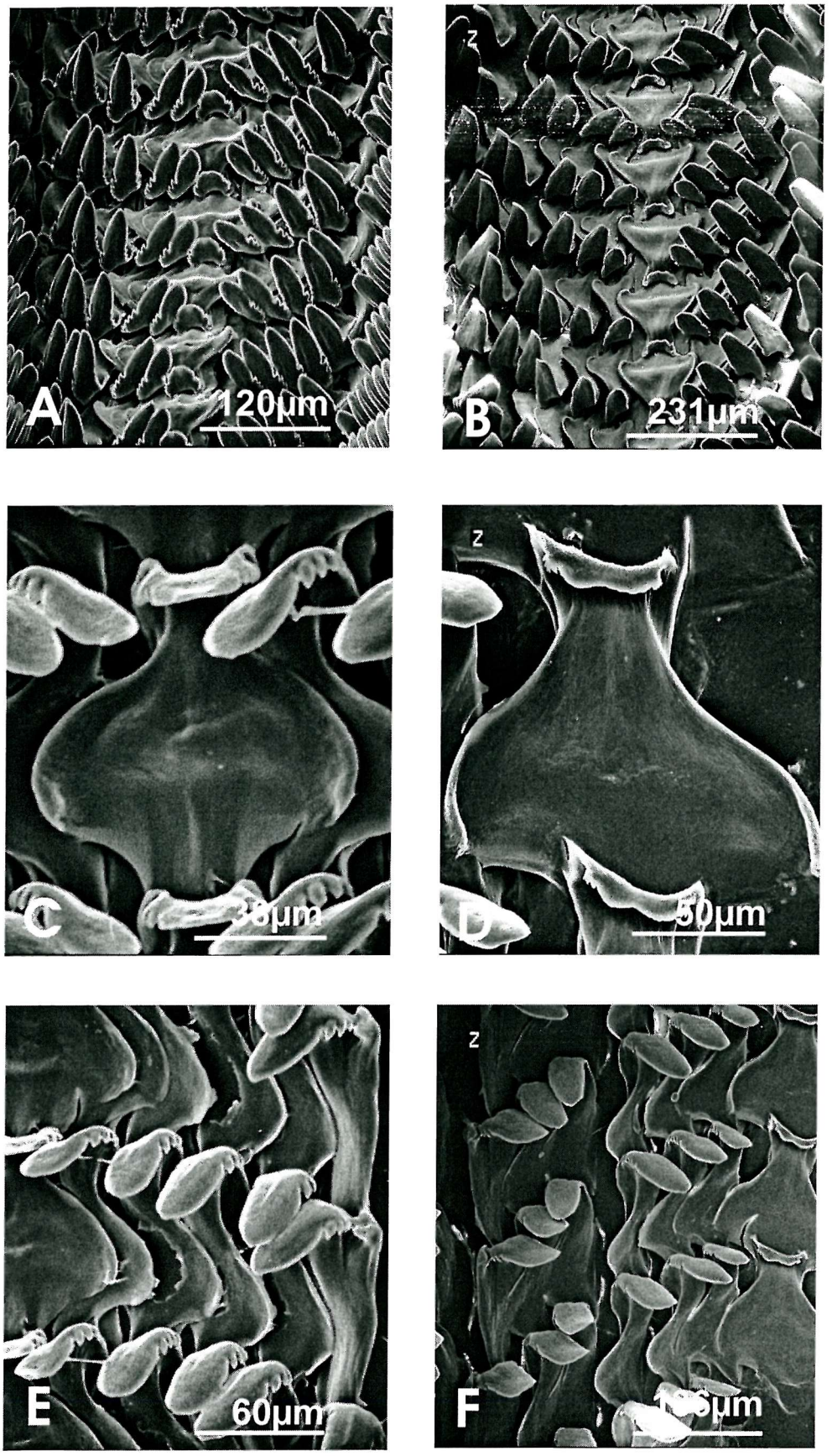


Figure 7.8. Scanning electron micrographs of radulae of *Osilinus articulatus*. **A, C** and **E** (Southern France; male, SL = 18.6mm, RL = 9.2mm, 60+ rows). **B, D** and **F** (Turkey; male, SL = 6mm, RL = 6.8mm, 60 rows). **A** and **B** Rachidian and lateral rows showing 5 and 6 lateral teeth pairs respectively. **C** and **D** showing intraspecific variation in rachidian morphology. **E** and **F** Half lateral rows showing 5 and 6 lateral teeth pairs respectively.

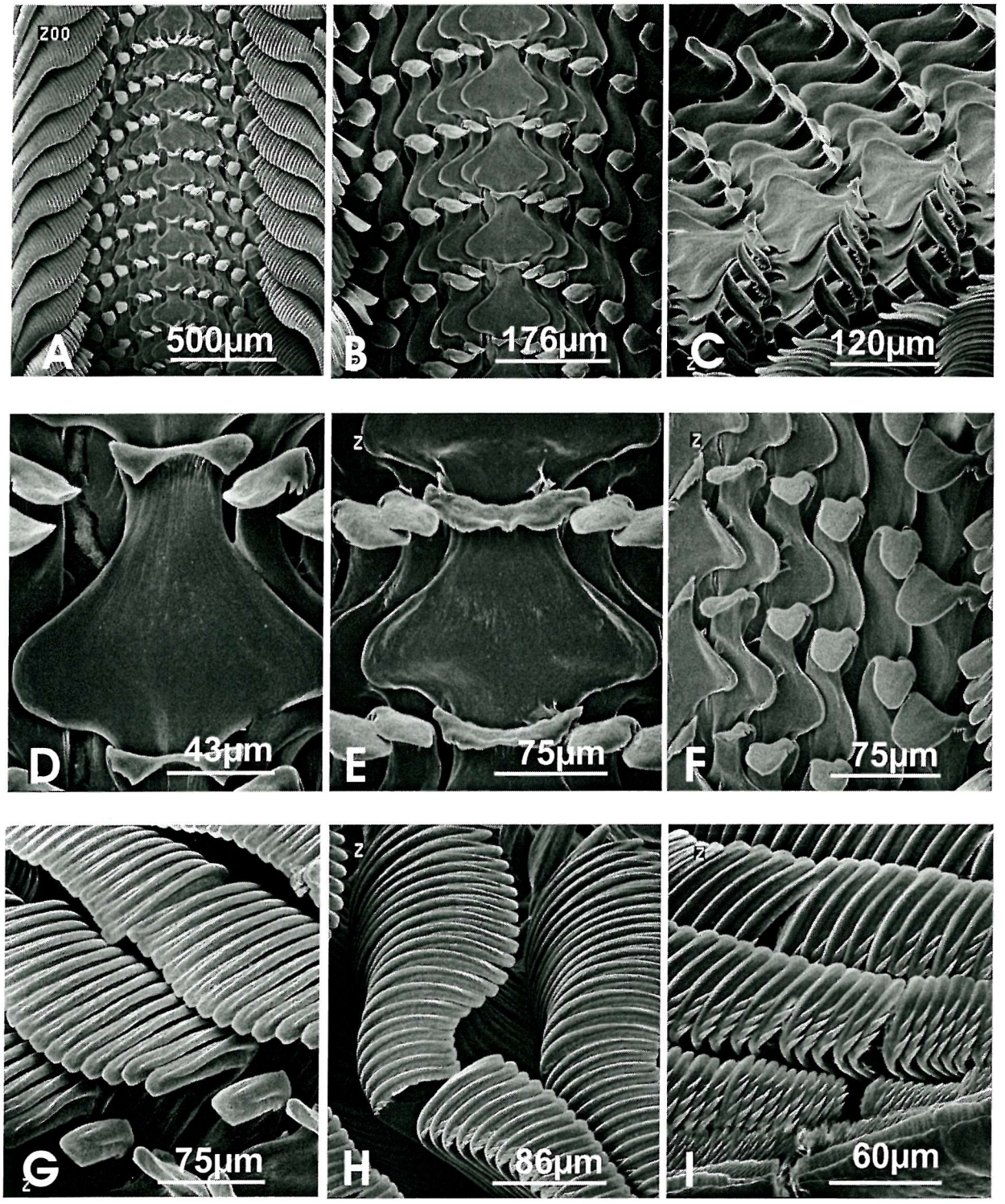


Figure 7.9. Scanning electron micrographs of radulae of *Osilinus atratus.* **A** and **F** (Maderia Island; male, SL = 14mm, RL = 11mm, 75 rows) **B, C** and **D** (Toston Reef, Fuerteventure; female, SL = 17.8mm, RL = 12.8mm, 58 rows). **E, G - I** (St Vincente Island, Cape Verde Islands, NHM Acc.no. 2324; male, SL = 14.8mm). **A** Whole radula width. **B** Rachidian and lateral teeth rows. **C** View at 45° showing reduced rachidan cusp, lateral teeth cusps and denticles. **D** and **E** Rachidian teeth showing intraspecific variation. **F** Half lateral row showing differentiated outer tooth with basal lobe. **G - I** Marginal teeth showing the dorsal rachis and cusp denticles.

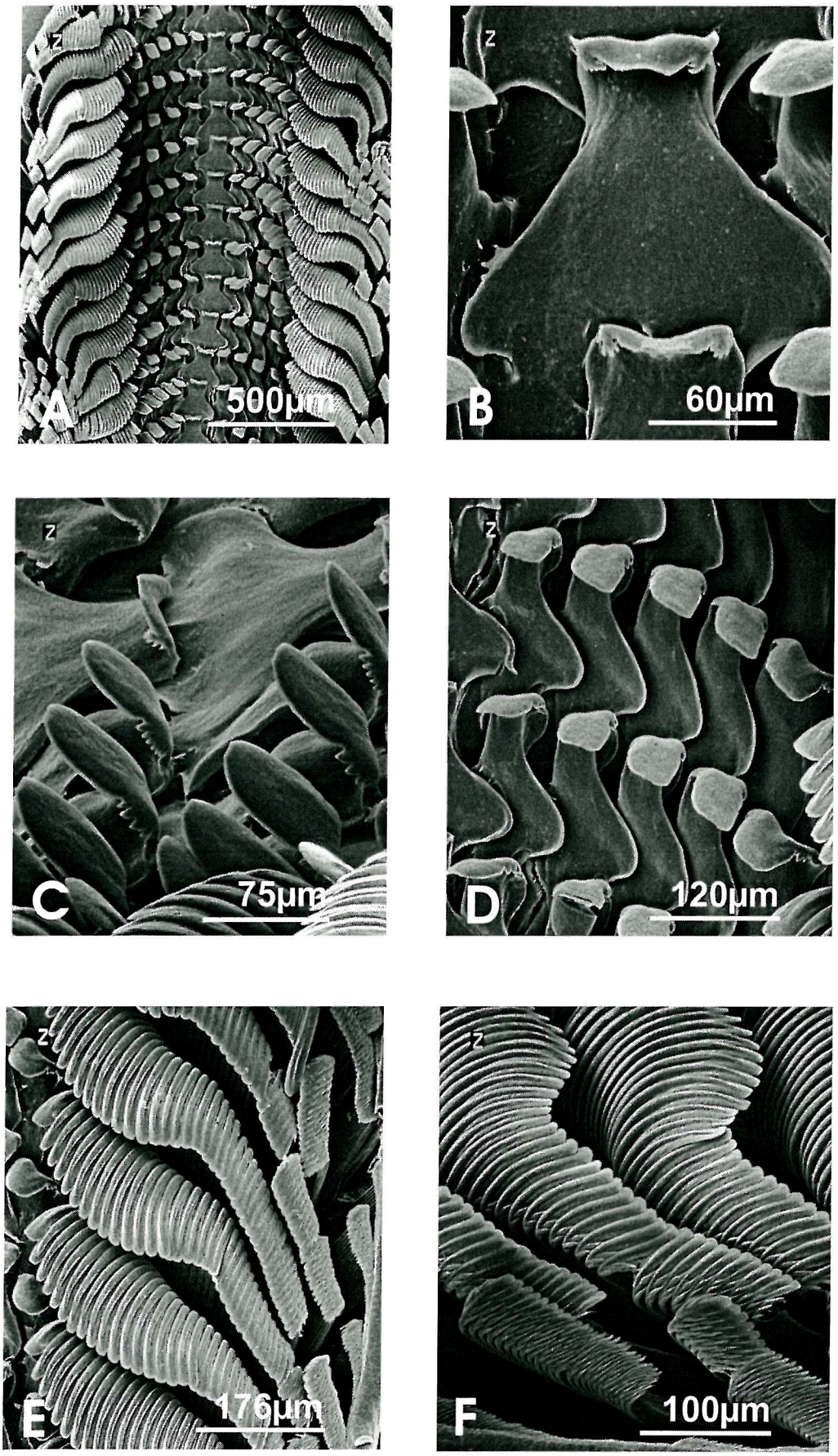


Figure 7.10. Scanning electron micrographs of radulae of *Osilinus edulis*. **A - F** (Southwest Portugal; female, SL = 14.8mm, RL = 12.5mm, 68 rows). **A** Radula width. **B** Rachidian. **C** View at 45° showing diminuitive rachidian cusp and lateral teeth cusps with denticles. **D** Half lateral row with differentiated outer tooth. **E** and **F** Marginal teeth.

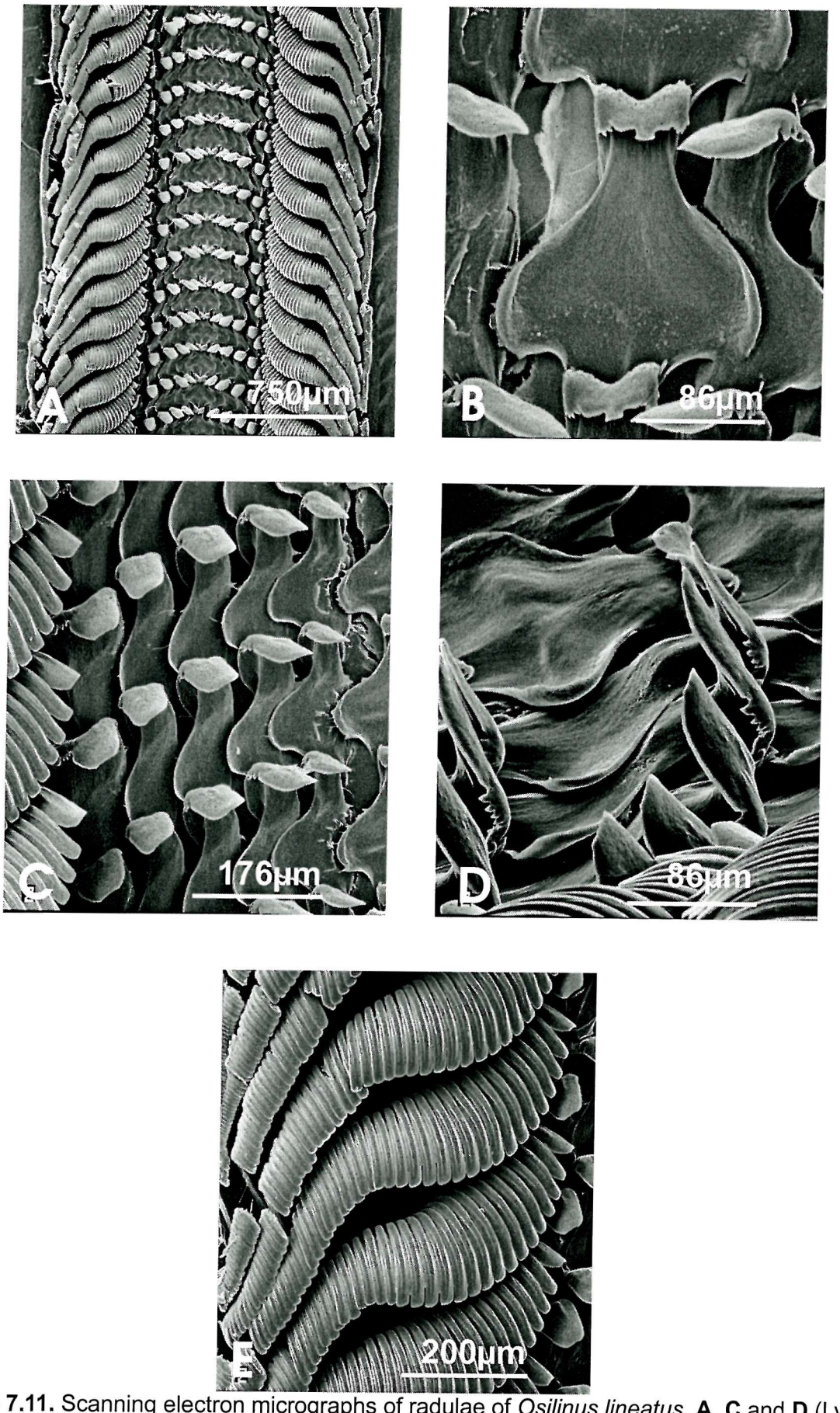


Figure 7.11. Scanning electron micrographs of radulae of *Osilinus lineatus.* **A, C** and **D** (Lyme Regis, UK; male, SL = 16.7mm, RL = 17mm, 93 rows). **B** and **E** (Lamorna Cove, Cornwall, UK; female, SL = 20mm). **A** Whole radula width. **B** Rachidian. **C** Half lateral row showing differentiated outer tooth. **D** Side view of radula at 45° showing reduced rachidan cusp and lateral teeth denticles. **E** Marginal teeth.

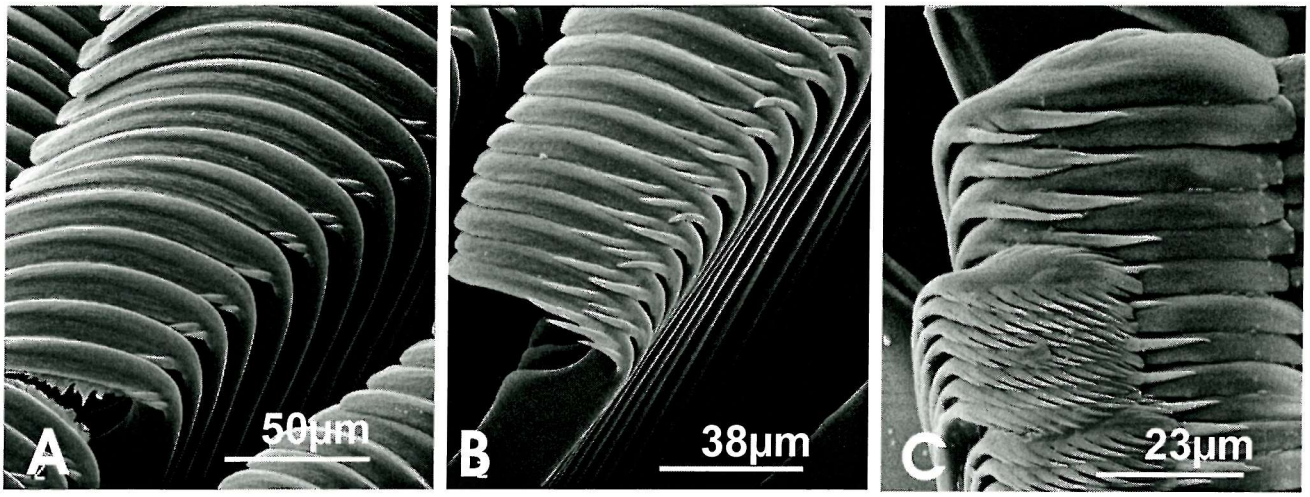


Figure 7.12. Scanning electron micrographs of radulae of *Osilinus lineatus.* (Lyme Regis, UK; male, SL = 16.7mm, RL = 17mm, 93 rows). **A - C** Showing the variation in size and extent of denticualtion of marginal teeth on a single radula. **A** Large inner marginal teeth with few denticles present at the cusp base. **B** Mid marginal teeth with slightly reduced cusp length and a greater number of larger denticles extending further along the cusp. **C** Small outer marginal teeth with many denticles extending along the entire cusp length.

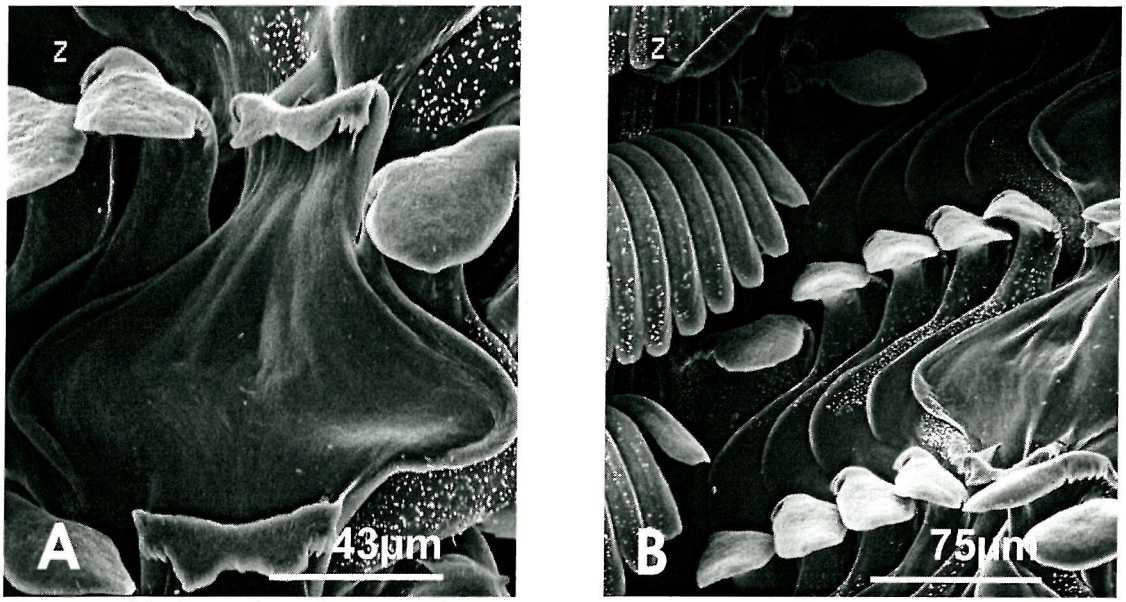


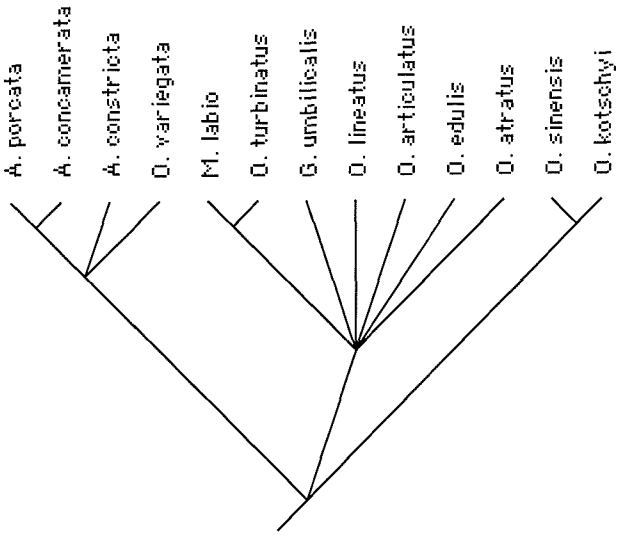
Figure 7.13. Scanning electron micrographs of radulae of *Osilinus turbinatus.* (La Rapiuta, Majorca, NHM collection; female, SL = 19.5mm, RL = 12.8mm, 65 rows). **A** Rachidian. **B** Half lateral row showing differentiated outer tooth.

7.5 Cladistic phylogeny based on radula characters

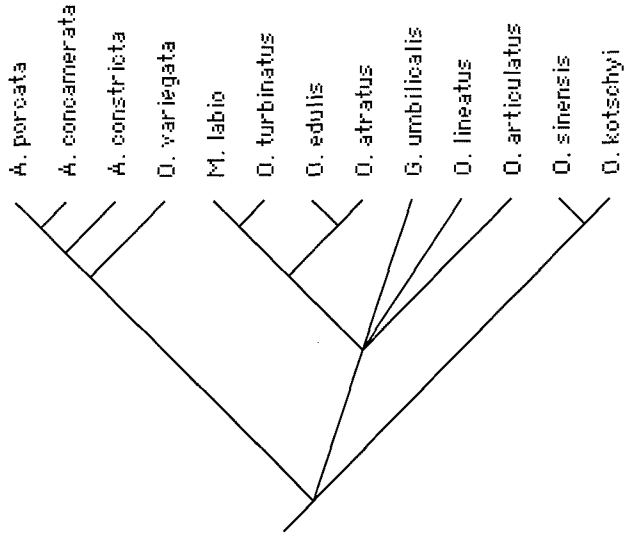
The 12 characters (Table 7.2) and their states in the six *Osilinus* species (ingroup) and seven outgroup species are given in table 7.3. The phylogenetic software PAUP*v4.0b10 (Swofford 1999) was used to analyse the data. All multistate characters were treated as unordered and unweighted. A heuristic search was performed with ten replicates of a random addition of sequences and the tree-bisection-reconnection (TBR) branch swapping algorithm and MULPARS in effect. A total of 13371 rearrangements found 16 most parsimonious trees with a length of 12 steps. The 16 trees were used to calculate the strict consensus and 50% majority-rule consensus trees (Figure 7.14).

Table 7.3: Character state matrix for the cladistic analysis of Gibbulini species. For description of character states see Table 7.2. Unknown or ambiguous characters states are indicated by ?

Species	Character number											
	1	2	3	4	5	6	7	8	9	10	11	12
Austrocochlea porcata	1	1	2	1	0	0	1	1	0	1	2	0
Austrocochlea concamerata	1	1	2	1	0	0	1	1	0	1	2	0
Austrocochlea constricta	1	0	2	1	0	0	1	1	0	1	2	0
Monodonta labio	0	1	1	0	2	0	1	1	0	1	2	0
Oxystele variegata	1	0	1	1	1	0	0	1	0	1	2	0
Oxystele sinensis	1	0	2	1	0	0	1	0	0	0	1	0
Gibbula umbilicalis	0	0	2	1	1	0	1	0	0	1	1	0
Osilinus kotschyi	1	0	2	1	1	1	1	0	1	0	1	0
Osilinus lineatus	0	0	1	1	1	0	1	0	0	1	1	0
Osilinus turbinatus	0	1	1	1	2	0	1	0	0	1	1	1
Osilinus articulatus	0	0	2	1	0	?	1	0	1	1	1	0
Osilinus edulis	0	1	0	1	0	0	1	0	0	1	1	0
Osilinus atratus	0	1	0	?	0	0	1	0	0	1	1	0



A.



B.

Figure 7.14. The phylogenetic relationships of *Osilinus* and selected gibbuline genera outgroup taxa based on radula characters. (A) strict consensus tree. (B) 50% majority-rule consensus tree.

Three clades are identified. One clade contains all three of the *Austrocochlea* species and the South African species *Oxystele variegata*. The second clade contains all of the Atlantic-Mediterranean *Osilinus* species, *Gibbula umbilicalis* and *Monodonta labio*. The third contains *O.kotschyi* and *O.sinensis*. The analysis failed to resolve *Osilinus* as a monophyletic group. The Mediterranean /Atlantic species were joined in a clade with *Monodonta labio* and *Gibbula umbilicalis,* separate from *Osilinus kotschyi*. Interestingly, based on these characters, *Oxystele* is not one group.

The phylogeny based on radular characters proposes *Osilinus* as a polyphyletic group including *Gibbula umbilicalis* and *Monodonta labio*. This suggests that in light of the mtDNA phylogenetic analysis clearly defining the Mediterranean-Atlantic species as monophyletic, the radula does not provide genuinely homologous characters that can resolve evolutionary relationships at the species-level within the Gibbulini tribe. This conclusion does not however render the coding of radula characters as uninformative. Such an approach can help to identify radula characters that are phylogenetically informative at the species and genus level within the Trochidae family, and those that are not useful, there by adding to the debate on the role of morphological characters in resolving phylogenetic relationships.

7.6 Evolution of radula characters

The characters states for all 12 radular characters used in the cladistic analysis are mapped onto the 16S mtDNA maximum likelihood 50% majority-rule consensus phylogeny (chapter 5) using the program MacClade version 3.0 (Maddison & Maddison, 1992) (see Figure 7.15). Characters 2 (rachidian neck-shaft width), 3 (neck-shaft wings), 5 (rachidian base plate shape), 9 (lateral tooth cusp shape) and 12 (shape of marginal tooth denticles) showed variation within the Atlantic/ Mediterranean *Osilinus*. The ancestral states could be deduced from characters 2, 5, 9 and 12, and therefore provide informative on the evolution of the radula. Only character 2 (rachidian neck shaft width), however, was phylogenetically informative within *Osilinus*. This character, however, showed parallelism in the Indian Ocean species *Monodonta labio*. The small rachidian cusp length state was observed in all the Atlantic/ Mediterranean trochids *Osilinus* and *Gibbula umbilicalis* and may be phylogenetically informative in a more inclusive morphological analysis of the Gibbulini tribe. This character also showed parallelism in the Indian Ocean species *Monodonta labio*.

Figure 7.15.

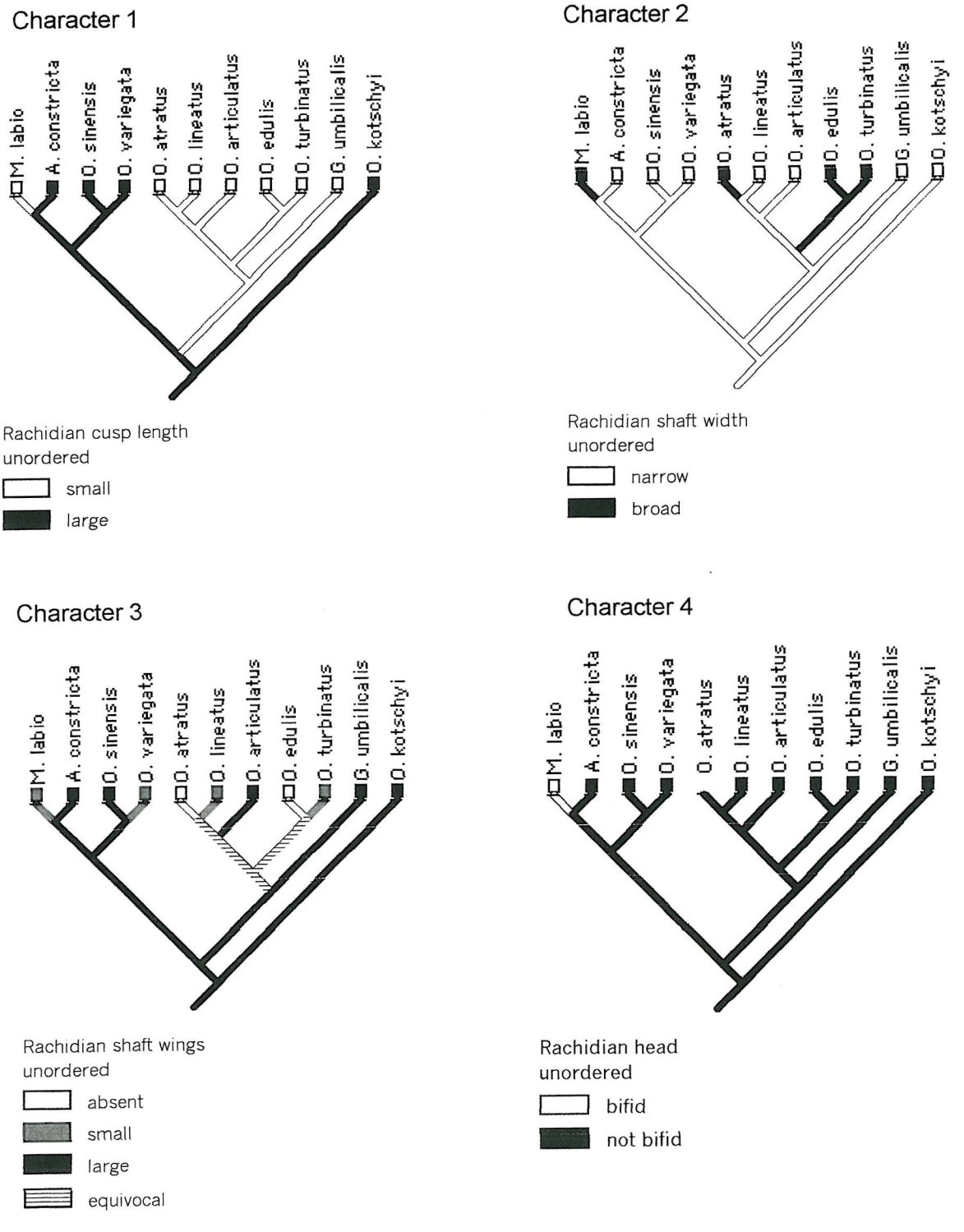
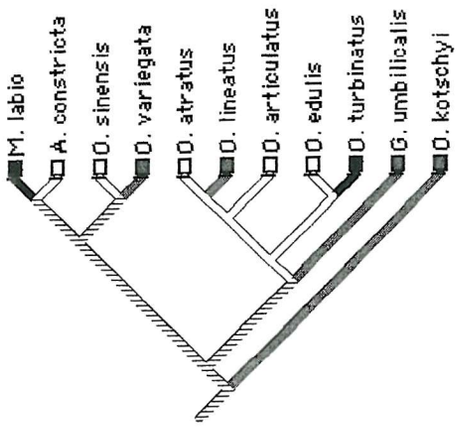

Character 1
M. labio
A. constricta
O. sinensis
O. variegata
O. atratus
O. lineatus
O. articulatus
O. edulis
O. turbinatus
G. umbilicalis
O. kotschyi
Rachidian cusp length
unordered
small
large
Character 2
M. labio
A. constricta
O. sinensis
O. variegata
O. atratus
O. lineatus
O. articulatus
O. edulis
O. turbinatus
G. umbilicalis
O. kotschyi
Rachidian shaft width
unordered
narrow
broad
Character 3
M. labio
A. constricta
O. sinensis
O. variegata
O. atratus
O. lineatus
O. articulatus
O. edulis
O. turbinatus
G. umbilicalis
O. kotschyi
Rachidian shaft wings
unordered
absent
small
large
equivocal
Character 4
M. labio
A. constricta
O. sinensis
O. variegata
O. atratus
O. lineatus
O. articulatus
O. edulis
O. turbinatus
G. umbilicalis
O. kotschyi
Rachidian head
unordered
bifid
not bifid

Figure 7.15. cont.

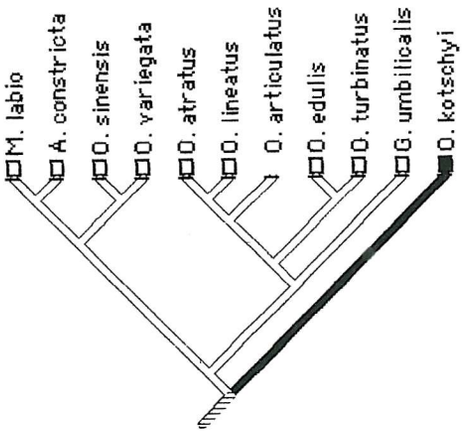
Character 5



Rachidian base plate
unordered

- straight
- convex
- concave
- equivocal

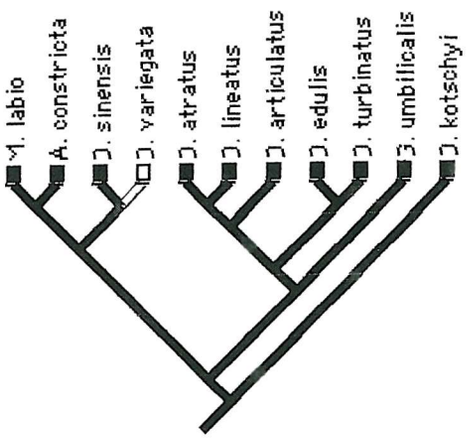
Character 6



Number of lateral teeth
unordered

- 5 pairs
- 6 pairs
- equivocal

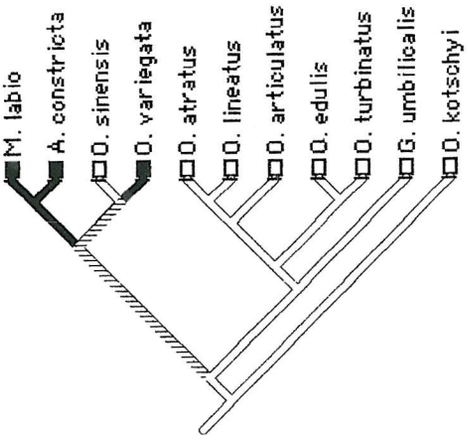
Character 7



Differentiation of outermost lateral
unordered

- basal lobe present on all laterals
- basal lobe present only on outermost lateral

Character 8

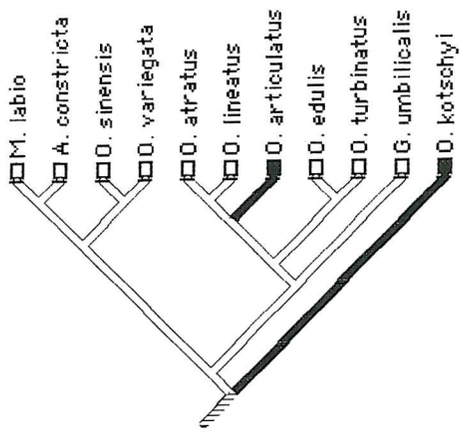


Denticles on lateral tooth cusp
unordered

- denticles at cusp base
- denticles along entire length
- equivocal

Figure 7.15. cont.

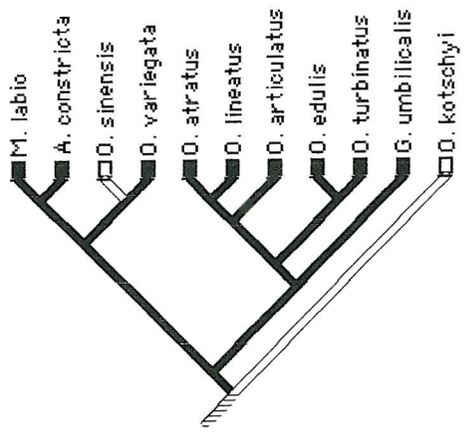
Character 9



Lateral tooth cusp shape
unordered

- blunt or chisel-shaped
- pointed or round
- equivocal

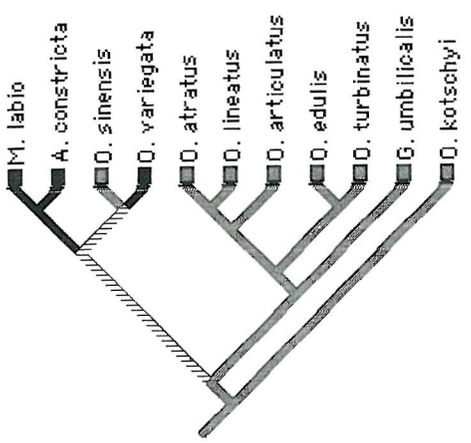
Character 10



Marginal tooth rachis
unordered

- absent
- present
- equivocal

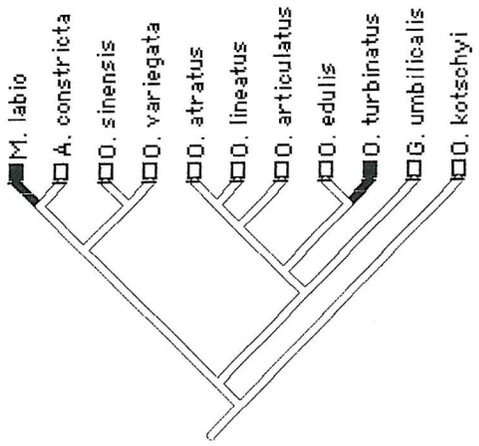
Character 11



Denticles on marginal tooth cusp
unordered

- denticles at cusp base
- denticles along entire length
- equivocal

Character 12



Shape of marginal tooth denticles
unordered

- long
- short

7.7 Discussion

The morphological phylogeny presented in this chapter shows limited congruence in light of the molecular phylogeny. Only rachidian cusp size appears to be homologous among the Atlantic/Mediterranean *Osilinus* species. Two states of rachidian cusp size were observed, small or large. *Monodonta labio* also shared a small rachidian cusp with the Atlantic/Mediterranean *Osilinus* species. This state therefore appears to be a derived character within the Gibbulini tribe that has undergone convergent evolution. However, this character could also be affected by ontogenetic factors (Waren, 1990), since both large and small cusps were observed in *O.articulatus.* Alternatively, ecological factors such as substrate type or feeding habits may influence rachidian cusp size, as shown in *Littoraria* (Reid, 1999b). Intraspecific variation in rachidian cusp shape was only evident in species with a reduced rachidian cusp (e.g. *M.labio, O.articulatus* and *O.atratus*). Species in which the rachidian cusp was analogous to the lateral teeth exhibited far more rigid rachidian morphology. This may be a reflection of the role of the rachidian in grazing, and the food substrate upon which is grazes.

The observation of rare abnormal teeth can be instructive in determining fundamental distinctions between teeth characters that are phylogenetically informative (Hickman, 1990). The observation of variation in lateral tooth number in *O.articulatus* along side the published radula morphology of *O.kotschyi* (Herbert, 1994) therefore presents problems for the use of lateral teeth number (along side marginal teeth number, e.g. Ponder & Lindberg 1997) as a radula character in cladistic analyses of gastropod molluscs.

The taxonomic status of *O.kotschyi* as a member of this genus can be questioned as a result of examination of radular morphology. The *O.kostchyi* radula morphology is not analogous to the Atlantic/ Mediterranean *Osilinus,* adding further evidence that its current taxonomic classification is misplaced. The *O.kotschyi* radula appeared intermediate to the outgroup taxa *Oxystele sinensis* and *A.constricta.* Although Herbert (1994) proposed *O.kotschyi* to be a pre-Miocene relic of the genus, the molecular and morphological work places this species as basal in the Gibbulini tribe in relation to the taxa considered in this work. Trochid radula morphology may therefore provide a phylogenetically informative character within the higher hierarchy of taxonomic resolution, such as the tribe and sub-family level.

Although the radula-based morphology did not support the Atlantic/Mediterranean *Osilinus* species in a monophyletic clade, the radula characters clearly united the Northeast Atlantic species *Osilinus* and *Gibbula* albeit in a polyphyletic clade inclusive of *Monodonta labio.* This reflects the use of the genus *Monodonta* before the reintroduction of *Osilinus* (type species *Trochus turbinatus* Born, 1778) as the appropriate genus name for the Atlantic/Med species (ICZN opinion 1930, 1999).

The morphological work presented here illustrates the variability in the phylogenetic usefulness of radula characters for cladistic analysis. Some of the characters may be appropriate for the

resolution of higher taxonomic relationships, or a more extensive analysis of the genera within the Gibbulini tribe. This work provides a starting point for the morphological analysis of the northeast Atlantic *Osilinus* trochids. Many more characters would need to be included, however, to produce a robust cladistic review. This may not be the most effective approach, however, to resolving the phylogeny of *Osilinus* or Gibbulini (Scotland *et al.,* 2003). The reconstruction of the phylogeny of *Osilinus* taxa and the gibbuline outgroup taxa using radula characters indicates that the rate of change of characters is not comparable to the rate of lineage branching (Reid, 1999b). Comparison of the radular-based morphology with the molecular phylogeny indicates that radula characters in the terminal taxa do not exhibit sufficient derived/apomorphic characters to reflect a common ancestry within *Osilinus.*

Chapter 8
General Discussion

The overall aim of my thesis was to present a model of allopatric speciation in the intertidal gastropod *Osilinus* at the Atlantic/Mediterranean interface. For the first time, the evolutionary relationships of *Osilinus* have been investigated and the taxonomic ambiguities associated with the species of this genus have been clarified. In this final chapter, the results from the whole thesis are interpreted and synthesized to provide a phylogeographic overview of the genus *Osilinus.*

8.1 Limitations of the work

The major limitation to resolving the interspecific relationships of the Atlantic/Mediterranean species of *Osilinus* was the lack of samples representing the entire geographic range of each species. This was particularly true of *O. atratus.* It was not possible to obtain *O. atratus* specimens from the Selvagens, Madeira (despite extensive searching), the Cape Verde Islands or Senegal, N. Africa. Molecular analysis of DNA sequences of specimens from these locations is required to establish the population structure of this species throughout the Atlantic Islands. The genetic population structure will provide an insight into gene flow between the Islands, and offer further insight to the mechanisms of speciation. An appropriately variable mitochondrial or nuclear gene would need to be identified to resolve the population structure: possible markers include Cytochrome *b* or the Internal Transcribed Spacer 2 (ITS2). Furthermore, the subspecies designations of the *O. atratus* and *O. edulis* populations on the Selvagens cannot be confirmed or rejected, and therefore remains a taxonomic ambiguity. The intraspecific relationships of the Selvagen Island *O. edulis* and *O. atratus* morphotypes to the rest of the Atlantic island populations are of interest: the Selvagens population may be ancestral to the more common morphotypes radiated throughout the younger Atlantic Islands. The sample size of *O. articulatus* was also very low. This limitation of the sequence data was most likely the cause of the lack of resolution of the position of *O. articulatus* within the phylogenetic relationships of *Osilinus* between the optimality criteria and the two gene fragments (16S and COI) (Chapter 6).

The phylogenetic trees presented in this work represent the ancestry of the mitochondrial genes COI and 16S. These trees are used to infer the relationships of the *Osilinus* species from which the gene sequences were taken. The branching points on these trees are taken as representative of speciation events. The gene tree and the species tree are not, however, the same (Nichols, 2001). The correct species tree can only be determined from a tree based on the DNA sequences of the entire genome, which is currently not feasible (Cummings, 1995) but is likely to be so; the complete mitochondrial genome sequence of the snail *Biomphalaria glabrata* was released on GenBank in 2004. Therefore a major limitation of this work is that it uses gene trees based on limited sequence data to infer the phylogenetic relationships of the species, and construct hypotheses on the speciation events represented as branching points on the gene tree.

Molecular divergence times can be used to infer the date of cladogenetic events (Cunningham & Collins, 1994). This requires the assumption that the data behaves in a clock-like manner (Avise, 1994). The phylogenetic analysis (Chapter 6) revealed that the 16S and COI mtDNA genes did not behave in such a manner for the taxa under investigation. This limits the ability to confidently test if the ages of nodes in a phylogeny topology are congruent with vicariant episodes (in this case palaeoceanographic events) that may have been responsible for divergence at these nodes. A general mitochondrial DNA clock (2-4% Myr $^{-1}$) is assumed for an approximate estimate of lineage branching times. Methods that do not assume a strict molecular clock have been recently proposed (Thorne *et al.,* 1998; Huelsenbeck *et al.,* 2000; Yoder & Zhang, 2000), although these methods have not been fully evaluated (Barraclough & Nee, 2001), and time pressures did not allow their use within this study.

8.2 Taxonomic classification of *Osilinus*

Limitations aside, the taxonomic confusion surrounding the *Osilinus* species has been clarified as a result of my work. The molecular phylogeny of chapter 5 suggests that the recent re-classification of *O. kotschyi* within *Osilinus* is wrong. The description of *O. kotschyi* as an ancient Tethyan relic of the Atlantic/Mediterranean species (Herbert, 1994) is not supported by common ancestry. This species may, however, be sister to all Atlantic/Mediterranean gibbuline taxa, or even basal within Gibbulini.

The number and taxonomy of the species within the Atlantic/Mediterranean *Osilinus* genus has been clarified by the interspecific phylogeny (chapter 6). The conservative working species designations I assumed at the start of my work, as a result of the taxonomic ambiguities, have been supported by the molecular phylogenetic analysis. *O. edulis, O. atratus, O. lineatus, O. turbinatus, O. articulatus* are defined as separate species based on high bootstrap support values for monophyly of each species throughout the analyses. *Osilinus* in the Atlantic/ Mediterranean does not, therefore, appear to represent a complex of sibling species (Hickman & McLean, 1990) conforming to the true definition of such species (Knowlton, 1994). Reproductive isolation of each species is also supported by their sympatric distributions: each species occurs in sympatry with another species represented in the phylogeny at at least one sample location. Reproductive isolation is also suggested by the sequence saturation curves and transition/ transversion plots (chapter 6). The micro- processes and macro-evolutionary processes appear to be decoupled in terms of genetic divergence within and between species respectively.

The greatest taxonomic confusion at the outset of my work was regarding the number of species occurring under the name *O. edulis* (Poppe & Guido, 1991; Crothers, 2001). The intraspecific phylogeny of *O. edulis* (chapter 6) and the morphological analysis (chapter 7) does not support an *O. edulis/ O.colubrinus/ O.sauciatus* species complex (Gofas *pers. comm.,* Nordsieck, 1982). The shell morphology shows that intermediate forms exist between the two morphotypes thought to typify the Portuguese specimens and the Atlantic Island/N. African specimens. There is no

biogeographic basis for the morphological division; a large degree of shell variation is exhibited in *O. edulis* populations throughout is range in the northeast Atlantic (chapter7). Furthermore, specimens of *O. edulis* from highly divergent shell morphs are genetically identical in their COI and 16S DNA sequences. The mtDNA phylogenies show no intraspecific clade structure supporting the geographic division between a Portuguese *versus* Atlantic Island/N. African subspecies within *O. edulis*.

Some population structure within *O. edulis* is evident, however, from the 16S and COI phylogenies. It appears that the Madeira population of *O. edulis* has undergone genetic differentiation, occurring uniquely in a clade shared only by Moroccan samples (as discussed in chapter 6). This may reflect two historic colonisation events of the Atlantic Islands by the mainland populations of *O. edulis* (O'Foighil, 2001). Conversely, the Madeiran population may be undergoing divergence due to barriers to dispersal between them and populations on the Canary Islands, and limited gene flow between the mainland populations. Alternatively, the Madeiran population of *O. edulis* may have undergone a population bottleneck as a result of range expansions and retractions during the Pleistocene Ice Ages. The cooler climate of North Europe as a result of ice sheet expansion caused a southward shift of the ranges of flora and fauna in the Northeast Atlantic (Hewitt, 1996). The genetic differentiation exhibited by the Madeira populations of *O. edulis* may therefore be a relic of a glacial refuge of this species on the Atlantic Islands, as the mainland populations of the Iberian continent retreated south during the transition from warmer interglacial to cooler glacial climatic conditions.

In contrast to *O. edulis*, the shell morphology of *O. turbinatus* supports the biogeographic patterning exhibited in the molecular phylogeny between the western and eastern Mediterranean basin populations (chapter 7). Although still recognized as a single species, further work is required on the population structure of *O. turbinatus* to establish the extent and pattern of gene flow within the Mediterranean. Intraspecific molecular analysis of a greater number of samples of this species representing its geographic distribution throughout the Mediterranean is required using genetic markers with greater resolving power. This would establish if the observed phylogenetic distinction of individuals from the two basins represents the extreme ends of isolation-by-distance occurring gradually between the two ends of the Mediterranean, or it is a reflection of a population subdivision between the two basins. Parallel intraspecific molecular analysis of the population structure of *O. articulatus* in the Mediterranean would provide an interesting comparison of gene flow and structure between these sympatric species. The intraspecific variation in the radula morphology of *O. articulatus* was the most extensive of the *Osilinus*. Population genetic analysis would establish if the plasticity in radular morphology is reflected in its genetic structure, or is a result of ecological and/ or feeding factors.

8.3 Mode of speciation in *Osilinus*

A consequence of determining the correct number and identification of species within this genus is the distinction of clear species boundaries. Knowledge of species boundaries is crucial to inferring speciation modes from geographic patterns. Thus, biogeography depends on the species concerned being correctly described and their distributional limits known. Classically, the vicariant and dispersal theories of biogeography assume that speciation is predominantly allopatric, and that sympatry is a result of dispersal (Avise, 1994). The development of phylogenetic approaches, and methods of phylogeography, have led to a more robust method of determining the geography of speciation (Losos & Glor, 2003). The temporal pattern of lineages (phylogenetic relationships) can be used to infer the mode of speciation: allopatric, peripatric or sympatric (Barraclough & Vogler, 2000). Peripatric speciation is when a small founder population, fragmented from a large parent population, diverges rapidly under the effects of genetic drift and inbreeding (Hartl & Clark, 1989).

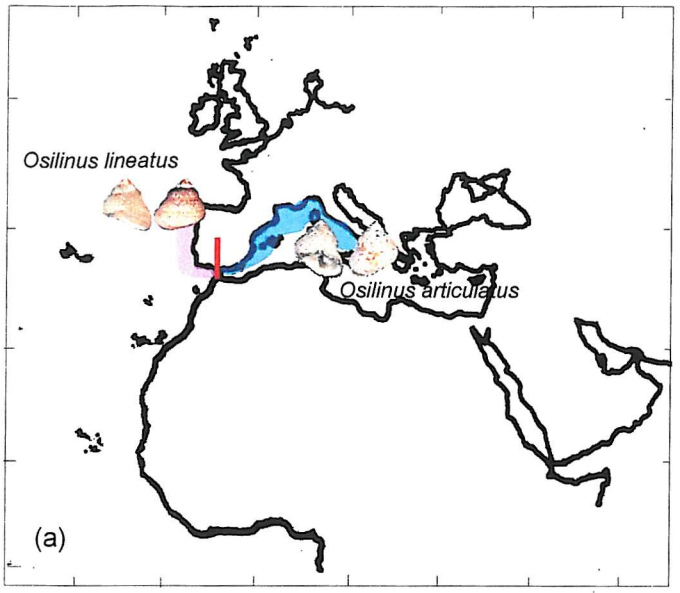
Species-level phylogenies, with all living members of a clade present, can provide a powerful tool for resolving the evolutionary relationships and thus investigating speciation events in a taxa (Barraclough & Nee, 2001). The molecular phylogenetic analysis presented here has partially resolved the evolutionary relationships of all living species of *Osilinus* (discussed in chapter 6). The spatial and temporal distributions of these phylogenies, in the context of historical events, have been used to infer the mode and processes of speciation of the taxa. This is the basis of a phylogeographic approach to understanding speciation.

My work suggests that two key speciation events have occurred within *Osilinus* in the Northeast Atlantic/ Mediterranean. The first major branching point in the phylogeny separates these two proposed radiations (chapter 6). One radiation has resulted in the species *O. edulis* and *O. turbinatus*. There is strong support for *O. edulis* and *O. turbinatus* as sister species in a monophyletic clade (a value of 100 is supports the clade in the combined maximum-likelihood bootstrap tree, figure 6.28). The second radiation has led to the evolution of *O. atratus, O. lineatus* and *O. articulatus*. These occur in a separate monophyletic clade albeit with lower bootstrap support values than for the *O. edulis* + *O. turbinatus* clade, and therefore cannot be relied upon until further phylogenetic analysis including more sequences provides a more robust branching pattern between these three species.

The geographical distribution of sister taxa determined by the interspecific phylogeny of *Osilinus* has been used to infer allopatric speciation as the main mode of evolution in this Mediterranean/ N. Atlantic gastropod. The allopatry of sister taxa infers allopatric speciation (Barraclough & Vogler, 2000). The biogeography of the sister taxa *O. edulis* + *O. turbinatus* and *O. lineatus* + *O. articulatus* is strong support for allopatric speciation by vicariance in these species pairs (Losos & Glor, 2003). According to branch length, and therefore inferred evolutionary time since a shared ancestor (assuming a fairly constant rate of mtDNA mutation/sequence divergence across

Osilinus) (Hillis *et al.,* 1996; Nichols, 2001) these two speciation events occurred concurrently. This suggests that the same historical event caused speciation in both the lineages. Alternatively, two different events occurred at similar times in the evolutionary history of *Osilinus.* It is likely, however, that the Messinian Salinity Crisis (MSC, see chapter 2) approximately 6 Ma (Benson, 1991), and subsequent isolation of the Mediterranean from the Atlantic was the historical event that caused geographical separation of the ancestral populations and initiated genetic divergence and the ensuing allopatric speciation in both lineages.

From my work, it is possible to suggest peripatric speciation as the mode of geographical speciation for the species *O. lineatus* and *O. atratus.* Peripatric speciation can be inferred when sister species do not overlap, and the range of one species is smaller than the range of another (Losos & Glor, 2003). This is true for the sister taxa *O. lineatus* and *O. atratus.* The speciation of *O. atratus* may therefore have arisen by a rare dispersal event. Colonisation of the Atlantic Islands by a founder population that overcame an existing geographic barrier (i.e. the distance from the continent to the Atlantic Islands) may have led to divergence from the geographically isolated mainland population in allopatry under genetic drift and differential selection pressures in the new environment. Peripatric speciation, as described above, mediated by the effect of founder populations was thought to be the main mode of speciation to explain species distributions before the development of cladistic and phenetic hypotheses (Dobzhansky, 1937, 1951; Mayr 1954). Since then it has been argued that the occurrence of peripatric speciation in nature is relatively rare as a geographical mode of speciation (e.g. Bush, 1994; Moya *et al.,* 1995 but see Slatkin, 1996, 1997). In a study inferring speciation mode from the geographical distribution of sister-taxa lineages, however, support for the peripatric model of speciation was confirmed (Barraclough & Vogler, 2000). An alternative explanation for the geographical distribution of the sister taxa *O. lineatus* and *O. atratus* is that low sea stands during glacial cycles of the Pleistocene Ice Ages and the consequent exposure of seamounts (Epp & Smoot, 1989) facilitated the range expansion of the ancestral population from the mainland to the Atlantic Islands via a stepping stone model (Kimura & Weiss, 1964). Subsequent rises in sea level would have created a physical barrier to gene flow if the distance between the continent and remaining Atlantic Islands was greater than larval dispersal ability. The island and mainland populations would subsequently have diverged and therefore speciated by vicariant allopatry. In the maximum-likelihood phylogeny (Figure 6.27) *O. atratus* is basal within the *O. lineatus* + *O. articulatus* + *O. atratus* clade. This suggests that the colonisation, and subsequent isolation of populations of *Osilinus* on the mainland and Atlantic Islands (which became *O. lineatus* and *O. atratus* respectively), may have preceded the speciation events as a result of the Messinian Salinity Crisis, see Figure 8.1.



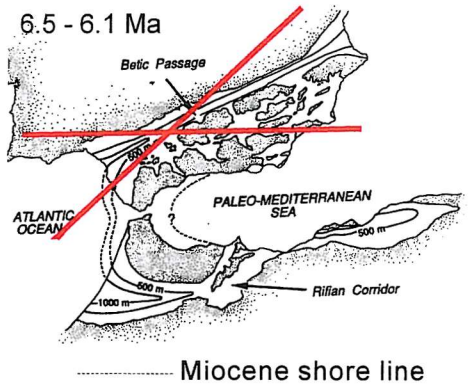
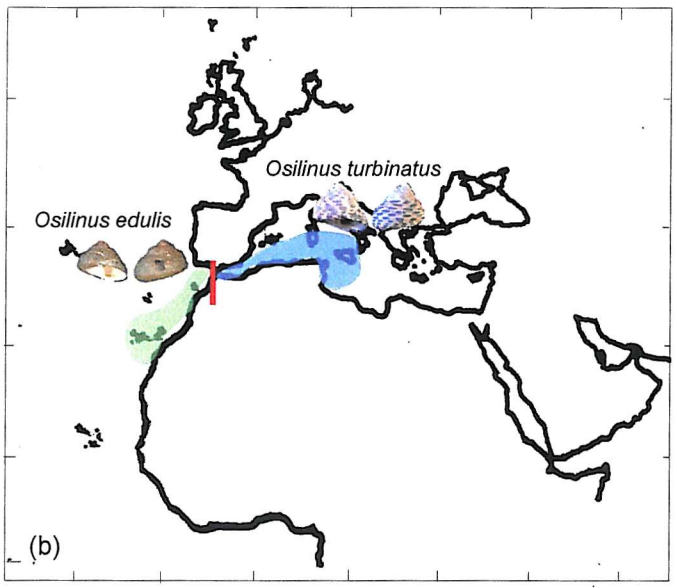


Figure 6.29(a) vicariant speciation model of *O. lineatus* and *O. articulatus*. The Betic Corridor closed between 6.5-6.1 Ma signalling the onset of the Messinian Salinity Crisis (MSC), and separating the previously continuous population of ancestral *Osilinus* taxa either side of the Iberian peninsula.



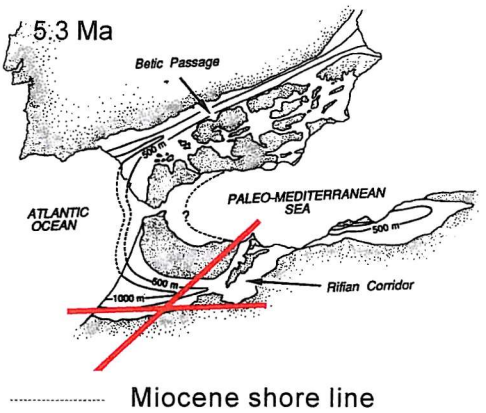
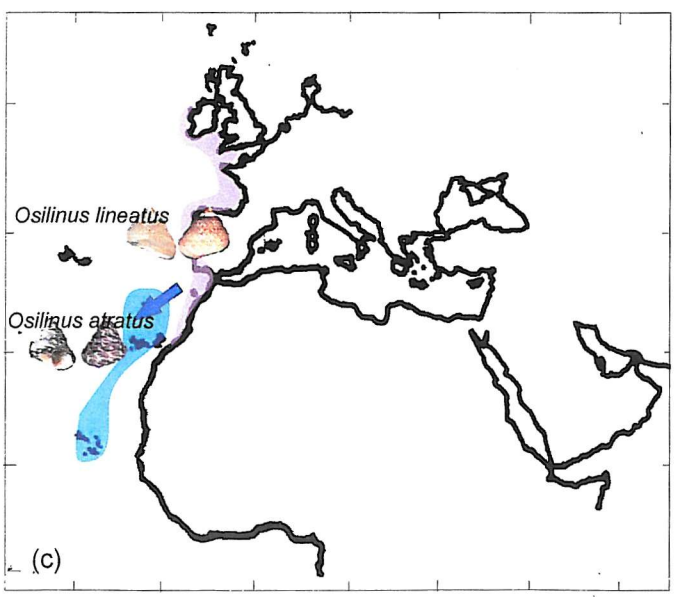


Figure 6.29(b) vicariant speciation model of *O. edulis* and *O. turbinatus*. The Rifian Corridor closed 5.3 Ma isolating the Mediterranean from the Atlantic Ocean, and dividing the previously continuous population of ancestral *Osilinus* taxa either side of what became the Gibraltar Straits.



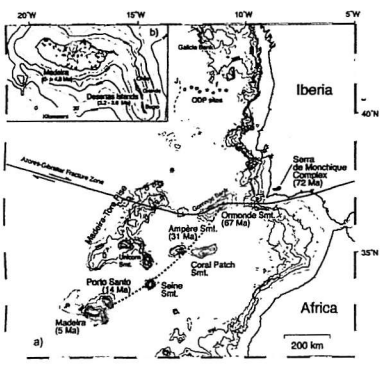


Figure 6.29(c) vicariant or dispersal speciation model of *O. lineatus* and *O. atratus*. The low level sea stands during the MSC facilitated colonisation, by ancestral *Osilinus* taxa, of the Atlantic Islands (Madeira, Canary Islands and Cape Verde Is) via exposed sea mounts. Consequent submerison of the seamounts created an isolation-by-distance barrier and the populations diverged. Alternatively the Atlantic Islands were colonised by a rare dispersal event after the MSC.

Figure 8.1. Radiation hypothesis of the evolutionary history of *Osilinus* in the Northeast Atlantic and Mediterranean

8.4 Development, dispersal and gene flow in *Osilinus*

The role of genetic drift, dispersal and gene flow, and natural selection can only be inferred from the mode of speciation occurring in a species. Hansen (1983) describes a link between developmental mode and rates of speciation and extinction in marine invertebrates. Gastropods with low dispersal potential tend to speciate more often than species with high dispersal potential, although this is not always true (Palumbi, 1992, 1994). A powerful tool in phylogeographic reconstruction is the mapping of mode of reproduction or larval dispersal potential characters on phylogenetic trees. This was not possible since the life history strategies of *Osilinus* are relatively unknown. *O. lineatus* has leicithotrophic larvae with a planktonic stage of 5 days to 2 weeks (Fretter & Graham, 1995). Despite the limited knowledge of life history strategies within *Osilinus,* the larval dispersal abilities, and therefore the mechanisms underlying the speciation of *Osilinus,* has been inferred from their phylogeography.

The lack of geographic structure within the *O. lineatus* intraspecific phylogeny indicates that gene flow occurs throughout its whole Atlantic range. A stepping stone-like model along the coastline between suitable rocky shores may facilitate this gene flow. The absence of *O. atratus* on the European coastline suggests that this species has limited larval dispersal compared to *O. edulis*, with which it is sympatric on the Atlantic Islands, and that also occurs on the continent. A diversity gradient of larval development types is present in fossil and recent biota of several gastropod families. There is a trend towards non-planktonic larval types (Lieberman *et al.,* 1993), which in general, are predominant in cold water (Thorson, 1950). The developmental strategies of Northeast Atlantic prosobranchs reflect this shift to non-planktotrophic larvae. Loss of planktotrophy can explain the large number of sibling species that differ predominantly in developmental strategy (Oliverio, 1996).

The current range of *O. edulis* reflects the combined range of *O. lineatus* and *O. atratus* (although *O. edulis* is not found as far north as *O. lineatus*). It may therefore be inferred that the distance between the Atlantic Islands and mainland is not an effective barrier to dispersal of *O. edulis* larvae. Conversely, the same distance appears to be an effective dispersal filter for *O. lineatus* and *O. atratus.* Barriers to migration are known to affect some taxa more than others (Vermeij, 1978, 1989ab, 1991ab). If species compete for similar ecological resources, an allopatric distribution may be maintained by competitive exclusion (Letcher *et al.,* 1994; Brown *et al.,* 1996; Gotelli *et al.,* 1997). The absence of *O atratus* on the mainland, may therefore be an example of isolation-by-distance, or exclusion by competition for limited ecological niche availability maintaining allopatry.

The intraspecific structure exhibited by *O. turbinatus* populations from the eastern and western Mediterranean basins suggests that this species has the most limited dispersal ability, and perhaps produces non-planktotrophic larvae. An increase in non-planktotrophic forms in the eastern Mediterranean basin is attributed to palaeoclimatic and glaciation events (Oliverio, 1996)

that produced unsuitable conditions for planktotrophic forms. The life history strategy, or larval dispersal ability cannot be inferred for *O. articulatus* since sample size was too small to resolve any intraspecific phylogenetic structure.

8.5 Conclusions and further work

This study partially resolved the evolutionary relationships within *Osilinus,* and presents a model of allopatric speciation in the Northeast Atlantic and Mediterranean. The taxonomic status of the inclusive species of this genus has been clarified. The biogeographic distributions and taxonomic descriptions of shell and radula morphology given here provide a guide to the identification of the species of this genus.

The limitations of this work, discussed above, provide immediate suggestions for further work. More extensive sampling of *O. turbinatus* and *O. articulatus* from the Mediterranean, and *O. atratus* from the Atlantic Islands and North Africa will help to resolve the ambiguities in the phylogenetic relationships within *Osilinus.* Further phylogenetic analysis of the data set including DNA sequences from these samples would establish the relative position of *O. articulatus* to the remaining taxa, and therefore add weight to the theories of allopatric speciation presented in my work.

There are major differences in the degree of intraspecific phylogeny structure and genetic variation between the species examined here, due partially to the limitation of the sequence data. This may be explained by different rates of molecular evolution within the 16S and COI mtDNA genes between taxa. The variation in intraspecific variation between the species may also, however, be a reflection of the evolution of different life history strategies within *Osilinus.* An investigation into the mode of reproduction and larval dispersal potential of all the *Osilinus* species is key to understanding the current biogeography and would offer further insight into mechanisms of speciation within these northeast Atlantic and Mediterranean trochids when superimposed on a fully resolved phylogeny.

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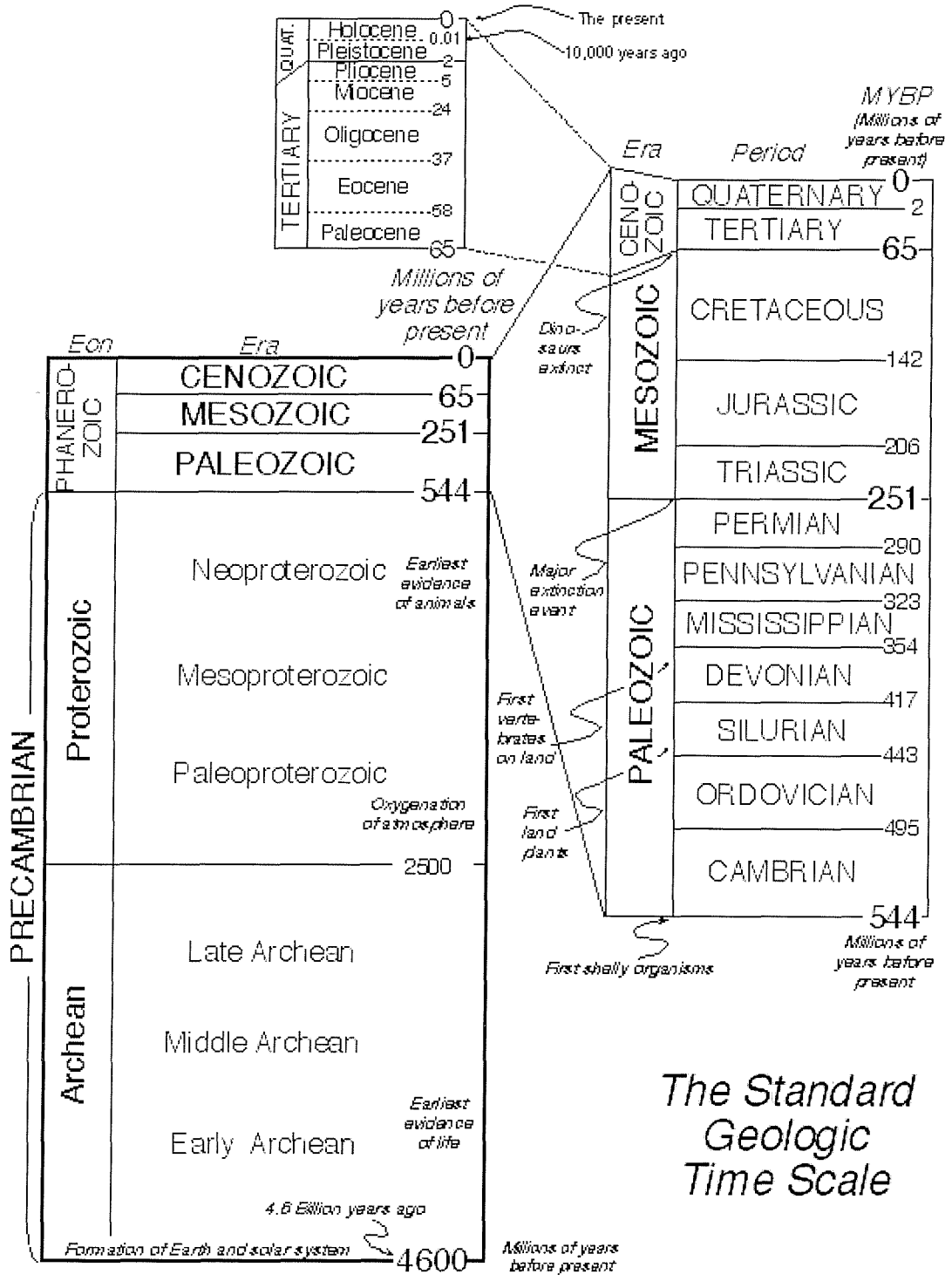
Appendices

Appendix A	Standard Geological Timescale
Appendix B	Molecular protocols and solution formulations
Appendix C	16S mt DNA sequence alignment file for the gibbuline outgroup taxa and *Osilinus* species used in chapter 5.
Appendix D	The partial COI mtDNA sequence alignment file for *Gibbula umbilicalis* and *Osilinus* species used in chapter 6.
Appendix E	The partial 16S mt DNA sequence alignment file for *Gibbula umbilicalis* and *Osilinus* species used in chapter 6.
Appendix F	The partial 16S and COI mt DNA sequence alignment file for *Gibbula umbilicalis* and *Osilinus* species used in chapter 6.

Appendix A

Standard Geological Timescale



Appendix B

Molecular protocols and solution formulations

I. DNA extraction protocol

- Prepare 3x 2.0ml clip top tubes and 1x 1.5ml clip top tubes for each sample. Sterilise and label
- Add 500μl CTAB extraction buffer and 1μl BME to each tube in fume cupboard. Incubate @ 60°C 30mins
- Wash 2-5mg tissue of each sample in 1M Tris-HCl and macerate with clean blade. Muscle tissue is the best source of DNA in molluscs. 100-200mg of tissue provides enough DNA.
- Add sample tissue to the incubated CTAB extraction buffer
- Add 5μl Proteinase K, incubate at 60°C 30mins
- Add further 5-10μl Pro K, incubate at 60°C 1hr
- Add 500μl Chloroform isoamyl 24:1 to samples. Mix in a rotation mixer for 5mins RT
- Centrifuge @ 14 000 rpm (Eppendorf 5404 centrifuge) 5mins RT
- Transfer aqueous phase to fresh 2.0ml tube with wide bore pipette
- Repeat steps 7-9 twice, the last time transferring aqueous phase to 1.5ml tube
- Add 700μl 100% ice-cold ethanol and 30μl Sodium Acetate. Invert tubes by hand 10+ times and place in -20°C for min 30mins to precipitate DNA
- Centrifuge @ 14 000 rpm @ 0°C 15mins, ensuring all hinges are pointing outwards
- Decant ethanol taking care not to dislodge DNA pellet
- Add 500μl 70% ice cold ethanol
- Centrifuge @ 14 000 2mins @ 0°C. Repeat wash
- Dry pellet on hot block at 70°C 10-20mins
- Resuspend DNA in 50μl TE or elution buffer (Qiagen)

Adapted and optimised from the CTAB extraction protocol of Sokolov (2000).

II. PCR protocol

Marker	50μl PCR reactions		PCR cycle conditions
16S	H_2O	18.5 µl	90s @ 94°C
	10x buffer	10 µl	
	Q solution	10 µl	60s @ 94°C
	dNTPs	200 μM each	60s @ 52°C x36
	16SAR	25 pmol	60s @ 72°C
	16SBR	25 pmol	
	Taq	0.5 U	10min @ 72°C
	DNA	20 ng	
			hold @ 4°C
CO1	H2O	18.5 µl	90s @ 95°C
	$MgCl_2$	25 mM	
	10x buffer	5 µl	60s @ 95°C
	dNTPs	200 μM each	80s @ 48°C x36
	Q	10 µl	70s @ 72°C (1sec increase/cycle)
	CO1AR	25 pmol	
	CO1BR	25 pmol	300s @ 74°C
	DNA	20 ng	
	Taq	0.5 U	hold @ 4°C
			Wilke & Davis (2000)
28S	H_2O	18.5 µl	3 mins @ 95°C
	10x buffer	10 µl	
	Q solution	10 µl	30s @ 94°C
	dNTPs	200 μM each	30s @ 52°C x35
	LSU 900F	25 pmol	120s @ 72°C
	LSU 1600R	25 pmol	
	Taq	0.5 U	10min @ 72°C
	DNA	20 ng	
			hold @ 4°C
			Littlewood *et al.*, (2000)

III. Visualisation of DNA fragments

DNA from the CTAB extractions and PCR cycles are visualised on a horizontal 1% agarose gel stained with 0.1% ethidium bromide. The buffer system used was 1x TAE.

IV. PCR Clean-up

Upon completion of the PCR, the product is cleaned using Qiagen QIAquick® PCR Purification Kit according to the manufacturers handbook (Qiagen, March 2001).

V.Cycle Sequencing

Thermal cycle sequencing protocol	
Reaction	Cycle conditions
8μl TRMM 5-20ng template DNA 3.2pmol Primer *q.s.* ddH2O 20μl total reaction volume	30 cycles of: 30s @ 95°C 15s @ 50°C 1min @ 70°C

The sequence cycle product is cleaned using the Qiagen DyeEx Spin Kit, according to the manufacturers handbook (Qiagen DyeExtm Handbook, October 2000).

VI. Sequencing using RapidLoad membrane comb on the ABIPrism 377™ sequencer

1. Prepare for pouring the polyacrylamide gel
   - Place kimwipes under frame of gel former
   - Clean glass plates
   - Place back plate on, add spacers (wet one side to create surface tension). Add front plate
   - Clamp middle sections of frame, insert comb and mark edges, add top clamp to align plates
   - Add bottom clamp with rubber seal
   - Remove top clamp & comb
2. Make gel
   - 200μl 10% APS (50mg APS, 500μl dH2O)
   - 40ml Gene Page plus 5.0% 6M urea
   - 28μl TEMED
3. Pour gel
   - Fill syringe and inject gel
   - Insert comb, add top clamp, and remove bottom clamp and rinse well.
   - Leave to set for 1.5hrs
4. Restart Mac computer and create new sample file entering your samples
5. Create new sequence run file
6. Start plate check. Allow electrophoresis to run for 2 min then pause
7. Add 600ml 1x TBE buffer to bottom tray. Connect hotplate to sequencer, resume plate check ensuring settings are correct (48/64 lanes, square tooth comb)
8. Prepare samples.
   - 2μl Microstop Red in lid of sample 1.5ml eppendorfs
   - Pulse spin in microcentrifuge
   - Vortex briefly
   - Pulse spin in microcentrifuge
   - Place Eppendorf tubes on a hotblock at 90°C for 3.5min
   - Hold on ice until ready to load
9. Warm up gel
   - Manual control

- Pump on
- Execute

10. Load samples on loading tray. Pipette 0.75μl of sample per loading well.
11. Start sequence Run for 15sec (note 30sec countdown before voltage/current registered)
12. Flush out the gel well with syringe using the buffer tank solution then add 500μl 5% Blue zone dye (20% Ficoll with Dextran Blue dye, Web Scientific Ltd) to fill well, ensuring no bubbles
13. Soak up the samples from the loading tray with the comb and load the comb on to the gel.
14. Add 600ml dH2O to top tank
15. Start the sequence run immediately for 2mins
16. Stop run and remove comb
17. Flush any excess Bluezone from the well area 3x taking care not to disturb the gel surface
18. Add 67ml 10x TBE to top buffer tank and mix, Restart run

VII. Sequence data extraction

1. Retrieving sequence data files
   - Click on <Macintosh HD>
   - Folder <ABI 377kl>
   - Folder <Runs>
   - Folder <"date and time of run">
   - File <Gel> get picture
   - Menu <Gel> <Track and Extract Lanes> (This is the <Sample Manager>)
   - Accept defaults. The processing takes about 20 minutes. Clean Sequencer now.
   - Check examples of sequences and check starting and end points of tracking. Adjust those to reduce junk background noise and file size. (Fill down a column by "Apple"+D). Mark Box "A" (for Adjust).
   - <Start>
2. Check lane tracking and hand track lanes that are not tracked well.
   - Menu <Gel> <Extract Lanes>, only extracts lanes that have been changed.
3. Save sequence data files to Zip disc.

VIII The Pearson/Fasta multiple sequence input format

One of the simplest examples of multiple sequence input is sequence data in the Pearson/Fasta format. This format looks like:

```
>locus1
GCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGC
GCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGC
GCGCGCGCGC
>locus2
ATATATATATATATATATATATATATATATATATATATATATATATATATATAT
ATATATATATATATATATATATATATATATATATATATATATATATATATATAT
ATATATATAT
```

The format can be described as follows. The first line should begin with a greater-than character ('>'), which indicates the start of a sequence. The first word after the '>' is the name of the sequence, any other words on this line are considered to be a comment/title for the sequence. All lines until the next line beginning with a '>' (or until there are no more lines) should contain the raw sequence data (no spaces or numbers) of the first sequence. Lines should be no longer than 80 characters, but 50 or 60 is most often the default line length for output. The next line beginning with a '>' (if there are any more lines) marks the start of the second sequence. Again, the first word after the '>' is the second sequence name and any additional words on this line are a comments or title for the second sequence. This second line beginning with a '>' should be followed by lines containing the sequence data up until another line beginning with a '>', (or until there are no more lines). Additional sequences can follow the second sequence in a similar fashion.

IX. Solution formulations

2x CTAB buffer
10g CTAB
140ml 5M NaCl
25ml 2M Tris-HCl, pH 8
20ml 0.5M EDTA
ddH_20 to 500ml

1X TAE buffer

0.04M Tris (Tris[hydroxymethyl]aminomethane)
0.04M Acetic Acid
0.001M EDTA (Ethylenediaminetetraacetic acid)

1M Tris-HCl ph 8

Dissolve 121.14g Tris in 800ml ddH_20
Add concentrate hydrochloric acid until the pH reaches 8 (approximately 42ml)
Add ddH_20 to a final volume of 1L.

TE buffer ph 8

10mM Tris-HCl
1mM EDTA

Appendix C

16S mt DNA sequence alignment file for the gibbuline outgroup taxa and *Osilinus* species used in chapter 5.

```
174nf_Oxytigrina        AAACGGCCGCGGTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAG
173nf_Oxytigrina        AAACGGCCGCGGTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAG
177nf_Oxytigrina        AAACGGCCGCGGTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAG
153nf_Oxysinensis       AAACGGCCGCGGTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAG
154nf_Oxysinensis       AAACGGCCGCGGTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAG
390nf_Oxysinensis       AAACGGCCGCGGTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAG
393nf_Oxyvariegat       AAACGGCCGCGGTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAG
149nf_Oxyvariegat       AAACGGCCGCGGTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAG
391nf_Oxyvariegat       AAACGGCCGCGGTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAG
Gumbilicalis127F        AAACGGCCGCGGTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAG
Gumbilicalis128F        AAACGGCCGCGGTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAG
Gumbilicalis129F        AAACGGCCGCGGTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAG
Oturbinatus096F         AAACGGCCGCGGTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAG
Oturbinatus121F         AAACGGCCGCGGTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAG
Oatratus067F            AAACGGCCGCGGTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAG
Olineatus001F           AAACGGCCGCGGTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAG
Oarticulatus093F        AAACGGCCGCGGTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAG
Oedulis182F             AAACGGCCGCGGTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAG
Oedulis183F             AAACGGCCGCGGTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAG
400nf_Okotschyi         AAACGGCCGCGGTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAG
401nf_Okotschyi         AAACGGCCGCGGTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAG
216nf_Aconstricta       AAACGGCCGCGGTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGTAG
404nf_Aconstricta       AAACGGCCGCGGTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGTAG
218nf_Aconstricta       AAACGGCCGCGGTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGTAG
403nf_Mlabio            AAACGGCCGCGGTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAG
402nf_Mlabio            AAACGGCCGCGGTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAG
                        ******************************************************** **

174nf_Oxytigrina        GCTGGTATGAATGGTTTGACGAGAGCCACGCTGTCTCTCCCA-AAATATCTAAAAATTAA
173nf_Oxytigrina        GCTGGTATGAATGGTTTGACGAGAGCCACGCTGTCTCTCCCA-AAATATCTAAAAATTAA
177nf_Oxytigrina        GCTGGTATGAATGGTTTGACGAGAGCCACGCTGTCTCTCCCA-AAATATCTAAAAATTAA
153nf_Oxysinensis       GCTGGTATGAATGGTTTGACGAGAGCCACGCTGTCTCCCCTA-AAATATTTAAAAATTAA
154nf_Oxysinensis       GCTGGTATGAATGGTTTGACGAGAGCCACGCTGTCTCCCCTA-AAATATTTAAAAATTAA
390nf_Oxysinensis       GCTGGTATGAATGGTTTGACGAGAGCCACGCTGTCTCCCCTA-AAATATTTAAAAATTAA
393nf_Oxystelespp       GCTGGTATGAATGGTTTGACGAAAGCCAGACTGTCTCCCCTG-AAATATTTAAAAATTAA
149nfOxyvariegata       GCTGGTATGAATGGTTTGACGAAAGCCAGACTGTCTCCCCTG-AAATATTTAAAAATTAA
391nf_Oxysinensis       GCTGGTATGAATGGTTTGACGAAAGCCAGACTGTCTCCCCTG-AAATATTTAAAAATTAA
Gumbilicalis127F        GCTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCTTTTA-AAATATTTAAAAATTAA
Gibbulaspp246F          GCTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCTTTTA-AAATATTTAAAAATTAA
Gumbilicalis128F        GCTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCTTTTA-AAATATTTAAAAATTAA
Gumbilicalis129F        GCTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCTTTTA-AAATATTTAAAAATTAA
Oturbinatus096F         GCTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCTTTCG-AAATATTTAAAAATTAA
Oturbinatus121F         GCTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCTTTCG-AAATATTTAAAAATTAA
Oatratus067F            GCTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCCTTCG-AAATATTTAAAAATTAA
Olineatus001F           GCTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCTTTCG-AAATATTTAAAAATTAA
Oarticulatus093F        GCTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCTTTCG-AAATATTTAAAAATTAA
Oedulis182F             GCTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCTTTTG-GAATATTTAAAAATTAA
Oedulis183F             GCTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCTTTTG-GAATATTTAAAAATTAA
400nf_Okotschyi         GCTGGTATGAATGGTTTGACGAGAGCTAAGCTGTCTCTTTTTTGAATATTTAGAAGTTAA
401nf_Okotschyi         GCTGGTATGAATGGTTTGACGAGAGCTAAGCTGTCTCTTTTTTGAATATTTAGAAATTAA
216nf_Aconstricta       GCTTGTATGAATGGTTTGACGAGAGCTAAGCTGTCTCTTTCT-TAATATTTAAAAATTAA
404f_Aconstricta        GCTTGTATGAATGGTTTGACGAGAGCTAAGCTGTCTCTTTCT-TAATATTTAAAAATTAA
218nf_Aconstricta       GCTTGTATGAATGGTTTGACGAGAGCTAAGCTGTCTCTTTCT-TAATATTTAAAAATTAA
403nf_Mlabio            GCTTGTATGAATGGTTTGACGAGGGCTAAGCTGTCTCTTTTA-AAATATGTAGAAATTAA
402f_Mlabio             GCTTGTATGAATGGTTTGACGAGGGCTAAGCTGTCTCTTTTA-AAATATGTAGAAATTAA
                        *** ******************  ** *  *******       ***** ** ** ****

174nf_Oxytigrina        CTTTTAGGTGAAGAGGCCTGAATATATTTAAGGGACAAGAAGACCCTGTTGAGCTTTAGT
173nf_Oxytigrina        CTTTTAGGTGAAGAGGCCTGAATATATTTAAGGGACAAGAAGACCCTGTTGAGCTTTAGT
177nf_Oxytigrina        CTTTTAGGTGAAGAGGCCTGAATATATTTAAGGGACAAGAAGACCCTGTTGAGCTTTAGT
153nf_Oxysinensis       CTTTTAGGTGAAGAGGCCTAAATATATTTAAGGGACAAGAAGACCCTGTTGAGCTTTAGT
154nf_Oxysinensis       CTTTTAGGTGAAGAGGCCTAAATATATTTAAGGGACAAGAAGACCCTGTTGAGCTTTAGT
390nf_Oxysinensis       CTTTTAGGTGAAGAGGCCTAAATATATTTAAGGGACAAGAAGACCCTGTTGAGCTTTAGT
393nf_Oxystelespp       CTTTTAGGTGAAGAGGCCTAAATTAATTTAAGGGACAAGAAGACCCTGTTGAGCTTTAGT
149nfOxyvariegata       CTTTTAGGTGAAGAGGCCTAAATTAATTTAAGGGACAAGAAGACCCTGTTGAGCTTTAGT
391nf_Oxysinensis       CTTTTAGGTGAAGAGGCCTAAATTAATTTAAGGGACAAGAAGACCCTGTTGAGCTTTAGT
Gumbilicalis127F        CTTTTAGGTGAAGAGGCCTAAATTTATTTAAGGGACAAGAAGACCCTGTTGAGCTTTAGT
Gibbulaspp246F          CTTTTAGGTGAAGAGGCCTAAATTTATTTAAGGGACAAGAAGACCCTGTTGAGCTTTAGT
Gumbilicalis128F        CTTTTAGGTGAAGAGGCCTAAATTTATTTAAGGGACAAGAAGACCCTGTTGAGCTTTAGT
Gumbilicalis129F        CTTTTAGGTGAAGAGGCCTAAATTTATTTAAGGGACAAGAAGACCCTGTTGAGCTTTAGT
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Oturbinatus096F        CCTTTAGGTGAAGAGGCCTGAATCTATTTAAGGGACAAGAAGACCCTGTTGAGCTTTAGT
Oturbinatus121F        CCTTTAGGTGAAGAGGCCTGAATTTATTTAAGGGACAAGAAGACCCTGTTGAGCTTTAGT
Oatratus067F           CTTTTAGGTAAAGAGGCCTAAATTTGTTTAAGGGACAAGAAGACCCTGTTGAGCTTTAGC
Olineatus001F          CTTTTAGGTAAAGAGGCCTAAATTCGTTTAAGGGACAAGAAGACCCTGTTGAGCTTTAGT
Oarticulatus093F       CTTTTAGGTAAAGAGGCCTAAATTTATTTAAGGGACAAGAAGACCCTGTTGAGCTTTAGT
Oedulis182F            CTTTTAGGTGAAGAGGCCTGAATTTATTTAAGGGACAAGAAGACCCTGTTGAGCTTTAGT
Oedulis183F            CTTTTAGGTGAAGAGGCCTGAATTTATTTAAGGGACAAGAAGACCCTGTTGAGCTTTAGT
400nf_Okotschyi        CTTTTAGGTGAAGAGGCCTAAATTTGTTTAAGGGACAAGAAGACCCTGTTGAGCTTTAGT
401nf_Okotschyi        CTTTTAGGTGAAGAGGCCTAAATTTGTTTAAGGGACAAGAAGACCCTGTTGAGCTTTAGT
216nf_Aconstricta      CTTTTAGGTGAAGAGGCCTAAATAAAACTAAGGGACGAGAAGACCCTATTGAGCTTTAGT
404f_Aconstricta       CTTTTAGGTGAAGAGGCCTAAATAAAACTAAGGGACGAGAAGACCCTATTGAGCTTTAGT
218nf_Aconstricta      CTTTTAGGTGAAGAGGCCTAAATAAAACTAAGGGACGAGAAGACCCTATTGAGCTTTAGT
403nf_Mlabio           CCTTTAGGTGAAGAGGCCTAAATTTGGCTGAGGGACAAGAAGACCCTATTGAGCTTTAGT
402f_Mlabio            CCTTTAGGTGAAGAGGCCTAAATTTGGCTGAGGGACAAGAAGACCCTATTGAGCTTTAGT
                       * ******* ********* ***     * ****** ********** ***********

174nf_Oxytigrina       GTAGTTACAAATAATTAAGCGAGTTTT--------ATTTGAC-AAAATAAAATGATGTTG
173nf_Oxytigrina       GTAGTTACAAATAATTAAGCGAGTTTT--------ATTTGAC-AAAATAAAATGATGTTG
177nf_Oxytigrina       GTAGTTACAAATAATTAAGCGAGTTTT--------ATTTGAC-AAAATAAAATGATGTTG
153nf_Oxysinensis      TTAGTTACAAATGATTAAGTAAATTTT--------GTTTAATTAAAATAAAATTATGTTG
154nf_Oxysinensis      TTAGTTACAAATGATTAAGTAAATTTT--------GTTTAATTAAAATAAAATTATGTTG
390nf_Oxysinensis      TTAGTTACAAATGATTAAGTAAATTTT--------GTTTAATTAAAATAAAATTATGTTG
393nf_Oxystelespp      AAAATTATAAATTGTAAAATGAACTTT--------ATTTAGTAAAATAGAATTAATGCGG
149nfOxyvariegata      AAAATTATAAATTGTAAAATGAATTTT--------ATTTAGTAAAATAGAATTAATGCGG
391nf_Oxysinensis      AAAATTATAAATTGTAAAATGAATTTT--------ATTTAGTAAAATAGAATTAATGCGG
Gumbilicalis127F       GATGTTGTAAAC-GATGAAGAATAAG-----------T-TATTATTGATTTGTTGTGTAT
Gibbulaspp246F         GATGTTGTAAAC-GATGAAGAATAAG-----------T-TATTATTGATTTGTTGTGTAT
Gumbilicalis128F       GATGTTGTAAAC-GATGAAGAATAAG-----------GTCATTATTGATTTGTTGTGTAT
Gumbilicalis129F       GATGTTGTAAAC-GATGAAGAATAAG-----------G-CATTATTGATTTGTTGTGTAT
Oturbinatus096F        GATTTTATAAATTTACAGGTAATCTAG----------TTAATGATTATAGTTCGGTATAT
Oturbinatus121F        GATTTTATAAATTTACAGGTAATCTAG----------TTAATGATTATAGTTCGGTATAT
Oatratus067F           GATCTTGTAAATTTTTGTGTAGTTT-----------------GCTTATATTT--GTATAT
Olineatus001F          GATCCTATAAATTGATGGGCAATTTA-----------TTGGTTGTTGTGTATTTGTGTAT
Oarticulatus093F       GATCCTGTAAGTTTACACGCAGCTTA-----------ATAACTGTTGTATTTTTGTGTAT
Oedulis182F            GACTTTATAAATTTATATATAATTTG-----------TTATTGATTATATTTTTGTATAT
Oedulis183F            GACTTTATAAATTTATATATAATTTG-----------TTATTGATTATATTTTTGTATAT
400nf_Okotschyi        GATTCTGCAAATATATATCTATTTGGCTAAAAATAAGTTAAATAGTTTGCTAAAATTTAA
401nf_Okotschyi        GATTCTGCAAATATATATCTATTTGGCTAAAAATAAGTTAAATAGTTTGCTAAAATTTAA
216nf_Aconstricta      GAAGCAATAAGTTTT-----GAGTGTA--------ATTAAATTTACGAACTGTTATATA-
404f_Aconstricta       GAAGCAATAAGTTTT-----GAGTGTA--------ATTAAATTTACGAACTGTTATATA-
218nf_Aconstricta      GAAGCAATAAGTTTT-----GAGTGTA--------ATTAAATTTACGAATTGTTATATA-
403nf_Mlabio           TTTGTAATAAATAAT-----GAGTTT----------TTGGTTTGTCCTATAA--ATGTA-
402f_Mlabio            TTTGTAATAAATAAT-----AAGTTT----------TTGGTTTGTCCTATAA--ATGTA-
                                 **                                            *

174nf_Oxytigrina       TA-CTTTGTATAACGTAAGTTTGTAATGAATCTTTAGTTGGGGC-GACTGAGGAACAATG
173nf_Oxytigrina       TA-CTTTGTATAACGTAAGTTTGTAATGAATCTTTAGTTGGGGC-GACTGAGGAACAATG
177nf_Oxytigrina       TA-CTTTGTATAACGTAAGTTTGTAATGAATCTTTAGTTGGGGC-GACTGAGGAACAATG
153nf_Oxysinensis      TG-CTTTGTATAACGTAAGTTTGTAATGAATCTTTAGTTGGGGC-GACTGAGGAACAAAA
154nf_Oxysinensis      TG-CTTTGTATAACGTAAGTTTGTAATGAATCTTTAGTTGGGGC-GACTGAGGAACAAAA
390nf_Oxysinensis      TG-CTTTGTATAACGTAAGTTTGTAATGAATCTTTAGTTGGGGC-GACTGAGGAACAAAA
393nf_Oxystelespp      CA-CTTTGTGTAGCAAAAGTTTATGATGAATCTTTAGTTGGGGC-GACTGAGGAACAATG
149nfOxyvariegata      CA-CTTTGTGTAGCAAAAGTTTATGATGAATCTTTAGTTGGGGC-GACTGAGGAACAATG
391nf_Oxysinensis      CA-CTTTGTGTAGCAAAAGTTTATGATGAATCTTTAGTTGGGGC-GACTGAGGAACAATG
Gumbilicalis127F       TT---AT-TATAATAAAAGTTTGCAGATAATCTTTAGTTGGGGC-GACTGAGGAACATAA
Gibbulaspp246F         TT---AT-TATAATAAAAGTTTGCAGATAATCTTTAGTTGGGGC-GACTGAGGAACATAA
Gumbilicalis128F       TT---AT-TATAATAAAAGTTTGCAGATAATCTTTAGTTGGGGC-GACTGAGGAACATAA
Gumbilicalis129F       TT---AT-CATAATAAAAGTTTGCAGATAATCTTTAGTTGGGGC-GACTGAGGAACATAC
Oturbinatus096F        TA---ATATATAACATAGGTTTATAGGTAATCTTTAGTTGGGGC-GACTGAGGAACACAT
Oturbinatus121F        TA---ATATATAACATAGGTTTATAGGTAATCTTTAGTTGGGGC-GACTGAGGAACACAT
Oatratus067F           TT---ATATATAACATAAGTTTATAGGTAATCTTTAGTTGGGGC-GACTGAGGAACATGA
Olineatus001F          T----ATATATAACGTAAGTTTAAAGGTAATCTTTAGTTGGGGC-GACTGAGGAACATGG
Oarticulatus093F       TT---ATATATAACATAAGTTTGTAGGTAATCTTTAGTTGGGGC-GACTAAGGAACATTA
Oedulis182F            TA---ATGTATAACTTAGGTTTATGAATAATCTTTAGTTGGGGC-GACTGAGGAACATAC
Oedulis183F            TA---ATGTATAACTTAGGTTTATGAATAATCTTTAGTTGGGGC-GACTGAGGAACATAC
400nf_Okotschyi        TTACTATGTATATTGATTATTTGTAGGGAATCTTTAGTTGGGGC-GACTAAGGAACATAT
401nf_Okotschyi        TTACTATGTATATTGATTATTTGTAGGGAATCTTTAGTTGGGGC-GACTAAGGAACATAT
216nf_Aconstricta      ----TTTATAGAGTTTTATTTTGTTGTGAATCTTTAGTTGGGGC-GACTGAGGAACAAAA
404f_Aconstricta       ----TTTATAGAGTTTTATTTTGTTGTGAATCTTTAGTTGGGGCCGACTGAGGAACAAAA
218nf_Aconstricta      ----TTTATAGAGTTTTATTTTGTTGTGAATCTTTAGTTGGGGC-GACTGAGGAACAAAA
403nf_Mlabio           ----TTTATATAGTACAATTTTGTTGCGGATTTTTAGTTGGGGC-GACTGAGGAACATAA
402f_Mlabio            ----TTTATATAGTACAATTTTGTTGCGGATTTTTAGTTGGGGC-GACTGAGGAACATAA
                            *    *       **        ** ************ **** *******

174nf_Oxytigrina       AAAGCTTCTTTTT--ATAAAACGTTTT-TGTTGTATTAATGAAG-TATAAGTTGATTTTT
173nf_Oxytigrina       AAAGCTTCTTTTT--ATAAAACGTTTT-TGTTGTATTAATGAAG-TATAAGTTGATTTTT
177nf_Oxytigrina       AAAGCTTCTTTTT--ATAAAACGTCTT-TGTTGTATTAATGAAG-TATAAGTTGATTTTT
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153nf_Oxysinensis       AAAGCTTCTTTTT-GATAAAATGT-TT-TGATATATTGATAAAGGTATAAATTAATTTTT
154nf_Oxysinensis       AAAGCTTCTTTTT-GATAAAATGT-TT-TGATATATTGATAAAGGTATAAATTAATTTTT
390nf_Oxysinensis       AAAGCTTCTTTTT-GATAAAATGT-TT-TGATATATTGATAAAGGTATAAATTAATTTTT
393nf_Oxystelespp       GAAGCTTCTTTTA--ATATGGTGACTTATGGATTATTGATAAGTTTATGAATTAATTTTT
149nfOxyvariegata       GAAGCTTCTTTTA--ATATGGTGACTTATGGATTATTGATAAGTTTATGAATTAATTTTT
391nf_Oxysinensis       GAAGCTTCTTTTA--ATATGGTGACTTACGGATTATTGATAAGTTTATGAATTAATTTTT
Gumbilicalis127F        AAAGCTTCCTTGTATATAAA--TTATTTAAGTAATTGAATAAATTAATGC-TTATGTGT-
Gibbulaspp246F          AAAGCTTCCTTGTATATAAA--TTATTTAAGTAATTGAATAAATTAATGC-TTATGTGT-
Gumbilicalis128F        AAAGCTTCCTTGTATATAAA--TTATTTAAGTAATTGAATAAATTAATGC-TTATGTGT-
Gumbilicalis129F        AAAGCTTCCCTGTATATAAA--TTATTTAAGTAATTGAATAAATTAATGC-TTATGTGT-
Oturbinatus096F         AAAGCTTCCTTGAATTTAAGAGCGTGTTAAGCAATTTAGTTTACTGATGCATT-TATGT-
Oturbinatus121F         AAAGCTTCCTTGAATTTAAGAGCGTGTTAAGCAATTTAGTTTACTGATGCATT-TATGT-
Oatratus067F            AAAGCTTCCTTGAACTTAAGAGTGTGTTAAACAATT-AGTTTATTGATGCGTT-TATGT-
Olineatus001F           AAAGCTTGCTTGAATTTAAAAGCATGTTGGGCAATT-AATTTGTTGATGCATTCTGCGT-
Oarticulatus093F        AAAGCTTCCTTGAATTTAGTAATAAGTTAAATTATTTAATTTTTTGATGCATT-TATGT-
Oedulis182F             AAAGCTTCCTTGAGTTCGAAGGCGTGTTGGCCTGGCTGATTTATTGATGCGTT-TGTGT-
Oedulis183F             AAAGCTTCCTTGAGTTCGAAGGCGTGTTGGCCTGGCTGATTTATTGATGCGTT-TGTGT-
400nf_Okotschyi         AAAGCTTCCTTTAATGAGTTGGTTGATTTAGTAACT-AGTATTTTGATGC-TGATATGT-
401nf_Okotschyi         AAAGCTTCCTTTAATGAGTTGGTTGATTTAGTAACT-AGTATTTTGATGC-TGATATGT-
216nf_Aconstricta       AAAGCTTCCTTTG-TATTATAAGATTT-TGATTTATTTGATATGATGTTAGCTAGCT---
404f_Aconstricta        AAAGCTTCCTTTG-TATTATAAGATTT-TGATTTATTTGATATGATGTTAGCTAGCT---
218nf_Aconstricta       AAAGCTTCCTTTG-TATTATAAGATTT-TGATTTATTTGATACGATGTTAGCTAGCT---
403nf_Mlabio            AAAGCTTCCTTTAACATAGGGTGGACT-TTGTGCACTGGGTGAAGAGTGAGTTCTTTCC-
402f_Mlabio             AAAGCTTCCTTTAACATAGGGTGGACT-TTGTGCACTGGGTGAAGAGTGAGTTCTTTCC-
                         ******   *               *                      *

174nf_Oxytigrina        ACTAATTATTTTGATCCAAATTTGTTTTGATTAAAGGAAAAAGTTACCACAGGGATAACA
173nf_Oxytigrina        ACTAATTATTTTGATCCAAATTTGTTTTGATTAAAGGAAAAAGTTACCACAGGGATAACA
177nf_Oxytigrina        ACTAATTATTTTGATCCAAATTTGTTTTGATTAAAGGAAAAAGTTACCACAGGGATAACA
153nf_Oxysinensis       ACTAATTATTTTGATCCAAAATTATTTTGATTAAAGGAAAAAGTTACCACAGGGATAACA
154nf_Oxysinensis       ACTAATTATTTTGATCCAAAATTATTTTGATTAAAGGAAAAAGTTACCACAGGGATAACA
390nf_Oxysinensis       ACTAATTATTTTGATCCAAAATTATTTTGATTAAAGGAAAAAGTTACCACAGGGATAACA
393nf_Oxystelespp       ACCGATTAATTTGATCCAAACTTGTTTTGATTAAAGGAAAAAGTTACCACAGGGATAACA
149nfOxyvariegata       ACCGATTAATTTGATCCAAACTTGTTTTGATTAAAGGAAAAAGTTACCACAGGGATAACA
391nf_Oxysinensis       ACCGATTAATTTGATCCAAACTTGTTTTGATTAAAGGAAAAAGTTACCACAGGGATAACA
Gumbilicalis127F        -----TA-TTTTGATCCAAATTATTTT-GATTAAGAGAAAAAGTTACCACAGGGATAACA
Gibbulaspp246F          -----TA-TTTTGATCCAAATTATTTT-GATTAAGAGAAAAAGTTACCACAGGGATAACA
Gumbilicalis128F        -----TA-TTTTGATCCAAATTATTTTTGATTAAGAGAAAAAGTTACCACAGGGATAACA
Gumbilicalis129F        -----TA-TTTTGATCCAAATTATTTT-GATTAAGAGAAAAAGTTACCAC-GGGATAACA
Oturbinatus096F         -----TAATTTTGATCCAATTTATTTT-GATTAAGAGAAAAAGTTACCACAGGGATAACA
Oturbinatus121F         -----TAATTTTGATCCAATTTATTTT-GATTAAGAGAAAAAGTTACCACAGGGATAACA
Oatratus067F            -----TAATTTTGATCCAATTTATTTT-GATTAAGAGAAAAAGTTACCACAGGGATAACA
Olineatus001F           -----TAATTTTGATCCAATCTATTTT-GATTAAGAGAAAAAGTTACCACAGGGATAACA
Oarticulatus093F        -----TAATTTTGATCCAATTTATTTT-GATTAAGAGAAAAAGTTACCACAGGGATAACA
Oedulis182F             -----TAATTTTGATCCAATTTATTTT-GATTAAGAGAAAAAGTTACCACAGGGATAACA
Oedulis183F             -----TAATTTTGATCCAATTTATTTT-GATTAAGAGAAAAAGTTACCACAGGGATAACA
400nf_Okotschyi         -----TA-TTTTGATCCAGGTTATTCT-GATCAAGAGAAAAAGTTACCACAGGGATAACA
401nf_Okotschyi         -----TA-TTTTGATCCAGGTTATTCT-GATCAAGAGAAAAAGTTACCACAGGGATAACA
216nf_Aconstricta       --------ATTTGATCCAGTA-TTTATTGATTAAGGGAAAAAGTTACCATAGGGATAACA
404f_Aconstricta        --------ATTTGATCCAGTA-TTTATTGATTAAGGGAAAAAGTTACCATAGGGATAACA
218nf_Aconstricta       --------ATTTGATCCAGTA-TTTATTGATTAAGGGAAAAAGTTACCATAGGGATAACA
403nf_Mlabio            ---GGTTAATGTGATCCAGTAGTTTATTGATTAAAGGAAAAAGTTACCATAGGGATAACA
402f_Mlabio             ---GGTTAATGTGATCCAGTAGTTTATTGATTAAAGGAAAAAGTTACCATAGGGATAACA
                                 * *******      * * *** **  *************  *********

174nf_Oxytigrina        -GCGTAATCTTTCTGGAGAGACCTTATCGAAAGAAGGGTTTGCGACCTC-GATGTTGGAC
173nf_Oxytigrina        -GCGTAATCTTTCTGGAGAGACCTTATCGAAAGAAGGGTTTGCGACCTC-GATGTTGGAC
177nf_Oxytigrina        -GCGTAATCTTTCTGGAGAGACCTTATCGAAAGAAGGGTTTGCGACCTC-GATGTTGGAC
153nf_Oxysinensis       -GCGTAATCTTTCTGGAGAGACCTTATCGAAAGAAGGGTTTGCGACCTC-GATGTTGGAC
154nf_Oxysinensis       -GCGTAATCTTTCTGGAGAGACCTTATCGAAAGAAGGGTTTGCGACCTC-GATGTTGGAC
390nf_Oxysinensis       -GCGTAATCTTTCTGGAGAGACCTTATCGAAAGAAGGGTTTGCGACCTC-GATGTTGGAC
393nf_Oxystelespp       -GCGTAATCTTTCTGGAGAGATCTTATCGAAAGAAGGGTTTGCGACCTC-GATGTTGGAC
149nfOxyvariegata       -GCGTAATCTTTCTGGAGAGATCTTATCGAAAGAAGGGTTTGCGACCTC-GATGTTGGAC
391nf_Oxysinensis       -GCGTAATCTTTCTGGAGAGATCTTATCGAAAGAAGGGTTTGCGACCTC-GATGTTGGAC
Gumbilicalis127F        -GCGTAATCTTTCTGGAGAGATCTTATCGAAAGAAGGGTTTGCGACCTC-GATGTTGGAC
Gibbulaspp246F          -GCGTAATCTTTCTGGAGAGATCTTATCGAAAGAAGGGTTTGCGACCTC-GATGTTGGAC
Gumbilicalis128F        -GCGTAATCTTTCTGGAGAGATCTTATCGAAAGAAGGGTTTGCGACCTC-GATGTTGGAC
Gumbilicalis129F        -GCGTAATCTTTCTGGAGAGATCTTATCGAAAGAAGGGTTTGCGACCTC-GATGTTGGAC
Oturbinatus096F         -GCGTAATCTTTCTGGAGAGATCTAATCGAAAGAAGGGTTTGCGACCTC-GATGTTGGAC
Oturbinatus121F         -GCGTAATCTTTCTGGAGAGATCTAATCGAAAGAAGGGTTTGCGACCTC-GATGTTGGAC
Oatratus067F            -GCGTAATCTTTCTGGAGAGATCTAATCGAAAGAAGGGTTTGCGACCTC-GATGTTGGAC
Olineatus001F           -GCGTAATCTTTCTGGAGAGATCTAATCGAAAGAAGGGTTTGCGACCTC-CATGTTGGAC
Oarticulatus093F        -GCGTAATCTTTCTGGAGAGATCTAATCGAAAGAAGGGTTTGCGACCTC-GATGTTGGAC
Oedulis182F             -GCGTAATCTTTCTGGAGAGATCTAATCGAAAGGAGGGTTTGCGACCTC-GATGTTGGAC
Oedulis183F             -GCGTAATCTTTCTGGAGAGATCTAATCGAAAGGAGGGTTTGCGACCTC-GATGTTGGAC
400nf_Okotschyi         -GCGTAATCTTTCTGGAGAGATCTTATCGAAAGGAGGGTTTGCGACCTC-GATGTTGGAC
401nf_Okotschyi         -GCGTAATCTTTCTGGAGAGATCTTATCGAAAGGAGGGTTTGCGACCTC-GATGTTGGAC
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216nf_Aconstricta      -GCGTAATCTTTCTGGAGAGATCATATCGAAAGGAGGGTTTGCGACCTC-GATGTTGGAC
404f_Aconstricta       AGCGTAATCTTTCTGGAGAGATCATATCGAAAGGAGGGTTTGCGACCTCCGATGTTGGAC
218nf_Aconstricta      -GCGTAATCTTTCTGGAGAGATCATATCGAAAGGAGGGTTTGCGACCTC-NATGTTGGAC
403nf_Mlabio           -GCGTAATCTTTCTGGAGAGATCTTATCGAAGGGAGGGTTTGCGACCTC-GATGTTGGAC
402f_Mlabio            -GCGTAATCTTTCTGGAGAGATCTTATCGAAGGGAGGGTTTGCGACCTC-GATGTTGGAC
                        ******************** *  ****** * ***************  *********

174nf_Oxytigrina       TAA
173nf_Oxytigrina       TAA
177nf_Oxytigrina       TAA
153nf_Oxysinensis      TAA
154nf_Oxysinensis      TAA
390nf_Oxysinensis      TAA
393nf_Oxystelespp      TAA
149nfOxyvariegata      TAA
391nf_Oxysinensis      TAA
Gumbilicalis127F       TAA
Gibbulaspp246F         TAA
Gumbilicalis128F       TAA
Gumbilicalis129F       TAA
Oturbinatus096F        TAA
Oturbinatus121F        TAA
Oatratus067F           TAA
Olineatus001F          TAA
Oarticulatus093F       TAA
Oedulis182F            TAA
Oedulis183F            TAA
400nf_Okotschyi        TAA
401nf_Okotschyi        TAA
216nf_Aconstricta      TAA
404f_Aconstricta       TAA
218nf_Aconstricta      TAA
403nf_Mlabio           TAA
402f_Mlabio            TAA
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Appendix D

The partial COI mtDNA sequence alignment file for *Gibbula umbilicalis* and *Osilinus* species used in chapter 6.

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0910.articulatusCalahondaMalag      TGATTCGTGCTGAGCTGGGGCAACCCGGTGCTCTTCTTGGAGACGACCAA
0930.articulatusCalahondaMalag      TGATTCGTGCTGAGCTGGGGCAACCCGGTGCTCTTCTTGGAGACGACCAA
3400.edulisFuerte6                  TAATTCGTGCTGAGCTAGGACAACCTGGTGCTCTTTTAGGAGACGACCAA
0640.edulisLaRamblaTener            TAATTCGTGCTGAGCTAGGACAACCTGGTGCTCTTTTAGGAGACGACCAA
2260.edulisBasqueCoastline          TAATTCGTGCTGAGCTAGGACAACCTGGTGCTCTTTTAGGAGACGACCAA
2640.edulisLanzarote3               TAATTCGTGCTGAGCTAGGACAACCTGGTGCTCTTTTAGGAGACGACCAA
3510.edulisGC2                      TAATTCGTGCTGAGCTAGGACAACCTGGTGCTCTTTTAGGAGACGACCAA
770.edulisLosGigantesTener          TAATTCGTGCTGAGCTAGGACAACCTGGTGCTCTTTTAGGAGACGACCAA
2720.edulisFuerte4                  TAATTCGTGCTGAGCTAGGACAACCTGGTGCTCTTTTAGGAGACGACCAA
4640.edulisPortugal7                TAATTCGTGCTGAGCTAGGACAACCTGGTGCTCTTTTAGGAGACGACCAA
3640.edulisGC5                      TAATTCGTGCTGAGCTAGGACAACCTGGTGCTCTTTTAGGAGACGACCAA
2630.edulisLanzarote3               TAATTCGTGCTGAGCTAGGACAACCTGGTGCTCTTTTAGGAGACGACCAA
0700.atratusLasGalletasTener        TAATTCGTGCTGAGCTAGGACAACCTGGTGCTCTTTTAGGAGACGACCAA
0320.edulisImouraneMoroc            TAATTCGTGCTGAGCTAGGACAACCTGGTGCTCTTTTAGGAGACGACCAA
4930.edulisPortugal11               TAATTCGTGCTGAGCTAGGACAACCTGGTGCTCTTTTAGGGGACGACCAA
4740.edulisPortugal8                TAATTCGTGCTGAGCTAGGACAACCTGGTGCTCTTTTAGGAGACGACCAA
0310.edulisImouraneMoroc            TAATTCGTGCTGAGCTAGGACAACCTGGTGCTCTTTTAGGAGACGACCAA
3550.edulisGC3                      TAGTTTAAGCTGAGCTAGGACAACCTGGTGCTCTTTTAGGAGACGACCAA
3540.edulisGC3                      TAATTCGTGCTGAGCTAGGACAACCTGGTGCTCTTTTAGGAGACGACCAA
2730.edulisFuerte4                  TAATTCGTGCTGAGCTAGGACAACCTGGTGCTCTTTTAGGAGACGACCAA
0530.edulisCapGhirMoroc             TAATTCGTGCTGAGCTAGGACAACCTGGTGCTCTTTTAGGAGACGACCAA
0630.edulisLaRamblaTener            TAATTCGTGCTGAGCTAGGACAACCTGGTGCTCTTTTAGGAGACGACCAA
3490.edulisGC2                      TAATTCGTGCTGAGCTAGGACAACCTGGTGCTCTTTTAGGAGACGACCAA
3390.edulisFuerte6                  TAATTCGTGCTGAGCTAGGACAACCTGGTGCTCTTTTAGGAGACGACCAA
3250.edulisLanz4                    TAATTCGTGCTGAGCTAGGACAACCTGGTGCTCTTTTAGGAGACGACCAA
0570.edulisElMedanoTener            TAATTCGTGCTGAGCTAGGACAACCTGGTGCTCTTTTAGGAGACGACCAA
0760.edulisLosGigantesTener         TAATTCGTGCTGAGCTAGGACAACCTGGTGCTCTTTTAGGAGACGACCAA
0420.edulisTinitMoroc               TAATTCGTGCTGAGCTAGGGCAACCTGGTGCTCTTTTAGGAGACGACCAA
0540.edulisCapGhirMoroc             TAATTCGTGCTGAGCTAGGGCAACCTGGTGCTCTTTTAGGAGACGACCAA
1820.edulisSeixalMadeira            TAATTCGTGCTGAGCTAGGGCAACCTGGTGCTCTTTTAGGAGACGACCAA
1830.edulisSaoVincentMadeira        TAATTCGTCCTGAGCTAGGGCAACCTGGTGCTCTTTTAGGAGACGACCAA
1920.edulisPrainhaBeachMadeira      TAATTCGTGCTGAGCTAGGGCAACCTGGTGCTCTTTTAGGAGACGACCAA
1970.edulisPontodoSolMadeira        TAATTCGTGCTGAGCTAGGGCAACCTGGTGCTCTTTTAGGAGACGACCAA
1240.turbinatusCoralBayCyprus       TAATTCGTGCTGAACTAGGACAACCCGGCGCTCTTCTCGGGGATGACCAA
1200.turbinatusCoralBayCyprus       TAATTCGTGCTGAACTAGGACAACCCGGCGCTCTTCTCGGGGATGACCAA
2560.turbinatusGreece               TAATTCCTGCAGAACTAAGACAACCCATCGCTCTTCTCGGGGATGACCAA
1020.turbinatusPuertoduguesaM       TAATTCGTGCTGAACTAGGACAACCCGGCGCTCTTCTCGGGGATGACCAA
1120.turbinatusCalahondaMalag       TAATTCGTGCTGAACAAGGACAACCCGGCGCTCTTCTCGGGGATGACCAA
1140.turbinatusCalahondaMalag       TAATTCGTGCTGAACTAGGACAACCCGGCGCTCTTCTCGGGGATGACCAA
1330.lineatusLymeRegis              TAATTCGTGCTGAGTTAGGACAACCCGGTGCTCTTCTCGGAGATGACCAA
1320.lineatusLymeRegis              TAATTCGTGCTGAGTTAGGACAACCCGGTGCTCTTCTCGGAGATGACCAA
4890.lineatusPortugal11             TAATTCGTGCTGAGTTAGGACAACCCGGTGCTCTTCTCGGAGATGACCAA
0280.lineatusImouraneMoroc          TAATTCGTGCTGAGTTAGGACAACCCGGTGCTCTTCTCGGAGATGACCAA
1390.lineatusPorthguerra            TAATTCGTGCTGAGTTAGGACAACCCGGTGCTCTTCTCGGAGATGACCAA
4600.lineatusPortugal7              TAATTCGTGCTGAGTTAGGACAACCCGGTGCTCTTCTCGGAGATGACCAA
2350.lineatusBasqueCoastline        TAATTCGTGCTGAGTTAGGACAACCCGGTGCTCTTCTCGGAGATGACCAA
2360.lineatusBasqueCoastline        TAATTCGTGCTGAGTTAGGACAACCCGGTGCTCTTCTCGGAGATGACCAA
4470.lineatusPortugal5              TAATTCGTGCTGAGTTAGGACAACCCGGTGCTCTTCTCGGAGATGACCAA
4950.lineatusPortugal11             TAATTCGTGCTGAGTTAGGACAACCCGGTGCTCTTCTCGGAGATGACCAA
1400.lineatusPorthguerra            TAATTCGTGCTGAGTTAGGACAACCCTGTGCTCTTCTCGGAGATGACCAA
0290.lineatusImouraneMoroc          TAATTCGTGCTGAGTTAGGACAACCCGGTGCTCTTCTCGGAGATGACCAA
4690.lineatusPortugal8              TAATTCGTGCTGAGTTAGGACAACCCGGTGCTCTTCTCGGAGATGACCAA
2510.lineatusGreece                 TAATTCGTGCTGAGTTAGGACAACCCGGTGCTCTTCTCGGAGATGACCAA
4140.lineatusBasqueCoastline        TAATTCGTGCTGAGTTAGGACAACCCGGTGCTCTTCTCGGAGACGACCAA
4510.lineatusPortugal3              TAATTCGTGCTGAGTTAGGACAACCCGGTGCTCTTCTCGGAGATGACCAA
4520.lineatusPortugal3              TAATTCGTGCTGAGTTAGGACAACCCGGTGCTCTTCTCGGAGATGACCAA
0360.lineatusTinitMoroc             TAATTCGTGCTGAGTTAGGACAACCTGGTGCTCTTCTCGGAGATGACCAA
4900.lineatusPortugal11             TAATTCGTGCTGAGTTAGGACAACCCGGTGCTCTTCTCGGAGATGACCAA
0690.atratusLasGalletasTener        TAATTCGTGCTGAGCTAGGGCAACCTGGTGCTCTTCTTGGAGATGACCAA
0680.atratusLasGalletasTener        TAATTCGTGCTGAGCTAGGGCAACCTGGTGCTCTTCTTGGAGATGACCAA
2770.atratusLanz2                   TAATTCGTGCTGAGCTAGGGCAACCTGGTGCTCTTCTTGGAGATGACCAA
2790.atratusLanz2                   TAATTCGTGCTGAGCTAGGGCAACCTGGTGCTCTTCTTGGAGATGACCAA
3210.atratusFuerte1                 TAATTCGTGCTGAGCTAGGGCAACCTGGTGCTCTTCTTGGAGATGACCAA
3300.atratusLanz4                   TAATTCGTGCTGAGCTAGGGCAACCTGGTGCTCTTCTTGGAGATGACCAA
0660.atratusLasGalletasTener        TAATTCGTGCTGAGCTAGGGCAACCTGGTGCTCTTCTTGGAGATGACCAA
2600.atratusLanzarote3              TAATTCGTGCTGAGCTAGGGCAACCTGGTGCTCTTCTTGGAGATGACCAA
336GibbumbjuvenilleLanz4            TAATTCGTGCTGAATTAGGACAGCCAGGAGCTCTCCTCGGCGATGACCAA
413GibbulasppBasqueCoastline        TAATTCGTGCTGAATTAGGACAGCCAGGAGCTCTCCTCGGCGATGACCAA
408GibbulasppBasqueCoastline        TAATTCGTGCTGAATTAGGACAGCCAGGAGCTCTCCTCGGCGATGACCAA
246GibbulasppBasqueCoastline        TAATTCGTGCTGAATTAGGACAGCCAGGAGCTCTCCTCGGCGATGACCAA
412GibbulasppBasqueCoastline        TAATTCGTGCTGAATTAGGACAGCCAGGAGCTCTCCTCGGCGATGACCAA
436G.umbilicalisPortugal6           TAATTCGTGCTGAATTAGGACAGCCAGGAGCTCTCCTCGGCGATGACCAA
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444G.umbilicalisPortugal4          TAATTCGTGCTGAATTAGGACAGCCAGGAGCTCTCCTCGGCGATGACCAA
245GibbulasppBasqueCoastline       TAATTCGTGCTGAATTAGGACAGCCAGGAGCTCTCCTCGGCGATGACCAA
                                   *  **    * **     * ** **    *****  * ** ** ******

091O.articulatusCalahondaMalag     CTATATAATGTAATTGTTACAGCTCACGCTTTTGTAATAATTTTTTTCTT
093O.articulatusCalahondaMalag     CTATATAATGTAATTGTTACAGCTCACGCTTTTGTAATAATTTTTTTCTT
340O.edulisFuerte6                 CTATATAATGTAATTGTTACAGCTCATGCGTTCGTAATAATTTTCTTTTT
064O.edulisLaRamblaTener           CTATATAATGTAATTGTTACAGCTCATGCGTTCGTAATAATTTTCTTTTT
226O.edulisBasqueCoastline         CTATACAATGTAATTGTTACAGCTCATGCGTTCGTAATAATTTTCTTTTT
264O.edulisLanzarote3              CTATATAACGTAATTGTTACAGCTCATGCGTTCGTAATAATTTTCTTTTT
351O.edulisGC2                     CTATATAATGTAATTGTTACAGCTCATGCGTTCGTAATAATTTTCTTTTT
77O.edulisLosGigantesTener         CTATATAATGTAATTGTTACAGCTCATGCGTTCGTAATAATTTTCTTTTT
272O.edulisFuerte4                 CTATATAATGTAATTGTTACAGCTCATGCGTTCGTAATAATTTTCTTTTT
464O.edulisPortugal7               CTATATAATGTAATTGTTACAGCTCATGCGTTCGTAATAATTTTCTTTTT
364O.edulisGC5                     CTATATAATGTAATTGTTACAGCTCATGCGTTCGTAATAATTTTCTTTTT
263O.edulisLanzarote3              CTATATAATGTAATTGTTACAGCTCATGCGTTCGTAATAATTTTCTTTTT
070O.atratusLasGalletasTener       CTATATAATGTAATTGTTACAGCTCATGCGTTCGTAATAATTTTCTTTTT
032O.edulisImouraneMoroc           CTATATAATGTAATTGTTACAGCTCATGCGTTCGTAATAATTTTCTTTTT
493O.edulisPortugal11              CTATATAATGTAATTGTTACAGCTCATGCGTTCGTAATAATTTTCTTTTT
474O.edulisPortugal8               CTATATAATGTAATTGTTACAGCTCATGCGTTCGTAATAATTTTCTTTTT
031O.edulisImouraneMoroc           CTATATAATGTAATTGTTACAGCTCATGCGTTCGTAATAATTTTCTTTTT
355O.edulisGC3                     CTATATAATGTAATTGTTACAGCTCATGCGTTCGTAATAATTTTCTTTTT
354O.edulisGC3                     CTATATAATGTAATTGTTACAGCTCATGCGTTCGTAATAATTTTCTTTTT
273O.edulisFuerte4                 CTATATAATGTAATTGTTACAGCTCATGCGTTCGTAATAATTTTCTTTTT
053O.edulisCapGhirMoroc            CTATATAATGTAATTGTTACAGCTCATGCGTTCGTAATAATTTTCTTTTT
063O.edulisLaRamblaTener           CTATATAATGTAATTGTTACAGCTCATGCGTTCGTAATAATTTTCTTTTT
349O.edulisGC2                     CTATATAATGTAATTGTTACAGCTCATGCATTCGTAATAATTTTCTTTTT
339O.edulisFuerte6                 CTATATAATGTAATTGTTACAGCTCATGCGTTCGTAATAATTTTCTTTTT
325O.edulisLanz4                   CTATATAATGTAATTGTTACAGCTCATGCGTTCGTAATAATTTTCTTTTT
057O.edulisElMedanoTener           CTATATAATGTAATTGTTACAGCTCATGCGTTCGTAATAATTTTCTTTTT
076O.edulisLosGigantesTener        CTATATAATGTAATTGTTACAGCTCATGCGTTCGTAATAATTTTCTTTTT
042O.edulisTinitMoroc              CTATATAATGTAATTGTTACAGCTCATGCGTTCGTAATAATTTTCTTTCT
054O.edulisCapGhirMoroc            CTATATAATGTAATTGTTACAGCTCATGCGTTCGTAATAATTTTCTTTCT
182O.edulisSeixalMadeira           CTATATAATGTAATTGTTACAGCTCATGCGTTCGTAATAATTTTCTTTCT
183O.edulisSaoVincentMadeira       CTATATAATGTAATTGTTACAGCTCATGCGTTCGTAATAATTTTCTTTCT
192O.edulisPrainhaBeachMadeira     CTATATAATGTAATTGTTACAGCTCATGCGTTCGTAATAATTTTCTTTCT
197O.edulisPontodoSolMadeira       CTATATAATGTAATTGTTACAGCTCATGCGTTCGTAATAATTTTCTTTCT
124O.turbinatusCoralBayCyprus      TTATATAATGTAATTGTTACAGCTCACGCATTCGTAATAATTTTCTTTTT
120O.turbinatusCoralBayCyprus      TTATATAATGTAATTGTTACAGCTCACGCATTCGTAATAATTTTCTTTTT
256O.turbinatusGreece              TTATATAATGTAATTGTTACAGCTCACGCATTCGTAATAATTTTCTTTTT
102O.turbinatusPuertoduguesaM      TTATATAATGTAATTGTTACAGCTCACGCATTCGTAATAATTTTCTTTTT
112O.turbinatusCalahondaMalag      TTATATAATGTAATTGTTACAGCTCACGCATTCGTAATAATTTTCTTTTT
114O.turbinatusCalahondaMalag      TTATATAATGTAATTGTTACAGCTCACGCATTCGTAATAATTTTCTTTTT
133O.lineatusLymeRegis             CTATACAATGTAATTGTTACAGCCCACGCTTTTGTAATAATTTTCTTTTT
132O.lineatusLymeRegis             CTATACAATGTAATTGTTACAGCCCACGCTTTTGTAATAATTTTCTTTTT
489O.lineatusPortugal11            CTATACAATGTAATTGTTACAGCCCACGCTTTTGTAATAATTTTCTTTTT
028O.lineatusImouraneMoroc         CTATACAATGTAATTGTTACAGCCCACGCTTTTGTAATAATTTTCTTTTT
139O.lineatusPorthguerra           CTATACAATGTAATTGTTACAGCTCACGCTTTTGTAATAATTTTCTTTTT
460O.lineatusPortugal7             CTATACAATGTAATTGTTACAGCTCACGCTTTTGTAATAATTTTCTTTTT
235O.lineatusBasqueCoastline       CTATACAATGTAATTGTTACAGCTCACGCTTTTGTAATAATTTTCTTTTT
236O.lineatusBasqueCoastline       CTATACAATGTAATTGTTACAGCTCACGCTTTTGTAATAATTTTCTTTTT
447O.lineatusPortugal5             CTATACAATGTAATTGTTACAGCTCACGCTTTTGTAATAATTTTCTTTTT
495O.lineatusPortugal11            CTATACAATGTAATTGTTACAGCTCACGCTTTTGTAATAATTTTCTTTTT
140O.lineatusPorthguerra           CTATACAATGTAATTGTTACAGCTCACGCTTTTGTAATAATTTTCTTTTT
029O.lineatusImouraneMoroc         CTATACAATGTAATTGTTACAGCCCACGCTTTTGTAATAATTTTCTTTTT
469O.lineatusPortugal8             CTATACAATGTAATTGTTACAGCCCACGCTTTTGTAATAATTTTCTTTTT
251O.lineatusGreece                CTATACAATGTAATTGTTACAGCCCACGCTTTTGTAATAATTTTCTTTTT
414O.lineatusBasqueCoastline       CTATACAACGTAATTGTTACAGCTCACGCTTTTGTAATAATTTTCTTTTT
451O.lineatusPortugal3             CTATACAATGTAATTGTTACAGCCCACGCTTTTGTAATAATTTTCTTTTT
452O.lineatusPortugal3             CTATACAATGTAATTGTTACAGCCCACGCTTTTGTAATAATTTTCTTTTT
036O.lineatusTinitMoroc            CTATACAATGTAATTGTTACAGCCCACGCTTTTGTAATAATTTTCTTTTT
490O.lineatusPortugal11            CTATACAATGTAATTGTTACAGCCCACGCTTTTGTAATAATTTTCTTTTT
069O.atratusLasGalletasTener       CTATATAATGTAATTGTTACAGCCCACGCTTTTGTAATGATTTTCTTTTT
068O.atratusLasGalletasTener       CTATATAATGTAATTGTTACAGCCCACGCTTTTGTAATGATTTTCTTTTT
277O.atratusLanz2                  CTATATAATGTAATTGTTACAGCCCACGCTTTTGTAATGATTTTCTTTTT
279O.atratusLanz2                  CTATATAATGTAATTGTTACAGCCCACGCTTTTGTAATGATTTTCTTTTT
321O.atratusFuerte1                CTATATAATGTAATTGTTACAGCCCACGCTTTTGTAATGATTTTCTTTTT
330O.atratusLanz4                  CTATATAATGTAATTGTTACAGCCCACGCTTTTGTAATGATTTTCTTTTT
066O.atratusLasGalletasTener       CTATATAATGTAATTGTTACAGCCCACGCTTTTGTAATGATTTTCTTTTT
260O.atratusLanzarote3             CTATATAATGTAATTGTTACAGCCCACGCTTTTGTAATGATTTTCTTTTT
336GibbumbjuvenilleLanz4           CTATATAATGTAATTGTAACAGCTCATGCTTTTGTAATAATTTTCTTTTT
413GibbulasppBasqueCoastline       CTATATAATGTAATTGTAACAGCTCATGCTTTTGTAATAATTTTCTTTTT
408GibbulasppBasqueCoastline       CTATATAATGTAATTGTAACAGCTCATGCTTTTGTAATAATTTTCTTTTT
246GibbulasppBasqueCoastline       CTATATAATGTAATTGTAACAGCTCATGCTTTTGTAATAATTTTCTTTTT
412GibbulasppBasqueCoastline       CTATATAATGTAATTGTAACAGCTCATGCTTTTGTAATAATTTTCTTTTT
436G.umbilicalisPortugal6          CTATATAATGTAATTGTAACAGCTCATGCTTTTGTAATAATTTTCTTTTT
444G.umbilicalisPortugal4          CTATATAATGTAATTGTAACAGCTCATGCTTTTGTAATAATTTTCTTTTT
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245GibbulasppBasqueCoastline        CTATATAATGTAATTGTAACAGCTCATGCTTTTGTAATAATTTTCTTTTT
                                     **** ** ******** ***** ** ** ** ***** ***** **  *

091O.articulatusCalahondaMalag      AGTTATACCTCTGATAATTGGCGGATTTGGTAACTGATTAATTCCCCTGA
093O.articulatusCalahondaMalag      AGTTATACCTCTGATAATTGGCGGATTTGGTAACTGATTAATTCCCCTGA
340O.edulisFuerte6                  AGTAATGCCTTTAATAATTGGTGGATTTGGTAATTGATTAATTCCCCTAA
064O.edulisLaRamblaTener            AGTAATGCCTTTAATAATTGGTGGATTTGGTAATTGATTAATTCCCCTAA
226O.edulisBasqueCoastline          AGTAATGCCTTTAATAATTGGTGGATTTGGTAATTGATTAATTCCCCTAA
264O.edulisLanzarote3               AGTAATGCCTTTAATAATTGGTGGATTTGGTAATTGATTAATTCCCCTAA
351O.edulisGC2                      AGTAATGCCTTTAATAATTGGTGGATTTGGTAATTGATTAATTCCCCTAA
77O.edulisLosGigantesTener          AGTAATGCCTTTAATAATTGGTGGATTTGGTAATTGATTAATTCCCCTAA
272O.edulisFuerte4                  AGTAATGCCTTTAATAATTGGTGGATTTGGTAATTGATTAATTCCCTTAA
464O.edulisPortugal7                AGTAATGCCTTTAATAATTGGTGGATTTGGTAATTGATTAATTCCCCTAA
364O.edulisGC5                      AGTAATGCCTTTAATAATTGGTGGATTTGGTAATTGATTAATTCCCCTAA
263O.edulisLanzarote3               AGTAATGCCTTTAATAATTGGTGGATTTGGTAATTGATTAATTCCCCTAA
070O.atratusLasGalletasTener        AGTAATGCCTTTAATAATTGGTGGATTTGGTAATTGATTAATTCCCCTAA
032O.edulisImouraneMoroc            AGTAATGCCTTTAATAATTGGTGGATTTGGTAATTGATTAATTCCCCTAA
493O.edulisPortugal11               AGTAATGCCTTTAATAATTGGTGGATTTGGTAATTGATTAATTCCCCTAA
474O.edulisPortugal8                AGTAATGCCTTTAATAATTGGTGGATTTGGTAATTGATTAATTCCCCTAA
031O.edulisImouraneMoroc            AGTAATGCCTTTAATAATTGGTGGATTTGGTAATTGATTAATTCCCCTAA
355O.edulisGC3                      AGTAATGCCTTTAATAATTGGTGGATTTGGTAATTGATTAATTCCCCTAA
354O.edulisGC3                      AGTAATGCCTTTAATAATTGGTGGATTTGGTAATTGATTAATTCCCCTAA
273O.edulisFuerte4                  AGTAATGCCTTTAATAATTGGTGGATTTGGTAATTGATTAATTCCCCTAA
053O.edulisCapGhirMoroc             AGTAATGCCTTTAATAATTGGTGGATTTGGTAATTGATTAATTCCCCTAA
063O.edulisLaRamblaTener            AGTAATGCCTTTAATAATTGGTGGATTTGGTAATTGATTAATTCCCCTAA
349O.edulisGC2                      AGTAATGCCTTTAATAATTGGTGGATTTGGTAATTGATTAATTCCCCTAA
339O.edulisFuerte6                  AGTAATGCCTTTAATAATTGGTGGATTTGGTAATTGATTAATTCCCCTAA
325O.edulisLanz4                    AGTAACGCCTTTAATAATTGGTGGATTTGGTAATTGATTAATTCCCCTAA
057O.edulisElMedanoTener            AGTAATGCCTTTAATAATTGGTGGATTTGGTAATTGATTAATTCCCCTAA
076O.edulisLosGigantesTener         AGTAATGCCTTTAATAATTGGTGGATTTGGTAATTGATTAATTCCCCTAA
042O.edulisTinitMoroc               AGTAATGCCTTTAATAATTGGTGGATTTGGTAATTGATTAATTCCCCTAA
054O.edulisCapGhirMoroc             AGTAATGCCTTTAATAATTGGTGGATTTGGTAATTGATTAATTCCCCTAA
182O.edulisSeixalMadeira            AGTAATGCCTTTAATAATTGGTGGATTTGGTAATTGATTAATTCCCCTAA
183O.edulisSaoVincentMadeira        AGTAATGCCTTTAATAATTGGTGGATTTGGTAACTGATTAATTCCCCTAA
192O.edulisPrainhaBeachMadeira      AGTAATGCCTTTAATAATTGGTGGATTTGGTAACTGATTAATTCCCCTAA
197O.edulisPontodoSolMadeira        AGTAATGCCTTTAATAATTGGTGGATTTGGTAACTGATTAATTCCCCTAA
124O.turbinatusCoralBayCyprus       AGTGATACCTTTAATAATTGGTGGATTTGGTAACTGATTAATTCCTTTAA
120O.turbinatusCoralBayCyprus       AGTGATACCTTTAATAATTGGTGGATTTGGTAACTGATTAATTCCTTTAA
256O.turbinatusGreece               AGTGATACCTTTAATAATTGGTGGATTTGGTAACTGATTAATTCCTTTAA
102O.turbinatusPuertoduguesaM       AGTGATACCTTTAATAATTGGTGGATTTGGTAACTGATTAATTCCTTTAA
112O.turbinatusCalahondaMalag       AGTTATACCTTTAATAATTGGTGGATTTGGTAACTGATTAATTCCTTTAA
114O.turbinatusCalahondaMalag       AGTTATACCTTTAATAATTGGTGGATTTGGTAACTGATTAATTCCTTTAA
133O.lineatusLymeRegis              AGTAATACCTTTAATGATTGGTGGGTTCGGTAACTGACTAATCCCTTTAA
132O.lineatusLymeRegis              AGTAATACCTTTAATGATTGGTGGGTTCGGTAACTGACTAATCCCTTTAA
489O.lineatusPortugal11             AGTAATACCTTTAATGATTGGTGGGTTCGGTAACTGACTAATCCCTTTAA
028O.lineatusImouraneMoroc          AGTAATACCTTTAATGATTGGTGGGTTCGGTAACTGACTAATCCCTTTAA
139O.lineatusPorthguerra            AGTAATACCTTTAATGATTGGTGGGTTCGGTAACTGACTAATCCCTTTAA
460O.lineatusPortugal7              AGTAATACCTTTAATGATTGGTGGGTTCGGTAACTGACTAATCCCTTTAA
235O.lineatusBasqueCoastline        AGTAATACCTTTAATGATTGGTGGGTTCGGTAACTGACTAATCCCTTTAA
236O.lineatusBasqueCoastline        AGTAATACCTTTAATGATTGGTGGGTTCGGTAACTGACTAATCCCTTTAA
447O.lineatusPortugal5              AGTGATACCTTTAATGATTGGTGGGTTCGGTAACTGACTAATCCCTTTAA
495O.lineatusPortugal11             AGTAATACCTTTAATGATTGGTGGGTTCGGTAACTGACTAATCCCTTTAA
140O.lineatusPorthguerra            AGTAATACCTTTAATGATTGGTGGGTTCGGTAACTGACTAATCCCTTTAA
029O.lineatusImouraneMoroc          AGTAATACCCTTAATGATTGGTGGATTCGGTAACTGACTAATCCCTTTAA
469O.lineatusPortugal8              AGTAATACCCTTAATGATTGGTGGATTCGGTAACTGACTAATCCCTTTAA
251O.lineatusGreece                 AGTAATACCCTTAATGATTGGTGGATTCGGTAACTGACTAATCCCTTTAA
414O.lineatusBasqueCoastline        AGTAATACCTTTAATGATTGGTGGGTTCGGTAACTGACTAATCCCTTTAA
451O.lineatusPortugal3              AGTAATACCTTTAATGATTGGTGGATTCGGTAACTGACTAATCCCTTTAA
452O.lineatusPortugal3              AGTAATACCTTTAATGATTGGTGGATTCGGTAACTGACTAATCCCTTTAA
036O.lineatusTinitMoroc             AGTAATACCTTTAATGATTGGTGGGTTCGGTAACTGGCTAATCCCTTTAA
490O.lineatusPortugal11             AGTAATACCTTTAATGATTGGTGGATTCGGTAACTGACTAATCCCTTTAA
069O.atratusLasGalletasTener        AGTGATACCTTTAATAATTGGTGGGTTCGGTAACTGGCTAATTCCCCTAA
068O.atratusLasGalletasTener        AGTGATACCTTTAATAATTGGTGGGTTCGGTAACTGGCTAATTCCCCTAA
277O.atratusLanz2                   AGTGATACCTTTAATAATTGGTGGGTTCGGTAACTGGCTAATTCCCCTAA
279O.atratusLanz2                   AGTGATACCTTTAATAATTGGTGGGTTCGGTAACTGGCTAATTCCCCTAA
321O.atratusFuerte1                 AGTGATACCTTTAATAATTGGTGGGTTCGGTAACTGGCTAATTCCCCTAA
330O.atratusLanz4                   AGTGATACCTTTAATAATTGGTGGGTTCGGTAACTGGCTAATTCCCCTAA
066O.atratusLasGalletasTener        AGTGATACCTTTAATAATTGGTGGGTTCGGTAACTGGCTAATTCCCCTAA
260O.atratusLanzarote3              AGTGATACCTTTAATAATTGGTGGGTTCGGTAACTGGCTAATTCCCTTAA
336GibbumbjuvenilleLanz4            AGTTATACCCCTAATAATTGGAGGATTTGGTAACTGACTAATTCCTTTAA
413GibbulasppBasqueCoastline        AGTTATACCCCTAATAATTGGAGGATTTGGTAACTGACTAATTCCTTTAA
408GibbulasppBasqueCoastline        AGTTATACCCCTAATAATTGGAGGATTTGGTAACTGACTAATTCCTTTAA
246GibbulasppBasqueCoastline        AGTTATACCCCTAATAATTGGAGGATTTGGTAACTGACTAATTCCTTTAA
412GibbulasppBasqueCoastline        AGTTATACCCCTAATAATTGGAGGATTTGGTAACTGACTAATTCCTTTAA
436G.umbilicalisPortugal6           AGTTATACCCCTAATAATTGGAGGATTTGGTAACTGACTAATCCCTTTAA
444G.umbilicalisPortugal4           AGTTATACCCCTAATAATTGGAGGATTCGGTAACTGACTAATTCCTTTAA
245GibbulasppBasqueCoastline        AGTTATACCCCTAATAATTGGAGGATTTGGTAACTGACTAATTCCTTTAA
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0910.articulatusCalahondaMalag    TGCTAGGAGCGCCCGACATGGCGTTTCCGCGACTAAACAATATAAGTTTC
0930.articulatusCalahondaMalag    TGCTAGGAGCGCCCGACATGGCGTTTCCGCGACTAAACAATATAAGTTTC
3400.edulisFuerte6                TACTAGGAGCACCTGATATAGCATTCCCTCGACTTAATAATATAAGTTTC
0640.edulisLaRamblaTener          TACTAGGAGCACCTGATATAGCATTCCCTCGACTTAATAATATAAGTTTC
2260.edulisBasqueCoastline        TACTAGGAGCACCTGATATAGCATTCCCTCGACTTAATAATATAAGTTTC
2640.edulisLanzarote3             TACTAGGAGCACCTGATATAGCATTCCCTCGACTTAATAATATAAGTTTC
3510.edulisGC2                    TACTAGGAGCACCTGATATAGCATTCCCTCGACTTAATAATATAAGTTTC
770.edulisLosGigantesTener        TACTAGGAGCACCTGATATAGCATTCCCTCGACTTAATAAYATAAGTTTC
2720.edulisFuerte4                TACTAGGAGCACCTGATATAGCATTCCCTCGACTTAATAATATAAGTTTC
4640.edulisPortugal7              TACTAGGAGCACCTGATATAGCATTCCCTCGACTTAATAATATAAGTTTC
3640.edulisGC5                    TACTAGGAGCACCTGATATAGCATTCCCTCGACTTAATAATATAAGTTTC
2630.edulisLanzarote3             TACTAGGAGCACCTGATATAGCATTCCCTCGACTTAATAATATAAGTTTC
0700.atratusLasGalletasTener      TACTAGGAGCACCTGATATAGCATTCCCTCGACTTAATAATATAAGTTTC
0320.edulisImouraneMoroc          TACTAGGAGCACCTGATATAGCATTCCCTCGACTTAATAATATAAGTTTC
4930.edulisPortugal11             TACTAGGAGCACCTGATATAGCATTCCCTCGACTTAATAATATAAGTTTC
4740.edulisPortugal8              TACTAGGAGCACCTGATATAGCATTCCCTCGACTTAATAATATAAGTTTC
0310.edulisImouraneMoroc          TACTAGGAGCACCTGATATAGCATTCCCTCGACTTAATAATATAAGTTTC
3550.edulisGC3                    TACTAGGAGCACCTGATATAGCATTCCCTCGACTTAATAATATAAGTTTC
3540.edulisGC3                    TACTAGGAGCACCTGATATAGCATTCCCTCGACTTAATAATATAAGTTTC
2730.edulisFuerte4                TACTAGGAGCACCTGATATAGCATTCCCTCGACTTAATAATATAAGTTTC
0530.edulisCapGhirMoroc           TACTAGGAGCACCTGATATAGCATTCCCTCGACTTAATAATATAAGTTTC
0630.edulisLaRamblaTener          TACTAGGAGCACCTGATATAGCATTCCCTCGACTTAATAATATAAGTTTC
3490.edulisGC2                    TACTAGGAGCACCTGATATAGCATTCCCTCGACTTAATAATATAAGTTTC
3390.edulisFuerte6                TACTAGGAGCACCTGATAT-GCATTCCCTCGACTTAATAATATAAGTTTC
3250.edulisLanz4                  TACTAGGAGCACCTGATATAGCATTCCCTCGACTTAATAATATAAGTTTC
0570.edulisElMedanoTener          TACTAGGAGCACCTGATATAGCATTCCCTCGACTTAATAATATAAGTTTC
0760.edulisLosGigantesTener       TACTAGGAGCACCTGATATAGCATTCCCTCGACTTAATAACATAAGTTTC
0420.edulisTinitMoroc             TACTAGGAGCACCCGATATGGCATTCCCTCGACTTAATAATATAAGTTTC
0540.edulisCapGhirMoroc           TACTAGGAGCACCCGATATGGCATTCCCTCGACTTAATAATATAAGTTTC
1820.edulisSeixalMadeira          TACTAGGAGCACCCGATATGGCATTCCCTCGACTTAATAATATAAGTTTC
1830.edulisSaoVincentMadeira      TACTAGGAGCACCTGATATGGCATTCCCTCGACTTAATAATATAAGTTTC
1920.edulisPrainhaBeachMadeira    TACTAGGAGCACCTGATATGGCATTCCCTCGACTTAATAATATAAGTTTC
1970.edulisPontodoSolMadeira      TACTAGGAGCACCTGATATGGCATTCCCTCGACTTAATAATATAAGTTTC
1240.turbinatusCoralBayCyprus     TACTAGGAGCACCAGATATAGCATTTCCCCGACTTAATAATATAAGTTTC
1200.turbinatusCoralBayCyprus     TACTAGGAGCACCAGATATAGCATTTCCCCGACTTAATAATATAAGTTTC
2560.turbinatusGreece             TACTAGGAGCACCAGATATAGCATTTCCCCGACTTAATAATATAAGTTTC
1020.turbinatusPuertoduguesaM     TACTAGGAGCACCAGATATAGCATTTCCCCGACTTAATAATATAAGTTTC
1120.turbinatusCalahondaMalag     TACTAGGAGCACCAGATATAGCATTTCCCCGACTTAATAATATAAGTTTC
1140.turbinatusCalahondaMalag     TACTAGGAGCACCAGATATAGCATTTCCCCGACTTAATAATATAAGTTTC
1330.lineatusLymeRegis            TACTAGGGGCACCCGATATAGCGTTCCCCCGACTAAATAATATAAGTTTC
1320.lineatusLymeRegis            TACTAGGGGCACCCGATATAGCGTTCCCCCGACTAAATAATATAAGTTTC
4890.lineatusPortugal11           TACTAGGGGCACCCGATATAGCGTTCCCCCGACTAAATAATATAAGTTTC
0280.lineatusImouraneMoroc        TACTAGGGGCACCCGATATAGCGTTCCCCCGACTAAATAATATAAGTTTC
1390.lineatusPorthguerra          TACTAGGGGCACCCGATATAGCGTTCCCCCGACTAAATAATATAAGTTTC
4600.lineatusPortugal7            TACTAGGGGCACCCGATATAGCGTTCCCCCGACTAAATAATATAAGTTTC
2350.lineatusBasqueCoastline      TACTAGGGGCACCCGATATAGCGTTCCCCCGACTAAATAATATAAGTTTC
2360.lineatusBasqueCoastline      TACTAGGGGCACCCGATATAGCGTTCCCCCGACTAAATAATATAAGTTTC
4470.lineatusPortugal5            TACTAGGGGCACCCGATATAGCGTTCCCCCGACTAAATAATATAAGTTTC
4950.lineatusPortugal11           TACTAGGGGCACCCGATATAGCGTTCCCCCGACTAAATAATATAAGTTTC
1400.lineatusPorthguerra          TACTAGGGGCACCCGATATAGCGTTTCCCCCGACTAAATAATATAAGTTTC
0290.lineatusImouraneMoroc        TACTAGGGGCACCCGATATAGCGTTTCCCCCGACTAAATAATATAAGTTTC
4690.lineatusPortugal8            TACTAGGGGCACCCGATATAGCGTTTCCCCCGACTAAATAATATAAGTTTC
2510.lineatusGreece               TACTAGGGGCACCCGATATAGCGTTTCCCCCGACTAAATAATATAAGTTTC
4140.lineatusBasqueCoastline      TACTAGGGGCACCCGATATAGCGTTCCCCCGACTAAATAATATAAGTTTC
4510.lineatusPortugal3            TACTAGGGGCACCCGATATGGCATTCCCCCGACTAAATAATATAAGTTTC
4520.lineatusPortugal3            TACTAGGGGCACCCGATATGGCATTCCCCCGACTAAATAATATAAGTTTC
0360.lineatusTinitMoroc           TACTAGGGGCACCCGATATAGCATTCCCCCGACTAAATAATATAAGTTTC
4900.lineatusPortugal11           TACTAGGGGCACCCGATATAGCGTTCCCCCGACTAAATAATATAAGTTTC
0690.atratusLasGalletasTener      TACTAGGGGCACCTGATATAGCGTTTCCGCGACTTAATAATATAAGTTTC
0680.atratusLasGalletasTener      TACTAGGGGCACCTGATATAGCGTTTCCGCGACTTAATAATATAAGTTTC
2770.atratusLanz2                 TACTAGGGGCACCTGATATAGCGTTTCCGCGACTTAATAATATAAGTTTC
2790.atratusLanz2                 TACTAGGGGCACCTGATATAGCGTTTCCGCGACTTAATAATATAAGTTTC
3210.atratusFuerte1               TACTAGGGGCACCTGATATAGCGTTTCCGCGACTTAATAATATAAGTTTC
3300.atratusLanz4                 TACTAGGGGCACCTGATATAGCGTTTCCCCGACTTAATAATATAAGTTTC
0660.atratusLasGalletasTener      TACTAGGAGCACCTGATATAGCGTTTCCGCGACTTAATAATATAAGTTTC
2600.atratusLanzarote3            TACTAGGGGCACCTGATATAGCGTTTCCGCGACTTAATAATATAAGTTTC
336GibbumbjuvenilleLanz4          TGCTAGGAGCACCTGATATAGCTTTCCCTCGACTTAATAACATAAGTTTT
413GibbulasppBasqueCoastline      TGCTAGGAGCACCTGATATAGCTTTCCCTCGACTTAATAACATAAGTTTT
408GibbulasppBasqueCoastline      TGCTAGGAGCACCTGATATAGCTTTCCCTCGACTTAATAACATAAGTTTT
246GibbulasppBasqueCoastline      TGCTAGGAGCACCTGATATAGCTTTCCCTCGACTTAATAACATAAGTTTT
412GibbulasppBasqueCoastline      TGCTAGGAGCACCTGATATAGCTTTCCCTCGACTTAATAACATAAGTTTT
436G.umbilicalisPortugal6         TGCTAGGAGCACCTGATATAGCTTTCCCTCGACTTAATAACATAAGTTTT
444G.umbilicalisPortugal4         TGCTAGGAGCACCTGATATAGCTTTTCCTCGACTTAATAACATAAGTTTT
245GibbulasppBasqueCoastline      TGCTAGGAGCACCTGATATAGCTTTCCCTCGACTTAATAACATAAGTTTT
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0910.articulatusCalahondaMalag      TGGCTCCTCCCTCCGTCTTTGACCCTTTTATTAGCCTCTTCTGCT-GTAG
0930.articulatusCalahondaMalag      TGGCTCCTCCCTCCGTCTTTGACCCTTTTATTAGCCTCTTCTGCT-GTAG
3400.edulisFuerte6                  TGACTTCTACCTCCTTCTTTAACCCTTCTATTAGCATCTTCCGCTTGTAG
0640.edulisLaRamblaTener            TGACTTCTACCTCCTTCTTTAACCCTTCTATTAGCATCTTCCGCT-GTAG
2260.edulisBasqueCoastline          TGACTTCTACCTCCTTCTTTAACCCTTCTATTAGCATCTTCCGCT-GTAG
2640.edulisLanzarote3               TGACTTCTACCTCCTTCCTTAACCCTTCTATTAGCATCTTCCGCT-GTAG
3510.edulisGC2                      TGACTTCTACCTCCTTCTTTAACCCTTCTATTAGCATCTTCCGCT-GTAG
770.edulisLosGigantesTener          TGACTTCTACCTCCTTCTTTAACCCTTCTATTAGCATCTTCCGCT-GTAG
2720.edulisFuerte4                  TGACTTCTACCTCCTTCTTTAACCCTTCTATTAGCATCTTCCGCT-GTAG
4640.edulisPortugal7                TGACTTCTACCTCCTTCTTTAACCCTTCTATTAACATCTTCCGCT-GTAG
3640.edulisGC5                      TGACTTCTACCTCCTTCTTTAACCCTTCTATTAGCATCTTCCGCT-GTAG
2630.edulisLanzarote3               TGACTTCTACCTCCTTCTTTAACCCTTCTATTAGCATCTTCCGCT-GTAG
0700.atratusLasGalletasTener        TGACTTCTACCTCCTTCTTTAACCCTTCTATTAGCATCTTCCGCT-GTAG
0320.edulisImouraneMoroc            TGACTTCTACCTCCTTCTTTAACCCTTCTATTAGCATCTTCCGCT-GTAG
4930.edulisPortugal11               TGACTTCTACCTCCTTCTTTAACCCTTCTATTAGCATCTTCCGCT-GTAG
4740.edulisPortugal8                TGACTTCTACCTCCTTCTTTAACCCTTCTATTAGCATCTTCCGCT-GTAG
0310.edulisImouraneMoroc            TGACTTCTACCTCCTTCTTTAACCCTTCTATTAGCATCTTCCGCT-GTAG
3550.edulisGC3                      TGACTTCTACCTCCTTCTTTAACCCTTCTATTAGCATCTTCCGCT-GTAG
3540.edulisGC3                      TGACTTCTACCTCCTTCTTTAACCCTTCTATTAGCATCTTCCGCT-GTAG
2730.edulisFuerte4                  TGACTTCTACCTCCTTCTTTAACCCTTCTATTAGCATCTTCCGCT-GTAG
0530.edulisCapGhirMoroc             TGACTTCTACCTCCTTCTTTAACCCTTCTATTAGCATCTTCCGCT-GTAG
0630.edulisLaRamblaTener            TGACTTCTACCTCCTTCTTTAACCCTTCTATTAGCATCTTCCGCT-GTAG
3490.edulisGC2                      TGACTTCTACCTCCTTCTTTAACCCTTCTATTAGCATCTTCCGCT-GTAG
3390.edulisFuerte6                  TGACTTCTACCTCCTTCTTTAACCCTTCTATTAGCATCTTCCGCT-GTAG
3250.edulisLanz4                    TGACTTCTACCTCCTTCTT-AACCCTTCTATTAGCATCTTCCGCT-GTAG
0570.edulisElMedanoTener            TGACTTCTACCTCCTTCTTTAACCCTTCTATTAGCATCTTCCGCT-GTAG
0760.edulisLosGigantesTener         TGACTTCTACCTCCTTCTTTAACCCTTCTATTAGCATCTTCCGCT-GTAG
0420.edulisTinitMoroc               TGACTTCTACCTCCTTCTTTAACCCTTCTATTAGCATCTTCCGCT-GTAG
0540.edulisCapGhirMoroc             TGACTTCTACCTCCTTCTTTAACCCTTTTATTAGCATCTTCCGCT-GTAG
1820.edulisSeixalMadeira            TGACTTCTACCTCCTTCTTTAACCCTTCTATTAGCATCTTCCGCT-GTAG
1830.edulisSaoVincentMadeira        TGACTTCTACCTCCTTCTTTAACCCTTCTATTAGCATCTTCCGCT-GTAG
1920.edulisPrainhaBeachMadeira      TGACTTCTACCTCCTTCTTTAACCCTTCTATTAGCATCTTCCGCT-GTAG
1970.edulisPontodoSolMadeira        TGACTTCTACCTCCTTCTTTAACCCTTCTATTAGCATCTTCCGCT-GTAG
1240.turbinatusCoralBayCyprus       TGGCTTCTTCCTCCTTCTTTAACTCTTTTATTAGCGTCTTCCGCT-GTAG
1200.turbinatusCoralBayCyprus       TGGCTTCTTCCTCCTTCTTTAACTCTTTTATTAGCGTCTTCCGCT-GTAG
2560.turbinatusGreece               TGGCTTCTTCCTCCTTCTTTAACTCTTTTATTAGCGTCTTCCGCT-GTAG
1020.turbinatusPuertoduguesaM       TGGCTTCTTCCTCCTTCTTTAACTCTTTTATTAGCGTCTTCCGCT-GTAG
1120.turbinatusCalahondaMalag       TGGCTTCTTCCTCCTTCTTTAACTCTTTTATTAGCCTCTTCCGCT-GTAG
1140.turbinatusCalahondaMalag       TGGCTTCTTCCTCCTTCTTTAACTCTTTTATTAGCCTCTTCCGCT-GTAG
1330.lineatusLymeRegis              TGACTTCTCCCTCCTTCTTTAACTCTTTTATTAGCCTC-TTCTGCCGTAG
1320.lineatusLymeRegis              TGACTTCTCCCTCCTTCTTTAACTCTTTTATTAGCCTC-TTCTGCCGTAG
4890.lineatusPortugal11             TGACTTCTCCCTCCTTCTTTAACTCTTTTATTAGCCTC-TTCTGCCGTAG
0280.lineatusImouraneMoroc          TGACTTCTCCCTCCTTCTTTAACCCTTTTATTAGCCTC-TTCTGCCGTAG
1390.lineatusPorthguerra            TGACTTCTCCCTCCTTCTTTAACCCTTTTATTAGCCTC-TTCTGCCGTAG
4600.lineatusPortugal7              TGACTTCTCCCTCCTTCTTTAACCCTTTTATTAGCCTC-TTCTGCCGTAG
2350.lineatusBasqueCoastline        TGACTTCTCCCTCCTTCTTTAACCCTTTTATTAGCCTC-TTCTGCCGTAG
2360.lineatusBasqueCoastline        TGACTTCTCCCTCCTTCTTTAACCCTTTTATTAGCCTC-TTCTGCCGTAG
4470.lineatusPortugal5              TGACTTCTCCCTCCTTCTTTAACCCTTTTATTAGCCTC-TTCTGCCGTAG
4950.lineatusPortugal11             TGACTTCTCCCTCCTTCTTTAACCCTTTTATTAGCCTC-TTCTGCCGTAG
1400.lineatusPorthguerra            TGACTTCTCCCTCCTTCTTTAACCCTTTTATTAGCCTC-TTCTGCCGTAG
0290.lineatusImouraneMoroc          TGACTTCTCCCTCCTTCTTTAACCCTTTTATTAGCCTC-TTCTGCCGTAG
4690.lineatusPortugal8              TGACTTCTCCCTCCTTCTTTAACCCTTTTATTAGCCTC-TTCTGCCGTAG
2510.lineatusGreece                 TGACTTCTCCCTCCTTCTTTAACCCTTTTATTAGCCTC-TTCTGCCGTAG
4140.lineatusBasqueCoastline        TGACTTCTCCCTCCTTCTTTAACCCTTTTATTAGCCTC-TTCTGCCGTAG
4510.lineatusPortugal3              TGACTTCTCCCTCCTTCTTTAACCCTTTTACTAGCCTC-TTCTGCCGTAG
4520.lineatusPortugal3              TGACTTCTCCCTCCTTCTTTAACCCTTTTACTAGCCTC-TTCTGCCGTAG
0360.lineatusTinitMoroc             TGACTTCTCCCTCCTTCTTTAACCCTTTTATTAGCCTC-TTCTGCCGTAG
4900.lineatusPortugal11             TGACTTCTCCCTCCTTCTTTAACCCTTTTATTAGCCTC-TTCTGCCGTAG
0690.atratusLasGalletasTener        TGACTTCTTCCCCCTTCTTTAACTCTCTTACTAGCTTC-TTCCGCTGTAG
0680.atratusLasGalletasTener        TGACTTCTTCCCCCTTCTTTAACTCTCTTACTAGCTTC-TTCCGCTGTAG
2770.atratusLanz2                   TGACTTCTTCCCCCTTCTTTAACTCTCTTACTAGCTTC-TTCCGCTGTAG
2790.atratusLanz2                   TGACTTCTTCCCCCTTCTTTAACTCTCTTACTAGCTTC-TTCCGCTGTAG
3210.atratusFuerte1                 TGACTTCTTCCCCCTTCTTTAACTCTCTTACTAGCTTCCTTCCGCTGTAG
3300.atratusLanz4                   TGACTTCTTCCCCCTTCTTTAACTCTCTTACTAGCTTC-TTCCGCTGTAG
0660.atratusLasGalletasTener        TGACTTCTTCCCCCTTCTTTAACTCTCTTACTAGCTTC-TTCCGCTGTAG
2600.atratusLanzarote3              TGACTTCTTCCCCCTTCTTTAACTCTCTTACTAGCTTC-TTCCGCTGTAG
336Gibbumbjuvenille Lanz4           TGGTTACTTCCCCCTTCTTTAACACTTTTATTAAGCTTCTTCAGCAGTAG
413GibbulasppBasqueCoastline        TGGTTACTTCCCCCTTCTTTAACACTTTTATTA-GCTTCTTCAGCAGTAG
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412GibbulasppBasqueCoastline        TGGCTACTTCCCCCTTCTTTAACACTTTTATTA-GCTTCTTCAGCAGTAG
436G.umbilicalisPortugal6           TGGCTACTTCCCCCTTCTTTAACACTTTTATTA-GCTTCTTCAGCAGTAG
444G.umbilicalisPortugal4           TGGCTACTTCCCCCTTCTTTAACACTTTTATTA-GCTTCTTCAGCAGTAG
245GibbulasppBasqueCoastline        TGGCTACTTCCCCCTTCTTTAACACTTTTATTA-GCTTCTTCAGCAGTAG
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2640.edulisLanzarote3               AAAGGGGAGTTGGAACAGGGT-GAACCGTGTATCCCCCTTTATCGGGAAA
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4690.lineatusPortugal8              AAAGCGGAGTTGGGACAGGAT-GAACTGTATATCCTCCTTTATCGGGAAA
2510.lineatusGreece                 AAAGCGGAGTTGGGACAGGAT-GAACTGTATATCCTCCTTTATCGGGAAA
4140.lineatusBasqueCoastline        AAAGCGGAGTTGGGACAGGAT-GAACTGTATATCCTCCTTTATCGGGAAA
4510.lineatusPortugal3              AAAGCGGAGTTGGGACAGGAT-GAACTGTATATCCTCCTTTATCGGGAAA
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0680.atratusLasGalletasTener        AAAGTGGAGTTGGTACCGGAT-GAACTGTGTATCCCCCTTTATCCGGAAA
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2790.atratusLanz2                   AAAGTGGAGTTGGTACCGGAT-GAACTGTGTATCCCCCTTTATCCGGAAA
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0660.atratusLasGalletasTener        AAAGTGGAGTTGGTACCGGAT-GAACTGTGTATCCCCCTTTATCCGGAAA
2600.atratusLanzarote3              AAAGTGGAGTTGGTACCGGAT-GAACTGTGTATCCCCCTTTATCCGGAAA
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436G.umbilicalisPortugal6           AAAGAGGAGTTGGTACGGGAT-GAACAGTTTACCCTCCGCTTTCAGGTAA
444G.umbilicalisPortugal4           AAAGAGGAGTTGGTACGGGAT-GAACAGTTTACCCTCCGCTTTCAGGTAA
245GibbulasppBasqueCoastline        AAAGAGGAGTTGGTACAGGAT-GAACAGTTTATCCTCCGCTTTCAGGTAA
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2260.edulisBasqueCoastline          CTTAGCTCACGCCGGTGCATCAGTTGACCTA-GCTATTTTTTCTCTCCAC
2640.edulisLanzarote3               CTTAGCTCACGCCGGTGCATCAGTTGACCTA-GCTATTTTTTCTCTCCAC
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770.edulisLosGigantesTener          CTTAGCTCACGCCGGTGCATCAGTTGACCTA-GCTATTTTTTCTCTCCAC
2720.edulisFuerte4                  CTTAGCTCACGCCGGTGCATCAGTTGACCTA-GCTATTTTTTCTCTCCAC
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2630.edulisLanzarote3               CTTAGCTCACGCCGGTGCATCAGTTGACCTA-GCTATTTTTTCTCTCCAC
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0320.edulisImouraneMoroc            CTTAGCTCACGCCGGTGCATCAGTTGACCTA-GCTATTTTTTCTCTCCAC
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4890.lineatusPortugal11             TTTAGCTCACGCCGGTGCCTCTGTTGATCTA-GCTATTTTTTCTCTTCAC
0280.lineatusImouraneMoroc          TTTAGCTCACGCCGGTGCCTCTGTTGATCTA-GCTATTTTTTCTCTTCAC
1390.lineatusPorthguerra            TTTAGCTCACGCCGGTGCCTCTGTTGATCTA-GCTATTTTTTCTCTTCAC
4600.lineatusPortugal7              TTTAGCTCACGCCGGTGCCTCTGTTGATCTA-GCTATTTTTTCTCTTCAC
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4950.lineatusPortugal11             TTTAGCTCACGCCGGTGCCTCTGTTGATCTA-GCTATTTTTTCTCTTCAC
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4690.lineatusPortugal8              TTTAGCTCACGCCGGTGCCTCTGTTGATCTA-GCTATTTTTTCTCTTCAC
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4510.lineatusPortugal3              TTTAGCTCACGCCGGTGCCTCTGTTGATCTA-GCTATTTTTTCTCTTCAC
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0360.lineatusTinitMoroc             TTTAGCTCACGCCGGTGCCTCTGTTGATCTA-GCTATTTTTTCTCTTCAC
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3300.atratusLanz4                   TCTAGCTCACGCCGGTGCGTCCGTTGACCTA-GCCATTTTTTCTCTTCAC
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2600.atratusLanzarote3              TCTAGCTCACGCCGGTGCGTCCGTTGACCTA-GCCATTTTTTCTCTTCAC
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436G.umbilicalisPortugal6           CTTGGCTCATGCAGGTGCTTCTGTTGACTTA-GCTATTTTTTCTTTACAC
444G.umbilicalisPortugal4           TTTGGCTCATGCAGGTGCTTCTGTTGACTTA-GCTATTTTTTCTTTACAC
245GibbulasppBasqueCoastline        CTTGGCTCATGCAGGTGCTTCTGTTGACTTA-GCTATTTTTTCTTTACAC
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0930.articulatusCalahondaMalag      TTGGCTGGTG-TTTCTTCTATTTTAGGGGCTG-CTAATTTTATTACAACA
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2260.edulisBasqueCoastline        TTAGCTGGTG-TTTCATCTATTTTAGGGGCCG-CTAACTTTATCACAACA
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770.edulisLosGigantesTener        TTAGCTGGTG-TTTCATCTATTTTAGGGGCCG-CTAACTTTATCACAACA
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4640.edulisPortugal7              TTAGCTGGTG-TTTCATCTATTTTAGGAGCCG-CTAACTTTATCACAACA
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1970.edulisPontodoSolMadeira      TTAGCTGGTG-TTTCATCTATTTTAGGGGCCG-CTAACTTTATCACAACA
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0280.lineatusImouraneMoroc        TTAGCCGGTG-TCTCTTCTATTTTAGGGGCTG-CTAATTTCATTACAACA
1390.lineatusPorthguerra          TTAGCCGGTG-TCTCTTCTATTTTAGGGGCTG-CTAATTTCATTACAACA
4600.lineatusPortugal7            TTAGCCGGTG-TCTCTTCTATTTTAGGGGCTG-CTAATTTCATTACAACA
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2360.lineatusBasqueCoastline      TTAGCCGGTG-TCTCTTCTATTTTAGGGGCTG-CTAATTTCATTACAACA
4470.lineatusPortugal5            TTAGCCGGTG-TCTCTTCTATTTTAGGGGCTG-CTAATTTCATTACAACA
4950.lineatusPortugal11           TTAGCCGGTG-TCTCTTCTATTTTAGGGGCTG-CTAATTTCATTACAACA
1400.lineatusPorthguerra          TTAGCCGGTG-TCTCTTCTATTTTAGGGGCTG-CTAATTTCATTACAACA
0290.lineatusImouraneMoroc        TTAGCCGGTG-TCTCTTCTATTTTAGGGGCCG-CTAATTTCATTACAACA
4690.lineatusPortugal8            TTAGCCGGTG-TCTCTTCTATTTTAGGGGCCG-CTAATTTCATTACAACA
2510.lineatusGreece               TTAGCCGGTG-TCTCTTCTATTTTAGGGGCTG-CTAATTTCATTACAACA
4140.lineatusBasqueCoastline      TTAGCCGGTG-TCTCTTCTATTTTAGGGGCTG-CTAATTTCATTACAACA
4510.lineatusPortugal3            TTAGCCGGTG-TCTCTTCTATTTTAGGAGCTG-CTAATTTCATTACAACA
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0680.atratusLasGalletasTener      TTAGCTGGTG-TCTCTTCTATTTTAGGGGCTG-CTAACTTCATTACAACG
2770.atratusLanz2                 TTAGCTGGTG-TCTCTTCTATTTTAGGGGCTG-CTAACTTCATTACAACG
2790.atratusLanz2                 TTAGCTGGTG-TCTCTTCTATTTTAGGGGCTG-CTAACTTCATTACAACG
3210.atratusFuerte1               TTAGCTGGTG-TCTCCTCTATCTTAGGGGCTG-CTAACTTCATTACAACG
3300.atratusLanz4                 TTAGCTGGTG-TCTCTTCTATTTTAGGGGCTG-CTAACTTCATTACAACG
0660.atratusLasGalletasTener      TTAGCTGGTG-TCTCTTCTATTTTAGGGGCTG-CTAACTTNATTACAACG
2600.atratusLanzarote3            TTAGCTGGTG-TCTCTTCTATTTTAGGGGCTG-CTAACTTCATTACAACG
336GibbumbjuvenilleLanz4          TTAGCTGGTG-TTTCTTCTATTCTAGGGGCTG-CAAATTTTATTACCACA
413GibbulasppBasqueCoastline      TTAGCTGGTG-TTTCTTCTATTCTAGGGGCTG-CAAATTTTATTACCACA
408GibbulasppBasqueCoastline      TTAGCTGGTG-TTTCTTCTATTCTAGGGGCTG-CAAATTTTATTACCACA
246GibbulasppBasqueCoastline      TTAGCTGGTG-TTTCTTCTATTCTAGGGGCTG-CAAATTTTATTACCACA
412GibbulasppBasqueCoastline      TTAGCTGGTG-TTTCTTCTATTCTAGGGGCTG-CAAATTTTATTACCACA
436G.umbilicalisPortugal6         TTAGCTGGTG-TTTCTTCTATTCTAGGGGCTG-CAAATTTTATTACCACA
444G.umbilicalisPortugal4         TTAGCTGGTG-TTTCTTCTATTTTAGGGGCTG-CAAATTTTATTACCACA
245GibbulasppBasqueCoastline      TTAGCTGGTG-TTTCTTCTATTCTAGGGGCTG-CAAATTTTATTACCACA
                                  ** ** **** * ** *****  **** ** * * ** ** ** **  *

0910.articulatusCalahondaMalag    GTAATTAATATGCGGTGGCAGGGCATGAAATTTGAACGACTACCTTTATT
0930.articulatusCalahondaMalag    GTAATTAATATGCGGTGGCAGGGCATGAAATTTGAACGACTACCTTTATT
3400.edulisFuerte6                GTGATTAATATACGATGACAAGGAATGAAGTTTGAACGACTACCTTTATT
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0640.edulisLaRamblaTener          GTGATTAATATACGATGACAAGGAATGAAGTTTGAACGACTACCTTTATT
2260.edulisBasqueCoastline        GTGATTAATATACGATGACAAGGAATGAAGTTTGAACGACTACCTTTATT
2640.edulisLanzarote3             GTGATTAATATACGATGACAAGGAATGAAGTTTGAACGACTACCTTTATT
3510.edulisGC2                    GTGATTAATATACGATGACAAGGAATGAAGTTTGAACGACTACCTTTATT
770.edulisLosGigantesTener        GTGATTAATATACGATGACAAGGAATGAAGTTTGAACGACTACCTTTATT
2720.edulisFuerte4                GTGATTAATATACGATGACAAGGAATGAAGTTTGAACGACTACCTTTATT
4640.edulisPortugal7              GTGATTAATATACGGTGACAAGGAATGAAGTTTGAACGACTACCTTTATT
3640.edulisGC5                    GTGATTAATATACGATGACAAGGAATGAAGTTTGAACGACTACCTTTATT
2630.edulisLanzarote3             GTGATTAATATACGATGACAAGGAATGAAGTTTGAACGACTACCTTTATT
0700.atratusLasGalletasTener      GTGATTAATATACGATGACAAGGAATGAAGTTTGAACGACTACCTTTATT
0320.edulisImouraneMoroc          GTGATTAATATACGATGACAAGGAATGAAGTTTGAACGACTACCTTTATT
4930.edulisPortugal11             GTGATTAATATACGATGACAAGGAATGAAGTTTGAACGACTACCTTTATT
4740.edulisPortugal8              GTGATTAATATACGATGACAAGGAATGAAGTTTGAACGACTACCTTTATT
0310.edulisImouraneMoroc          GTGATTAATATACGATGACAAGGAATGAAGTTTGAACGACTACCTTTATT
3550.edulisGC3                    GTGATTAATATACGATGACAAGGAATGAAGTTTGAACGACTACCTTTATT
3540.edulisGC3                    GTGATTAACATACGATGACAAGGAATGAAGTTTGAACGACTACCTTTATT
2730.edulisFuerte4                GTGATTAATATACGATGACAAGGAATGAAGTTTGAACGACTACCTTTATT
0530.edulisCapGhirMoroc           GTGATTAATATACGATGACAAGGAATGAAGTTTGAACGACTACCTTTATT
0630.edulisLaRamblaTener          GTGATTAATATACGATGACAAGGAATGAAGTTTGAACGACTACCTTTATT
3490.edulisGC2                    GTGATTAATATACGATGACAAGGAATGAAGTTTGAACGACTACCTTTATT
3390.edulisFuerte6                GTGATTAATATACGATGACAAGGAATGAAGTTTGAACGACTACCTTTATT
3250.edulisLanz4                  GTGATTAATATACGATGACAAGGAATGAAGTTTGAACGACTACCTTTATT
0570.edulisElMedanoTener          GTGATTAATATACGATGACAAGGAATGAAGTTTGAACGACTACCTTTATT
0760.edulisLosGigantesTener       GTGATTAATATACGATGACAAGGAATGAAGTTTGAACGACTACCTTTATT
0420.edulisTinitMoroc             GTGATTAATATACGATGACAAGGAATGAAGTTTGAACGACTACCTTTATT
0540.edulisCapGhirMoroc           GTGATTAATATACGATGACAAGGAATGAAGTTTGAACGACTACCTTTATT
1820.edulisSeixalMadeira          GTGATTAATATACGATGACAAGGAATGAAGTTTGAACGACTACCTTTATT
1830.edulisSaoVincentMadeira      GTGATTAATATACGATGACAAGGAATGAAGTTTGAACGACTACCTTTATT
1920.edulisPrainhaBeachMadeira    GTGATTAATATACGATGACAAGGAATGAAGTTTGAACGACTACCTTTATT
1970.edulisPontodoSolMadeira      GTGATTAATATACGATGACAAGGAATGAAGTTTGAACGACTACCTTTATT
1240.turbinatusCoralBayCyprus     GTAATTAATATACGGTGACAAGGAATAAAATTTGAACGATTACCTTTATT
1200.turbinatusCoralBayCyprus     GTAATTAATATACGGTGACAAGGAATAAAATTTGAACGATTACCTTTATT
2560.turbinatusGreece             GTAATTAATATACGGTGACAAGGAATAAAATTTGAACGATTACCTTTATT
1020.turbinatusPuertoduguesaM     GTAATTAATATACGGTGACAAGGAATAAAATTTGAACGATTGCCTTTATT
1120.turbinatusCalahondaMalag     GTAATTAATATACGGTGACAAGGAATAAAATTTGAACGATTGCCTTTATT
1140.turbinatusCalahondaMalag     GTAATTAATATACGGTGACAAGGAATAAAATTTGAACGATTGCCTTTATT
1330.lineatusLymeRegis            GTAATTAACATACGATGACAAGGAATAAAATTTGAACGACTACCTTTATT
1320.lineatusLymeRegis            GTAATTAACATACGATGACAAGGAATAAAATTTGAACGACTACCTTTATT
4890.lineatusPortugal11           GTAATTAACATACGATGACAAGGAATAAAATTTGAACGACTACCTTTATT
0280.lineatusImouraneMoroc        GTAATTAACATACGATGACAAGGAATAAAATTTGAACGACTACCTTTATT
1390.lineatusPorthguerra          GTAATTAACATACGATGACAAGGAATAAAATTTGAACGACTACCTTTATT
4600.lineatusPortugal7            GTAATTAACATACGATGACAAGGAATAAAATTTGAACGACTACCTTTATT
2350.lineatusBasqueCoastline      GTAATTAACATACGATGACAAGGAATAAAATTTGAACGACTACCTTTATT
2360.lineatusBasqueCoastline      GTAATTAACATACGATGACAAGGAATAAAATTTGAACGACTACCTTTATT
4470.lineatusPortugal5            GTAATTAACATACGATGACAAGGAATAAAATTTGAACGACTACCTTTATT
4950.lineatusPortugal11           GTAATTAACATACGATGACAAGGAATAAAATTTGAACGACTACCTTTATT
1400.lineatusPorthguerra          GTAATTAACATACGATGACAAGGAATAAAATTTGAACGACTACCTTTATT
0290.lineatusImouraneMoroc        GTAATTAACATACGATGACAAGGAATAAAATTTGAACGACTACCTTTATT
4690.lineatusPortugal8            GTAATTAACATACGATGACAAGGAATAAAATTTGAACGACTACCTTTATT
2510.lineatusGreece               GTAATTAACATACGATGACAAGGAATAAAATTTGAACGACTACCTTTATT
4140.lineatusBasqueCoastline      GTAATTAACATACGATGACAAGGAATAAAATTTGAACGACTACCTTTATT
4510.lineatusPortugal3            GTAATTAACATACGATGACAAGGAATAAAATTTGAGCGACTACCTTTATT
4520.lineatusPortugal3            GTAATTAACATACGATGACAAGGAATAAAATTTGAGCGACTACCTTTATT
0360.lineatusTinitMoroc           GTAATTAACATACGATGACAAGGAATAAAATTTGAACGACTACCTTTATT
4900.lineatusPortugal11           GTAATTAACATACGATGACAAGGAATAAAATTTGAACGACTACCTTTATT
0690.atratusLasGalletasTener      GTAATTAACATACGGTGACAAGGAATAAAATTTGAACGACTACCTTTATT
0680.atratusLasGalletasTener      GTAATTAACATACGGTGACAAGGAATAAAATTTGAACGACTACCTTTATT
2770.atratusLanz2                 GTAATTAACATACGGTGACAAGGAATAAAATTTGAACGACTACCTTTATT
2790.atratusLanz2                 GTAATTAACATACGGTGACAAGGAATAAAATTTGAACGACTACCTTTATT
3210.atratusFuerte1               GTAATTAACATACGGTGACAAGGAATAAAATTTGAACGACTACCTTTATT
3300.atratusLanz4                 GTAATTAACATACGGTGACAAGGAATAAAATTTGAACGACTACCTTTATT
0660.atratusLasGalletasTener      GTAATTAACATACGGTGACAAGGAATAAAATTTGAACGACTACCTTTATT
2600.atratusLanzarote3            GTAATTAACATACGGTGACAAGGAATAAAATTTGAACGACTACCTTTATT
336GibbumbjuvenilleLanz4          GTAATTAATATACGATGACAAGGAATGAAATTTGAGCGGTTACCTCTTTT
413GibbulasppBasqueCoastline      GTAATTAATATACGATGACAAGGAATGAAATTTGAGCGGTTACCTCTTTT
408GibbulasppBasqueCoastline      GTAATTAATATACGATGACAAGGAATGAAATTTGAGCGGTTACCTCTTTT
246GibbulasppBasqueCoastline      GTAATTAATATACGATGACAAGGAATGAAATTTGAGCGGTTACCTCTTTT
412GibbulasppBasqueCoastline      GTAATTAATATACGATGACAAGGAATGAAATTTGAGCGGTTACCTCTTTT
436G.umbilicalisPortugal6         GTAATTAATATACGATGACAAGGAATGAAATTTGAGCGGTTACCTCTTTT
444G.umbilicalisPortugal4         GTAATTAATATACGATGACAAGGAATGAAATTTGAGCGGTTACCTCTTTT
245GibbulasppBasqueCoastline      GTAATTAATATACGATGACAAGGAATGAAATTTGAGCGGTTACCTCTTTT
                                  ** ***** ** ** ** ** ** ** ** ***** **  * *** * **

0910.articulatusCalahondaMalag    TGTCTGGTCAGTAAAAATTA--CAGCCATTTTACTTTT-ACTTTC-ACTA
0930.articulatusCalahondaMalag    TGTCTGGTCAGTAAAAATTA--CAGCCATTTTACTTTT-ACTTTC-ACTA
3400.edulisFuerte6                TGTCTGATCAGTAAAAAGTCATCAGCTATTTTACTTCT-ACTTTC-ATTA
0640.edulisLaRamblaTener          TGTCTGATCAGTAAAAATCA--CAGCTATTTTACTTCT-ACTTTC-ATTA
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2260.edulisBasqueCoastline          TGTCTGATCAGTAAAAATCA--CAGCTATTTTACTTCT-ACTTTC-ATTA
2640.edulisLanzarote3               TGTCTGATCAGTAAAAATCA--CAGCTATTTTACTTCT-ACTTTC-ATTA
3510.edulisGC2                      TGTCTGATCAGTAAAAATCA--CAGCCATTTTACTTCT-ACTTTC-ATTA
770.edulisLosGigantesTener          TGTCTGATCAGTAAAAATCA--CAGCTATTTTACTTCT-ACTTTC-ATTA
2720.edulisFuerte4                  TGTCTGATCAGTAAAAATCA--CAGCTATTTTACTTCT-ACTTTC-ATTA
4640.edulisPortugal7                TGTCTGATCAGTAAAAATCA--CAGCTATTTTACTTCT-ACTTTC-ATTA
3640.edulisGC5                      TGTCTGATCAGTAAAAATCA--CAGCTCTTCTACTTCT-ACTTTC-ATTA
2630.edulisLanzarote3               TGTCTGATCAGTAAAAATCA--CAGCTATTCTACTTCT-ACTTTC-ATTA
0700.atratusLasGalletasTener        TGTCTGATCAGTAAAAATCA--CAGCTATTTTACTTCT-ACTTTC-ATTA
0320.edulisImouraneMoroc            TGTCTGATCAGTAAAAATCA--CAGCTATTTTACTTCT-ACTTTC-ATTG
4930.edulisPortugal11               TGTCTGATCAGTAAAAATCA--CAGCTATTTTACTTCT-ACTTTC-ATTA
4740.edulisPortugal8                TGTCTGATCAGTAAAAATCA--CAGCTATTTTACTTCT-ACTTTC-ATTA
0310.edulisImouraneMoroc            TGTCTGATCAGTAAAAATCA--CAGCTATTTTACTTCT-ACTTTC-ATTA
3550.edulisGC3                      TGTCTGATCAGTAAAAATCA--CAGCTATTTTACTTCTCACTTTC-ATTA
3540.edulisGC3                      TGTCTGATCAGTAAAAATCA--CAGCTATTTTACTTCT-ACTTTC-ATTA
2730.edulisFuerte4                  TGTCTGATCAGTAAAAATCA--CAGCTATTTTACTTCT-ACTTTT-ATTA
0530.edulisCapGhirMoroc             TGTCTGATCAGTAAAAATCA--CAGCTATTTTACTTCT-ACTTTC-ATTA
0630.edulisLaRamblaTener            TGTCTGATCAGTAAAAATCA--CAGCTATTTTACTTCT-ACTCTC-ATTA
3490.edulisGC2                      TGTCTGATCAGTAAAAATCA--CAGCTATTTTACTTCT-ACTTTC-ATTA
3390.edulisFuerte6                  TGTCTGATC-GTAAAAATCA--CAGCTATTTTACTTCT-ACTTTC-ATTA
3250.edulisLanz4                    TGTCTGATCAGTAAAAATCA--CAGCTATTTTACTTCT-ACTTTC-ATTA
0570.edulisElMedanoTener            TGTCTGATCAGTAAAAATCA--CAGCTATTTTACTTCT-ACTTTC-ATTA
0760.edulisLosGigantesTener         TGTCTGATCAGTAAAAATCA--CAGCTATTTTACTTCT-ACTTTC-ATTA
0420.edulisTinitMoroc               TGTCTGATCAGTAAAAATCA--CAGCTATTTTACTTCT-ACTTTC-ATTA
0540.edulisCapGhirMoroc             TGTCTGATCAGTAAAAATCA--CAGCTATTTTACTTCT-ACTTTC-ATTA
1820.edulisSeixalMadeira            TGTCTGATCAGTAAAAATCA--CAGCTATTTTACTTCT-GCTTTC-ATTA
1830.edulisSaoVincentMadeira        TGTCTGATCAGTAAAAATCA--CAGCTATTTTACTTCT-ACTTTC-ATTA
1920.edulisPrainhaBeachMadeira      TGTCTGATCAGTAAAAATCA--CAGCTATTTTACTTCT-ACTTTC-ATTA
1970.edulisPontodoSolMadeira        TGTCTGATCAGTAAAAATCA--CAGCTATTTTACTTCT-ACTTTC-ATTA
1240.turbinatusCoralBayCyprus       TGTCTGATCCGTAAAAATCA--CAGCTATTTTGCTTTT-GCTTTC-ATTA
1200.turbinatusCoralBayCyprus       TGTCTGATCCGTAAAAATCA--CAGCTATTTTGCTTTT-GCTTTC-ATTA
2560.turbinatusGreece               TGTCTGATCCGTAAAAATCA--CAGCTATTTTGCTTTT-GCTTTT-ATTA
1020.turbinatusPuertoduguesaM       TGTCTGATCCGTAAAAATCA--CAGCTATTTTGCTTTT-ACTTTC-ATTA
1120.turbinatusCalahondaMalag       TGTCTGATCCGTAAAAATCA--CAGCTATTTTGCTTTT-ACTTTC-ATTA
1140.turbinatusCalahondaMalag       TGTCTGATCCGTAAAAATCA--CAGCTATTTTGCTTTT-ACTTTC-ATTA
1330.lineatusLymeRegis              TGTTTGATCAGTAAAAATCA--CAGCTATCTTACTCCT-ACTTTT-ATTA
1320.lineatusLymeRegis              TGTTTGATCAGTAAAAATCA--CAGCTATCTTACTCCT-ACTTTTCATTA
4890.lineatusPortugal11             TGTTTGATCAGTAAAAATCA--CAGCTATCTTACTCCT-ACTTTC-ATTA
0280.lineatusImouraneMoroc          TGTTTGATCAGTAAAAATCA--CAGCTATCTTACTCCT-ACTTTC-ATTG
1390.lineatusPorthguerra            TGTTTGATCAGTAAAAATCA--CAGCTATCTTACTCCT-ACTTTC-ATTA
4600.lineatusPortugal7              TGTTTGATCAGTAAAAATCA--CAGCTATCTTACTCCT-ACTTTC-ATTA
2350.lineatusBasqueCoastline        TGTTTGATCAGTAAAAATCA--CAGCTATCTTACTCCT-ACTTTC-ATTA
2360.lineatusBasqueCoastline        TGTTTGATCAGTAAAAATCA--CAGCTATCTTACTCCT-ACTTTT-ATTA
4470.lineatusPortugal5              TGTTTGATCAGTAAAAATCA--CAGCTATCTTACTCCT-ACTTTC-ATTA
4950.lineatusPortugal11             TGTTTGATCAGTAAAAATCA--CAGCTATCTTACTCCT-ACTTTC-ATTA
1400.lineatusPorthguerra            TGTTTGATCAGTAAAAATCA--CAGCTATCTTACTCCT-ACTTTC-ATTA
0290.lineatusImouraneMoroc          TGTTTGGTCAGTAAAAATCA--CAGCTATCTTACTCCT-ACTTTC-ATTA
4690.lineatusPortugal8              TGTTTGGTCAGTAAAAATCA--CAGCTATCTTACTCCT-ACTTTC-ATTA
2510.lineatusGreece                 TGTTTGGTCAGTAAAAATCA--CAGCTATCTTACTCCT-ACTTTC-ATTA
4140.lineatusBasqueCoastline        TGTTTGATCAGTAAAAATCA--CAGCTATCTTACTCCT-ACTTTC-ATTA
4510.lineatusPortugal3              TGTTTGATCAGTAAAAATCA--CAGCTATCTTACTCCT-ACTTTC-ATTA
4520.lineatusPortugal3              TGTTTGATCAGTAAAAATCA--CAGCTATCTTACTCCT-ACTTTC-ATTA
0360.lineatusTinitMoroc             TGTTTGATCAGTAAAAATCA--CAGCTATCTTACTCCT-ACTTTC-ATTA
4900.lineatusPortugal11             TGTTTGATCAGTAAAAATCA--CAGCTATCTTACTCCT-ACTTTC-ATTA
0690.atratusLasGalletasTener        TGTTTGGTCAGTAAAGATTA--CAGCTATTCTACTTTT-ACTTTC-ACTG
0680.atratusLasGalletasTener        TGTTTGGTCAGTAAAGATTA--CAGCTATTCTACTTTT-ACTTTC-ACTG
2770.atratusLanz2                   TGTTTGGTCAGTAAAGATTA--CAGCTATTCTACTTTT-ACTTTC-ACTG
2790.atratusLanz2                   TGTTTGGTCAGTAAAGATTA--CAGCTATTCTACTTTT-ACTTTC-ACTG
3210.atratusFuerte1                 TGTTTGGTCAGTAAAGATTA--CAGCTATTCTACTTTT-ACTTTC-ACTG
3300.atratusLanz4                   TGTTTGGTCAGTAAAGATTA--CAGCTATTCCACTTTT-CCTTTC-ACTG
0660.atratusLasGalletasTener        TGTTTGGTCAGTAAAGATTA--CAGCTATTCTACTTTT-ACTTTC-ACTG
2600.atratusLanzarote3              TGTTTGGTCAGTAAAGATTA--CAGCTATTCTACTTTT-ACTTTC-ACTG
336GibbumbjuvenilleLanz4            TGTTTGATCAGTAAAAATTA--CTGCCATCTTGCTTTT-ATTATC-ATTA
413GibbulasppBasqueCoastline        TGTTTGATCAGTAAAAATTA--CTGCCATCTTACTTTT-ATTATC-ATTA
408GibbulasppBasqueCoastline        TGTTTGATCAGTAAAAATTA--CTGCCATCTTACTTTT-ATTATC-ATTA
246GibbulasppBasqueCoastline        TGTTTGATCAGTAAAAATTA--CTGCCATCTTACTTTT-ATTATC-ATTA
412GibbulasppBasqueCoastline        TGTTTGATCAGTAAAAATTA--CTGCCAT-TTACTTTT-ATTATC-ATTA
436G.umbilicalisPortugal6           TGTTTGATCAGTAAAAATTA--CTGCCATCTTACTTTT-ATTATC-ATTA
444G.umbilicalisPortugal4           TGTTTGATCAGTAAAAATTA--CTGCCATCTTACTTTT-ATTATC-ATTA
245GibbulasppBasqueCoastline        TGTTTGATCAGTAAAAATTA--CTGCCATCTTACTTTT-ATTATC-ATTA
                                    *** ** ** ***** *     * **  *    **  *   * *  * *

0910.articulatusCalahondaMalag      CCAG-TTTTAGC-TGGAG-CTATTACTATACTTTTAAC
0930.articulatusCalahondaMalag      CCAG-TTTTAGC-TGGGG-CTATTACTATACTTTTAAC
3400.edulisFuerte6                  CCAG-TTTTAGC-TGGAG-CTATTACCATACTTTTAAC
0640.edulisLaRamblaTener            CCAG-TTTTAGC-TGGAG-CTATTACCATACTTTTAAC
2260.edulisBasqueCoastline          CCAG-TTTTAGC-TGGAG-CTATTACCATACTTTTAAC
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2640.edulisLanzarote3              CCAG-TTTTAGC-TGGAG-CTATTACCATACTTTTAAC
3510.edulisGC2                     CCAG-TTTTAGC-TGGAG-CTATTACCATACTTTTAAC
770.edulisLosGigantesTener         CCAG-TTTTAGC-TGGAG-CTATTACCATACTTTTAAC
2720.edulisFuerte4                 CCAG-TTTTAGC-TGGAG-CTATTACCATACTTTTAAC
4640.edulisPortugal7               CCAG-TTTTAGC-TGGAG-CTATTACCATACTTTTAAC
3640.edulisGC5                     CCAG-TTTTAGC-TGGAG-CTATTACCATACTTTTAAC
2630.edulisLanzarote3              CCAG-TTTTAGC-TGGAG-CTATTACCATACTTTTAAC
0700.atratusLasGalletasTener       CCAG-TTTTAGC-TGGAG-CTATTACCATACTTTTAAC
0320.edulisImouraneMoroc           CCAG-TTTTAGC-TGGAG-CTATTACCATACTTTTAAC
4930.edulisPortugal11              CCAG-TTTTAGC-TGGAG-CTATTACCATACTTTTAAC
4740.edulisPortugal8               CCAG-TTTTAGC-TGGAG-CTATTACCATACTTTTAAC
0310.edulisImouraneMoroc           CCAG-TTTTAGC-TGGAG-CTATTACCATACTTTTAAC
3550.edulisGC3                     CCAG-TTTTAGC-TGGAG-CTATTACCATACTTTTAAC
3540.edulisGC3                     CCAG-TTTTAGCCTGGAG-CTATTACCATACTTTTAAC
2730.edulisFuerte4                 CCAG-TTTTAGC-TGGAG-CTAT-ACCATAGTTTTAAC
0530.edulisCapGhirMoroc            CCAG-TTTTAGC-TGGAG-CTATTACCATACTTTTAAC
0630.edulisLaRamblaTener           CCAG-TTTTAGC-TGGAG-CTATTACCATACTTTTAAC
3490.edulisGC2                     CCAG-TTTTAGC-TGGAG-CTATTACCATACTTTTAAC
3390.edulisFuerte6                 CCAG-TTTTAGC-TGGAG-CTATTACCATACTTTTAAC
3250.edulisLanz4                   CCAG-TTTTAGC-TGGAG-CTATTACCATACTTTTAAC
0570.edulisElMedanoTener           CCAG-TTTTAGC-TGGAG-CTATTACCATACTTTTAAC
0760.edulisLosGigantesTener        CCAG-TTTTAGC-TGGAG-CTATTACCATACTTTTAAC
0420.edulisTinitMoroc              CCAG-TTTTAGC-TGGAG-CTATTACCATACTTTTAAC
0540.edulisCapGhirMoroc            CCAG-TTTTAGC-TGGAG-CTATTACCATACTTTTAAC
1820.edulisSeixalMadeira           CCAG-TTTTAGC-TGGAG-CTATTACCATACTTTTAAC
1830.edulisSaoVincentMadeira       CCAG-TTTTAGC-TGGAG-CTATTACCATACTTTTAAC
1920.edulisPrainhaBeachMadeira     CCAG-TTTTAGC-TGGAG-CTATTACCATACTTTTAAC
1970.edulisPontodoSolMadeira       CCAG-TTTTAGC-TGGAG-CTATTACCATACTTTTAAC
1240.turbinatusCoralBayCyprus      CCAG-TTTTAGC-CGGAG-CCATTACTATGCTTTTAAC
1200.turbinatusCoralBayCyprus      CCAG-TTTTAGC-CGGAG-CCATTACTATGCTTTTAAC
2560.turbinatusGreece              CCAG-TTTTAGC-CGGAG-CCATTACTATGCTTTTAAC
1020.turbinatusPuertoduguesaM      CCAG-TTTTAGC-CGGAG-CCATTACTATGCTTTTAAC
1120.turbinatusCalahondaMalag      CCAG-TTTTAGC-CGGAG-CCATTACTATGCTTTTAAC
1140.turbinatusCalahondaMalag      CCAG-TTTTAGC-CGGAG-CCATTACTATGCTTTTAAC
1330.lineatusLymeRegis             CCAG-TTTTAGC-TGGGG-CTATTACTATGCTTTTAAC
1320.lineatusLymeRegis             CCAG-TTTTAGC-TGGGG-CTATTACTATGCTTTTAAC
4890.lineatusPortugal11            CCAG-TTTTAGC-TGGGG-CTATTACTATGCTTTTAAC
0280.lineatusImouraneMoroc         CCAG-TTTTAGC-TGGGG-CTATTACTATGCTTTTAAC
1390.lineatusPorthguerra           CCAGCTTTTAGC-TGGGG-CTATTACTATGCTTTTAAC
4600.lineatusPortugal7             CCAG-TTTTAGC-TGGGG-CTATTACTATGCTTTTAAC
2350.lineatusBasqueCoastline       CCAG-TTTTAGC-TGGGG-CTATTACTATGCTTTTAAC
2360.lineatusBasqueCoastline       CCAG-TTTTAGC-TGGGG-CTATTACTATGCTTTTAAC
4470.lineatusPortugal5             CCAG-TTTTAGC-TGGGG-CTATTACTATGCTTTTAAC
4950.lineatusPortugal11            CCAG-TTTTAGC-TGGGG-CTATTACTATGCTTTTAAC
1400.lineatusPorthguerra           CCAG-TTTTAGC-TGGGG-CTATTACTATGCTTTTAAC
0290.lineatusImouraneMoroc         CCAG-TTTTAGC-TGGGG-CTATTACTATGCTTTTAAC
4690.lineatusPortugal8             CCAG-TTTTAGC-TGGGG-CTATTACTATGCTTTTAAC
2510.lineatusGreece                CCAG-TTTTAGC-TGGGG-CTATTACTATGCTTTTAAC
4140.lineatusBasqueCoastline       CCAG-TTTTAGC-TGGGG-CTATTACTATGCTTTTAAC
4510.lineatusPortugal3             CCAG-TTTTAGC-TGGGG-CTATTACTATGCTTTTAAC
4520.lineatusPortugal3             CCAG-TTTTAGC-TGGGG-CTATAACTATGCTTTTAAC
0360.lineatusTinitMoroc            CCAG-TTTTAGC-TGGGG-CTATTACTATGCTTTTAAC
4900.lineatusPortugal11            CCAG-TTTTAGC-TGGGG-CTATTACTATGCTTTTAAC
0690.atratusLasGalletasTener       GCAG-TTTTAGC-CGGTG-CTATTACTATACTTTTAAC
0680.atratusLasGalletasTener       CCAG-TTTTAGC-CGGTGGCTATTACTATACTTTTAAC
2770.atratusLanz2                  CCAG-TTTTAGC-CGGTG-CTATTACTATACTTTTAAC
2790.atratusLanz2                  CCAG-TTTTAGC-CGGTG-CTATTACTATACTTTTAAC
3210.atratusFuerte1                CCAG-TTTTAGC-CGGTG-CTATTACTATACTTTTAAC
3300.atratusLanz4                  CCAG-TTTTAGC-CGGTG-CTATTACTATACTTTTATA
0660.atratusLasGalletasTener       CCAG-TTTTAGC-CGGTGGCTATTACTATACTTTTAAC
2600.atratusLanzarote3             CCAG-TTTTAGT-TGGTG-CTATTACTATACTTTTAAC
336GibbumbjuvenilleLanz4           CCAG-TTTTAGC-CGGTG-CCATTACAATACTATTAAC
413GibbulasppBasqueCoastline       CCAG-TTTTAGC-CGGTG-CCATTACAATACTATTAAC
408GibbulasppBasqueCoastline       CCAG-TTTTAGC-CGGTG-CCATTACAATACTATTAAC
246GibbulasppBasqueCoastline       CCAG-TTTTAGC-CGGTG-CCATTACAATACTATTAAC
412GibbulasppBasqueCoastline       CCAG-TTTTAGC-CGGTG-CCATTACAATACTATTAAC
436G.umbilicalisPortugal6          CCAG-TTTTAGC-CGGTG-CCATTACAATACTATTAAC
444G.umbilicalisPortugal4          CCAG-TTTTAGC-CGGTG-CCATTACAATACTATTAAC
245GibbulasppBasqueCoastline       CCAG-TTTTAGC-CGGTG-CCATTACAATACTATTAAC
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Appendix E

The partial 16S mt DNA sequence alignment file for *Gibbula umbilicalis* and *Osilinus* species used in chapter 6.

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001FOlineatusMoroc4                -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
008FOlineatusMoroc4                -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
046FOlineatusCapGhirMoroc          -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
047FOlineatusCapGhirMoroc          -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
005FOlineatusMoroc4                -AAACGGCCGCGGTACCCTAACCCGTGCAAAGGTAGCATAATCATTTGCC
039FOlineatusTifnitMoroc           -AAACGGCCGCGGTACCCTAACCCGTGCAAAGGTAGCATAATCATTTGCC
038FOlineatusTifnitMoroc           -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
024FOunknownImouraneMoroc          -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
040FOlineatusTifnitMoroc           -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
027FOlineatusImouraneMoro          -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
048FOlineatusCapGhirMoroc          AAACGGCCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
028FOlineatusImouraneMoro          AAACGGCCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
036FOlineatusTifnitMoroc           AAACGGCCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
049FOlineatusCapGhirMoroc          AAACGGCCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
037FOlineatusTifnitMoroc           AAACGGCCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
026FOlineatusImouraneMoroc         -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
236fOlineatusBasqueCoastline       -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
029FOlineatusImouraneMoro          -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
045FOedulisTifnitMoroc             -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
139fOlineatusPorthguerra           -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
70FOatratusLasGalletasTener        -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
385fOatratusGC7                    -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
069FOatratusLasGalletasTener       -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
066FOatratusLasGalletasTener       -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
307FOatratusFuerte5                -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
384OatratusGC7                     -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
364fOatratusGC5                    -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
068FOatratusLasGalletasTener       -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
277FOatratusLanz2                  -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
268fOatratusFuerte4                -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
297FOatratusFuerte3                -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
067FOatratusLasGalletasTener       -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
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093FOarticulatusCalahondaMalag     -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
106FOarticulatusTorreCadiz         -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
064FOedulisLaRamblaTener           -AAACGGCCGCGGTACCCTAACCCGTGCAAAGGTAGCATAATCATTTGCC
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302FOedulisFuerte5                 -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
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018FoedulisMoroc4                  -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
295FOedulisLanz1                   -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
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310FOedulis                        -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
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079FOedulisLosGigantesTener        -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
034FOedulisImouraneMoro            -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
294OedulisLanz1                    -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
325FOedulisLanz4                   -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
370fOedulisGC5                     -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
076FOedulisLosGigantesTener        -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
71FOedulisLasGalletasTener         -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
282FOedulisLanz2                   -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
225fOedulisBasqueCoastline         -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
017FoedulisMoroc4                  -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
077FOedulisLosGigantesTener        -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
023FOunknownImouraneMoroc          -AACGGCCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
324FOedulisLanz4                   -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
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226fOedulisBasqueCoastline        -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
285FOedulisLanz2                  -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
196fOedulisPontadoSolMadeira      -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
020FoedulisMoroc4                 -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
065FOedulisLaRamblaTener          -AAACGGCCGCGGTACCCTAACCCGTGCAAAGGTAGCATAATCATTTGCC
182fOedulisSeixalMadeira          -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
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183fOedulisSaoVincentMadeira      -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
191fOedulisPrainhaBeachMadeira    -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
096FOturbinatusTorreCadiz         -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
256fOturbinatusGreece             -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
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Okotschyi202of                    -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
400nfOkotschyi                    -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
401nfOkotschyi                    -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
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406fGibbulasppBasque              -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
374fGibbulasppGC5                 -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
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412fGibbulasppBasque              -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
145fGumbilicalisLymeRegis         -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
131fGumbilicalisPorthguerra       -AAACGGCCGCGGTACCCTAACC-GTGCAAAGGTAGCATAATCATTTGCC
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046FOlineatusCapGhirMoroc         TTTTAATTGGAGGCTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCT
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005FOlineatusMoroc4               TTTTAATTGGAGGCTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCT
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027FOlineatusImouraneMoro         TTTTAATTGGAGGCTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCT
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026FOlineatusImouraneMoroc        TTTTAATTGGAGGCTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCT
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045FOedulisTifnitMoroc            TTTTAATTGGAGGCTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCT
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70FOatratusLasGalletasTener       TTTTAATTGGAGGCTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCT
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307FOatratusFuerte5               TTTTAATTGGAGGCTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCT
384OatratusGC7                    TTTTAATTGGAGGCTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCT
364fOatratusGC5                   TTTTAATTGGAGGCTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCT
068FOatratusLasGalletasTener      TTTTAATTGGAGGCTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCT
277FOatratusLanz2                 TTTTAATTGGAGGCTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCT
268fOatratusFuerte4               TTTTAATTGGAGGCTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCT
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067FOatratusLasGalletasTener      TTTTAATTGGAGGCTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCC
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145fGumbilicalisLymeRegis            TTTTAATTGGAGGCTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCT
131fGumbilicalisPorthguerra          TTTTAATTGGAGGCTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCT
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046FOlineatusCapGhirMoroc            TTCG-AAATATTTAAAAATTAACTTTTAGGTAAAGAGGCCTAAATTCGTT
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005FOlineatusMoroc4                  TTCG-AAATATTTAAAAATTAACTTTTAGGTAAAGAGGCCTAAATTCGTT
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026FOlineatusImouraneMoroc           TTCG-AAATATTTAAAAATTAACTTTTAGGTAAAGAGGCCTAAATTCGTT
236fOlineatusBasqueCoastline         TTCG-AAATATTTAAAAATTAACTTTTAGGTAAAGAGGCCTAAATTCGTT
029FOlineatusImouraneMoro            TTCG-AAATATTTAAAAATTAACTTTTAGGTAAAGAGGCCTAAATTCGTT
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139fOlineatusPorthguerra             TTCG-AAATATTTAAAAATTAACTTTTAGGTAAAGAGGCCTAAATTCGTT
70FOatratusLasGalletasTener          TTCG-AAATATTTAAAAATTAACTTTTAGGTAAAGAGGCCTAAATTTGTT
385fOatratusGC7                      TTCG-AAATATTTAAAAATTAACTTTTAGGTAAAGAGGCCTAAATTTGTT
069FOatratusLasGalletasTener         TTCG-AAATATTTAAAAATTAACTTTTAGGTAAAGAGGCCTAAATTTGTT
066FOatratusLasGalletasTener         TTCG-AAATATTTAAAAATTAACTTTTAGGTAAAGAGGCCTAAATTTGTT
307FOatratusFuerte5                  TTCG-GAATATTTAAAAATTAACTTTTAGGTAAAGAGGCCTAAATTTGTT
384OatratusGC7                       TTCG-AAATATTTAAAAATTAACTTTTAGGTAAAGAGGCCTAAATTTGTT
364fOatratusGC5                      TTCG-AAATATTTAAAAATTAACTTTTAGGTAAAGAGGCCTAAATTTGTT
068FOatratusLasGalletasTener         TTCG-AAATATTTAAAAATTAACTTTTAGGTAAAGAGGCCTAAATTTGTT
277FOatratusLanz2                    TTCG-AAATATTTAAAAATTAACTTTTAGGTAAAGAGGCCTAAATTTGTT
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268fOatratusFuerte4                TTCG-AAATATTTAAAAATTAACTTTTAGGTAAAGAGGCCTAAATTTGTT
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067FOatratusLasGalletasTener       TTCG-AAATATTTAAAAATTAACTTTTAGGTAAAGAGGCCTAAATTTGTT
108FOarticulatusTorreCadiz         TTCG-AAATATTTAAAAATTAACTTTTAGGTAAAGAGGCCTAAATTTATT
109FOarticulatusTorreCadiz         TTCG-AAATATTTAAAAATTAACTTTTAGGTAAAGAGGCCTAAATTTATT
093FOarticulatusCalahondaMalag     TTCG-AAATATTTAAAAATTAACTTTTAGGTAAAGAGGCCTAAATTTATT
106FOarticulatusTorreCadiz         TTTG-AAATATTTAAAAATTAACTTTTAGGTAAAGAGGCCTAAATTTATT
064FOedulisLaRamblaTener           TTTG-GAATATTTAAAAATTAACTTTTAGGTGAAGAGGCCTGAATTTATT
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302FOedulisFuerte5                 TTTG-GAATATTTAAAAATTAACTTTTAGGTGAAGAGGCCTGAATTTATT
360foedulisGC4                     TTTG-GAATATTTAAAAATTAACTTTTAGGTGAAGAGGCCTGAATTTATT
407fOedulisBasque                  TTTG-GAATATTTAAAAATTAACTTTTAGGTGAAGAGGCCTGAATTTATT
061FOedulisLaRamblaTener           TTTG-GAATATTTAAAAATTAACTTTTAGGTGAAGAGGCCTGAATCTATT
346fOedulisGC1                     TTTG-GAATATTTAAAAATTAACTTTTAGGTGAAGAGGCCTGAATTTATT
060FOedulisElMedanoTener           TTTG-GAATATTTAAAAATTAACTTTTAGGTGAAGAGGCCTGAATTTATT
075FOedulisLasGalletasTener        TTTG-GAATATTTAAAAATTAACTTTTAGGTGAAGAGGCCTGAATTTATT
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033FOedulisImouraneMoro            TTTG-GAATATTTAAAAATTAACTTTTAGGTGAAGAGGCCTGAATTTATT
018FoedulisMoroc4                  TTTG-GAATATTTAAAAATTAACTTTTAGGTGAAGAGGCCTGAATTTATT
295FOedulisLanz1                   TTTG-GAATATTTAAAAATTAACTTTTAGGTGAAGAGGCCTGAATTTATT
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073FOedulisLasGalletasTener        TTTG-GAATATTTAAAAATTAACTTTTAGGTGAAGAGGCCTGAATTTATT
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044FOedulisTifnitMoroc             TTTG-GAATATTTAAAAATTAACTTTTAGGTGAAGAGGCCTGAATTTATT
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079FOedulisLosGigantesTener        TTTG-GAATATTTAAAAATTAACTTTTAGGTGAAGAGGCCTGAATTTATT
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294OedulisLanz1                    TTTG-GAATATTTAAAAATTAACTTTTAGGTGAAGAGGCCTGAATTTATT
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370fOedulisGC5                     TTTG-GAATATTTAAAAATTAACTTTTAGGTGAAGAGGCCTGAATTTATT
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71FOedulisLasGalletasTener         TTTG-GAATATTTAAAAATTAACTTTTAGGTGAAGAGGCCTGAATTTATT
282FOedulisLanz2                   TTTG-GAATATTTAAAAATTAACTTTTAGGTGAAGAGGCCTGAATTTATT
225fOedulisBasqueCoastline         TTTG-GAATATTTAAAAATTAACTTTCAGGTGAAGAGGCCTGAATTTATT
017FoedulisMoroc4                  TTTG-GAATATTTAAAAATTAACTTTCAGGTGAAGAGGCCTGAATTTATT
077FOedulisLosGigantesTener        TTTG-GAATATTTAAAAATTAACTTTTAGGTGAAGAGGCCTGAATTTATT
023FOunknownImouraneMoroc          TTTG-GAATATTTAAAAATTAACTTTTAGGTGAAGAGGCCTGAATTTATT
324FOedulisLanz4                   TTTG-GAATATTTAAAAATTAACTTTTAGGTGAAGAGGCCTGAATTTATT
062FOedulisLaRamblaTener           TTTG-GAATATTTAAAAATTAACTTTTAGGTGAAGAGGCCTGAATTTATT
226fOedulisBasqueCoastline         TTTG-GAATATTTAAAAATTAACTTTTAGGTGAAGAGGCCTGAATTTATT
285FOedulisLanz2                   TTTG-GAATATTTAAAAATTAACTTTTAGGTGAAGAGGCCTGAATTTATT
196fOedulisPontadoSolMadeira       TTTG-GAATATTTAAAAATTAACTTTTAGGTGAAGAGGCCTGAATTTATT
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065FOedulisLaRamblaTener           TTTG-GAATATTTAAAAATTAACTTTTAGGTGAAGAGGCCTGAATTTATT
182fOedulisSeixalMadeira           TTTG-GAATATTTAAAAATTAACTTTTAGGTGAAGAGGCCTGAATTTATT
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183fOedulisSaoVincentMadeira       TTTG-GAATATTTAAAAATTAACTTTTAGGTGAAGAGGCCTGAATTTATT
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096FOturbinatusTorreCadiz          TTCG-AAATATTTAAAAATTAACCTTTAGGTGAAGAGGCCTGAATCTATT
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Gumbilicalis128F                   TTTA-AAATATTTAAAAATTAACTTTTAGGTGAAGAGGCCTAAATTTATT
Gumbilicalis129F                   TTTA-AAATATTTAAAAATTAACTTTTAGGTGAAGAGGCCTAAATTTATT
245fGibbulasppBasque               TTTA-AAATATTTAAAAATTAACTTTTAGGTGAAGAGGCCTAAATTTATT
406fGibbulasppBasque               TTTA-AAATATTTAAAAATTAACTTTCAGGTGAAGAGGCCTAAATTTATT
374fGibbulasppGC5                  TTTA-AAATATTTAAAAATTAACTTTTAGGTGAAGAGGCCTAAATTTATT
338FO.edulis?juvLanz4              TTTA-AAATATTTAAAAATTAACTTTTAGGTGAAGAGGCCTAAATTTATT
412fGibbulasppBasque               TTTA-AAATATTTAAAAATTAACTTTTAGGTGAAGAGGCCTAAATTTATT
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131fGumbilicalisPorthguerra        TTTA-AAATATTTAAAAATTAACTTTTAGGTGAAGAGGCCTAAATTTATT
                                   **    ******** ** ***** ** **** **** **** ***   **

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008FOlineatusMoroc4                TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGATCCTATAAATTGATGG
046FOlineatusCapGhirMoroc          TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGATCCTATAAATTGATGG
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005FOlineatusMoroc4                TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGATCCTATAAATTGATGG
039FOlineatusTifnitMoroc           TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGATCCTATAAATTGATGG
038FOlineatusTifnitMoroc           TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGATCCTATAAATTGATGG
024FOunknownImouraneMoroc          TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGATCCTATAAATTGATGG
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040FOlineatusTifnitMoroc	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGATCCTATAAATTGATGG
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048FOlineatusCapGhirMoroc	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGATCCTATAAATTGATGG
028FOlineatusImouraneMoro	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGATCCTATAAATTGATGG
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037FOlineatusTifnitMoroc	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGATCCTATAAATTAATGG
026FOlineatusImouraneMoroc	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGATCCTATAAATTAATGG
236fOlineatusBasqueCoastline	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGATCCTATAAATTGATGG
029FOlineatusImouraneMoro	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGATCCTATAAATTGATGG
045FOedulisTifnitMoroc	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGATCCTATAAATTGATGG
139fOlineatusPorthguerra	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGATTCTATAAATTGATGG
70FOatratusLasGalletasTener	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGCGATCTTGTAAATTTTTGT
385fOatratusGC7	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGCGATCTTGTAAATTTTTGT
069FOatratusLasGalletasTener	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGCGATCTTGTAAATTTTTGT
066FOatratusLasGalletasTener	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGCGATCTTGTAAATTTTTGT
307FOatratusFuerte5	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGCGATCTTGTAAATTTTTGT
384OatratusGC7	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGCGATCTTGTAAATTTTTGT
364fOatratusGC5	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGCGATTTTGTAAATTTTTGT
068FOatratusLasGalletasTener	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGCGATCTTGTAAACTTTTGT
277FOatratusLanz2	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGATCTTGTAAATTTTTGT
268fOatratusFuerte4	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGATCTTGTAAATTTTTGT
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067FOatratusLasGalletasTener	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGCGATCTTGTAAATTTTTGT
108FOarticulatusTorreCadiz	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGATCCTGTAAGTTTACAC
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093FOarticulatusCalahondaMalag	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGATCCTGTAAGTTTACAC
106FOarticulatusTorreCadiz	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGATCCTGTAAGTTTACAG
064FOedulisLaRamblaTener	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGACTTTATAAATTTATAT
369fOedulisGC5	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGACTTTATAAATTTATAT
303FOedulisFuerte5	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGACTTTATAAATTTATAT
013FoedulisMoroc4	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGACTTTATAAATTTATAT
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407fOedulisBasque	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGACTTTATAAATTTATAT
061FOedulisLaRamblaTener	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGACTTTATAAATTTATAT
346fOedulisGC1	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGACTTTATAAATTTATAT
060FOedulisElMedanoTener	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGACTTTATAAATTTATAT
075FOedulisLasGalletasTener	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGACTTTATAAATTTATAT
316FOedulisFuerte1	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGACTTTATAAATTTATAT
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012FoedulisMoroc4	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGACTTTATAAATTTATAT
310FOedulis	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGACTTTATAAATTTATAT
073FOedulisLasGalletasTener	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGACTTTATAAATTTATAT
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034FOedulisImouraneMoro	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGACTTTATAAATTTATAT
294OedulisLanz1	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGACTTTATAAATTTATAT
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370fOedulisGC5	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGACTTTATAAATTTATAT
076FOedulisLosGigantesTener	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGACTTTATAAATTTATAT
71FOedulisLasGalletasTener	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGACTTTATAAATTTATAT
282FOedulisLanz2	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGACTTTATAAATTTATAT
225fOedulisBasqueCoastline	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGACTTTATAAATTTATAT
017FoedulisMoroc4	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGACTTTATAAATTTATAT
077FOedulisLosGigantesTener	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGACTTTATAAATTTATAT
023FOunknownImouraneMoroc	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGACTTTATAAATTTATAT
324FOedulisLanz4	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGACTTTATAAATTTATAT
062FOedulisLaRamblaTener	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGACTTTATAAATTTATAT
226fOedulisBasqueCoastline	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGACTTTATAAATTTATAT
285FOedulisLanz2	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGACTTTATAAATTTATAT
196fOedulisPontadoSolMadeira	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGACTTTATAAATTTATAT
020FoedulisMoroc4	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGACTTTATAAATTTATAT
065FOedulisLaRamblaTener	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGACTTTATAAATTTATAT
182fOedulisSeixalMadeira	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGACTTTATAAATTTATAT
197FOedulisPontaSolMadeira	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGACTTTATAAATTTATAT
183fOedulisSaoVincentMadeira	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGACTTTATAAATTTATAT
191fOedulisPrainhaBeachMadeira	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGACTTTATAAATTTATAT
096FOturbinatusTorreCadiz	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGATTTTATAAATTTACAG
256fOturbinatusGreece	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGATTTTATAAATTTACAG
198ofOkotschyi	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGATTCTGCAAATATATAT
Okotschyi202of	TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGATTCTGCAAATTTATAT

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Gumbilicalis128F                TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGATGTTGTAAACGATGAA
Gumbilicalis129F                TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGATGTTGTAAACGATGAA
245fGibbulasppBasque            TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGATGTTGTAAACGATGAA
406fGibbulasppBasque            TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGATGTTGTAAACGATGAA
374fGibbulasppGC5               TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGATGTTGTAAACGACGAA
338FO.edulis?juvLanz4           TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGATGTTGTAAACGATGGA
412fGibbulasppBasque            TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGATGTTGTAAACGATGAA
145fGumbilicalisLymeRegis       TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTAATGTTGTAAACGATGAA
131fGumbilicalisPorthguerra     TAAGGGACAAGAAGACCCTGTTGAGCTTTAGTGATGTTGTAAACGATGAA
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001FOlineatusMoroc4             GCAATTT--------------ATTGGTTGTTGTGTATTTGTGTATTATAT
008FOlineatusMoroc4             GCAATTT--------------ATTGGTTGTTGTGTATTTGTGTATTATAT
046FOlineatusCapGhirMoroc       GCAACTT--------------ATTGGTTGTTGTGTATTTGTGTATTATAT
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045FOedulisTifnitMoroc          GCGATTT--------------ATTGGTTGTTGTGTATTTGTGTATTATAT
139fOlineatusPorthguerra        GCAATTT--------------ATTGGTTGTTGTGTATTTGTGTATTATAT
70FOatratusLasGalletasTener     GCAGTTT--------------GCT--------TATATTTGTATATTTGTA
385fOatratusGC7                 GCAGTTT--------------GCT--------TATATTTGTATATTTGTA
069FOatratusLasGalletasTener    GCAGTTT--------------GCT--------TATATTTGTATATTTGTA
066FOatratusLasGalletasTener    GCAGTTT--------------GCT--------TATATTTGTATATTTGTA
307FOatratusFuerte5             GCAGTTT--------------GCT--------TATATTTGTATATTTGTA
384OatratusGC7                  GCAGTTT--------------GCT--------TATATTTGTATATTTGTA
364fOatratusGC5                 GCAGTTT--------------GCT--------TATATTTGTATATTTGTA
068FOatratusLasGalletasTener    GCAGTTT--------------GTT--------TATATTTGTATATTTGTA
277FOatratusLanz2               GCAGTTT--------------GCT--------TATATTTGTATATTTGTA
268fOatratusFuerte4             GCAGTTT--------------GCT--------TATATTTGTATATTTGTA
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067FOatratusLasGalletasTener    GTAGTTT--------------GCT--------TATATTTGTATATTTATA
108FOarticulatusTorreCadiz      GCAGCTT--------------AATAACTGGTGTATTTTTGTGTATTTATA
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093FOarticulatusCalahondaMalag  GCAGCTT--------------AATAACTGTTGTATTTTTGTGTATTTATA
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064FOedulisLaRamblaTener        ATAATTT--------------GTTATTGATTATATTTTTGTATATTAATG
369fOedulisGC5                  ATAATTT--------------GTTATTGGTTATATTTTTGTATATTAATG
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061FOedulisLaRamblaTener        ATAATTT--------------GTTATTGATTATATTTTTGTATATTAATG
346fOedulisGC1                  ATAATTT--------------GTTATTGATTATATTTTTGTATATTAATG
060FOedulisElMedanoTener        ATAATTT--------------GTTATTGATTATATTTTTGTATATTAATG
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316FOedulisFuerte1              ATAATTT--------------GTTATTGATTATATTTTTGTATATTAATG
057FOedulisElMedanoTener        ATAATTT--------------GTTATTGATTATATTTTTGTATATTAATG
033FOedulisImouraneMoro         ATAATTT--------------GTTATTGATTATATTTTTGTATATTAATG
018FoedulisMoroc4               ATAATTT--------------GTTATTGATTATATTTTTGTATATTAATG
295FOedulisLanz1                ATAGTTT--------------GTTATTGATTATATTTTTGTATATTAATG
012FoedulisMoroc4               ATAGTTT--------------GTTATTGATTATATTTTTGCATATTAATG
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073FOedulisLasGalletasTener     ATAATTT--------------GTTATTGATTATATTTTTGTATATTAATG
080FOedulisLosGigantesTener     ATAATTT--------------GTTATTGATTATATTTTTGTATATTAATG
044FOedulisTifnitMoroc          ATAATTT--------------GTTATTGATTATATTTTTGTATATTAATG
074FOedulisLasGalletasTener     ATAATTT--------------GTTATTGATTATATTTTTGTATATTAATG
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076FOedulisLosGigantesTener     ATAATTT--------------GTTATTGATTATATTTTTGTATATTAATG
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282FOedulisLanz2                    ATAATTT--------------GTTATTGATTATATTTTTGTATATTAATG
225fOedulisBasqueCoastline          ATAATTT--------------GTTATTGATTATATTTTTGTATATTAATG
017FoedulisMoroc4                   ATAATTT--------------GTTATTGATTATATTTTTGTATATTAATG
077FOedulisLosGigantesTener         ATAATTT--------------GTTATTGATTATATTTTTGTATATTAATG
023FOunknownImouraneMoroc           ATAATTT--------------GTTATTGATTATATTTTTGTATATTAATG
324FOedulisLanz4                    ATAATTT--------------GTTATTGATTATATTTT-GTATATTAATG
062FOedulisLaRamblaTener            ATAATTT--------------GTTATTGATTATATTTTTGTATATTAATG
226fOedulisBasqueCoastline          ATAATTT--------------GTTATTGATTATATTTTTGTATATTAATG
285FOedulisLanz2                    ATAATTT--------------GTTATTGATTATATTTTTGTATATTAATG
196fOedulisPontadoSolMadeira        ATAATTT--------------GTTATTGATTATATTTTTGTATATTAATG
020FoedulisMoroc4                   ATAATTT--------------GTTATTGATTATATTTTTGTATATTAATG
065FOedulisLaRamblaTener            ATAATTT--------------GTTATTGATTATATTTTTGTATATTAATG
182fOedulisSeixalMadeira            ATAATTT--------------GTTATTGATTATATTTTTGTATATTAATG
197FOedulisPontaSolMadeira          ATAATTT--------------GTTATTGATTATATTTTTGTATATTAATG
183fOedulisSaoVincentMadeira        ATAATTT--------------GTTATTGATTATATTTTTGTATATTAATG
191fOedulisPrainhaBeachMadeira      ATAATTT--------------GTTATTGATTATATTTTTGTATATTAATG
096FOturbinatusTorreCadiz           GTAATCTA-------------GTTAATGATTATAGTTCGGTATATTAATA
256fOturbinatusGreece               GTAATCTA-------------GTTAATGATTATAGTTCGGTATATTAATA
198ofOkotschyi                      CTATTTGGCTAAAAATAAGTTAAATAGTTTGCTAAAATTTAATTACTATG
Okotschyi202of                      CTATTTGGCTAAAAATAAGTTAAATAGTTTGCTAAAATTCAATTACTATG
400nfOkotschyi                      CTATTTGGCTAAAAATAAGTTAAATAGTTTGCTAAAATTTAATTACTATG
401nfOkotschyi                      CTATTTGGCTAAAAATAAGTTAAATAGTTTGCTAAAATTTAATTACTATG
Gumbilicalis128F                    GAATAAG---------------GTCATTATTGATTTGTTGTGTATTTAT-
Gumbilicalis129F                    GAATAAG---------------G-CATTATTGATTTGTTGTGTATTTAT-
245fGibbulasppBasque                GAATAAG---------------T-TATTATTGATTTGTTGTGTATTTAT-
406fGibbulasppBasque                GAATAAG---------------T-TATTATTGATTTGTTGTGTATTTAT-
374fGibbulasppGC5                   GAATAAG---------------T-TATTATTGATTTGTTGTGTATTTAT-
338FO.edulis?juvLanz4               GAATAAG---------------T-TATTATTGATTTGTTGTGTATTTAT-
412fGibbulasppBasque                GAATAAG---------------T-TATTATTGATTTGTTGTGTATTTAT-
145fGumbilicalisLymeRegis           GAATAAG---------------T-TATTATTGATTTGTTGTGTATTTAC-
131fGumbilicalisPorthguerra         AAATAAG---------------T-TATTATTGATTTGTTGTGTATTTAT-
                                                                                    *

001FOlineatusMoroc4                 ATAAC-GTAAGTTTAAAGGTAATCTTTAGTTGGGGC-GACTGAGGAACAT
008FOlineatusMoroc4                 ATAACCGTAAGTTTAAAGGTAATCTTTAGTTGGGGC-GACTGAGGAACAT
046FOlineatusCapGhirMoroc           ATAACCGTAAGTTTAAAGGCAATCTTTAGTTGGGGC-GACTGAGGAACAT
047FOlineatusCapGhirMoroc           ATAAC-GTAAGTTTAAAGGCAATCTTTAGTTGGGGCCGACTGAGGAACAT
005FOlineatusMoroc4                 ATAAC-GTAAGTTTAAAGGTAATCTTTAGTTGGGGC-GACTGAGGAACAT
039FOlineatusTifnitMoroc            ATAAC-GTAAGTTTAAAGGTAATCTTTAGTTGGGGC-GACTGAGGAACAT
038FOlineatusTifnitMoroc            ATAAC-GTAAGTTTAAAGGTAATCTTTAGTTGGGGC-GACTGAGGAACAT
024FOunknownImouraneMoroc           ATAAC-GTAAGTTTAAAGGTAATCTTTAGTTGGGGC-GACTGAGGAACAT
040FOlineatusTifnitMoroc            ATAAC-GTAAGTTTAAAGGTAATCTTTAGTTGGGGC-GACTGAGGAACAT
027FOlineatusImouraneMoro           ATAAC-GTAAGTTTAAAGGTAATCTTTAGTTGGGGC-GACTGAGGAACAT
048FOlineatusCapGhirMoroc           ATAAC-GTAAGTTTAAAGGCAATCTTTAGTTGGGGC-GACTGAGGAACAT
028FOlineatusImouraneMoro           ATAAC-GTAAGTTTAAAGGTAATCTTTAGTTGGGGC-GACTGAGGAACAT
036FOlineatusTifnitMoroc            ATAAC-GTAAGTTTAAAGGTAATCTTTAGTTGGGGC-GACTGAGGAACAT
049FOlineatusCapGhirMoroc           ATAAC-GTAAGTTTAAAGGTAATCTTTAGTTGGGGC-GACTGAGGAACAT
037FOlineatusTifnitMoroc            ATAAC-GTAAGTTTAAAGGTAATCTTTAGTTGGGGC-GACTGAGGAACAT
026FOlineatusImouraneMoroc          ATAAC-GTAAGTTTAAAGGTAATCTTTAGTTGGGGC-GACTGAGGAACAT
236fOlineatusBasqueCoastline        ATAAC-GTAAGTTTAAAGGTAATCTTTAGTTGGGGC-GACTGAGGAACAT
029FOlineatusImouraneMoro           ATAAC-GTAAGTTTAAAGGTAATCTTTAGTTGGGGC-GACTGAGGAACAT
045FOedulisTifnitMoroc              ATAAC-GTAAGTTTAAAGGTAATCTTTAGTTGGGGC-GACTGAGGAACAT
139fOlineatusPorthguerra            ATAAC-GTAAGTTTAAAGGTAATCTTTAGTTGGGGC-GACTGAGGAACAT
70FOatratusLasGalletasTener         TATAACATAAGTTTATAGGTAATTTTTAGTTGGGGC-GACTGAGGAACAT
385fOatratusGC7                     TATAACATAAGTTTATAGGTAATTTTTAGTTGGGGC-GACTGAGGAACAT
069FOatratusLasGalletasTener        TATAACATAAGTTTATAGGTAATCTTTAGTTGGGGC-GACTGAGGAACAT
066FOatratusLasGalletasTener        TATAACATAAGTTTATAGGTAATCTTTAGTTGGGGC-GACTGAGGAACAT
307FOatratusFuerte5                 TATAACATAAGTTTATAGGTAATCTTTAGTTGGGGC-GACTGAGGAACAT
384OatratusGC7                      TATAACATAAGTTTATAGGTAATCTTTAGTTGGGGC-GACTGAGGAACAT
364fOatratusGC5                     TATAACATAAGTTTATAGGTAATCTTTAGTTGGGGC-GACTGAGGAACAT
068FOatratusLasGalletasTener        TATAACATAAGTTTATAGGTAATCTTTAGTTGGGGC-GACTGAGGAACAT
277FOatratusLanz2                   TATAACATAAGTTTATAGGTAATCTTTAGTTGGGGC-GACTGAGGAACAT
268fOatratusFuerte4                 TATAACATAAGTTTATAGGTAATCTTTAGTTGGGGC-GACTGAGGAACAT
297FOatratusFuerte3                 TATAACATAAGTTTATAGGTAATCTTTAGTTGGGGC-GACTGAGGAACAT
067FOatratusLasGalletasTener        TATAACATAAGTTTATAGGTAATCTTTAGTTGGGGC-GACTGAGGAACAT
108FOarticulatusTorreCadiz          TATAACATAAATTTGTAGGTAATCTTTAGTTGGGGC-GACTGAGGAACAT
109FOarticulatusTorreCadiz          TATAACATAAATTTGTAGGTAATCTTTAGTTGGGGC-GACTGAGGAACAT
093FOarticulatusCalahondaMalag      TATAACATAAGTTTGTAGGTAATCTTTAGTTGGGGC-GACTAAGGAACAT
106FOarticulatusTorreCadiz          TATAACATAAGTTTGTAGGTAATCTTTAGTTGGGGC-GACTGAGGAACAT
064FOedulisLaRamblaTener            TATAACTTAGGTTTATGAATAATCTTTAGTTGGGGC-GACTGAGGAACAT
369fOedulisGC5                      TATAACTTAGGTTTATGAATAATCTTTAGTTGGGGC-GACTGAGGAACAT
303FOedulisFuerte5                  TATAACTTAGGTTTATGAATAATCTTTAGTTGGGGC-GACTGAGGAACAT
013FoedulisMoroc4                   TATAACTTAGGTTTATGAATAATCTTTAGTTGGGGC-GACTGAGGAACAT
302FOedulisFuerte5                  TATAACTTAGGTTTATGAATAATCTTTAGTTGGGGC-GACTGAGGAACAT
360foedulisGC4                      TATAACTTAGGTTTATAAATAATCTTTAGTTGGGGC-GACTGAGGAACAT
407fOedulisBasque                   TATAACTTAGGTTTATGAATAATCTTTAGTTGGGGC-GACTGAGGAACAT
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061FOedulisLaRamblaTener          TATAACTTAGGTTTATGAATAATCTTTAGTTGGGGC-GACTGAGGAACAT
346fOedulisGC1                    TATAACTTAGGTTTATGAATAATCTTTAGTTGGGGC-GACTGAGGAACAT
060FOedulisElMedanoTener          TATAACTTAGGTTTATGAATAATCTTTAGTTGGGGC-GACTGAGGAACAT
075FOedulisLasGalletasTener       TATAACTTAGGTTTATGAATAATCTTTAGTTGGGGC-GACTGAGGAACAT
316FOedulisFuertel                TATAACTTAGGTTTATGAATAATCTTTAGTTGGGGC-GACTGAGGAACAT
057FOedulisElMedanoTener          TATAACTTAGGTTTATGAATAATCTTTAGTTGGGGC-GACTGAGGAACAT
033FOedulisImouraneMoro           TATAACTTAGGTTTATGAATAATCTTTAGTTGGGGC-GACTGAGGAACAT
018FoedulisMoroc4                 TATAACTTAGGTTTATGAATAATCTTTAGTTGGGGC-GACTGAGGAACAT
295FOedulisLanz1                  TATAACTTAGGTTTATGAATAATCTTTAGTTGGGGC-GACTGAGGAACAT
012FoedulisMoroc4                 TATAACTTAGGTTTATGAATAATCTTTAGTTGGGGC-GACTGAGGAACAT
310FOedulis                       TATAACTTAGGTTTATGAATAATCTTTAGTTGGGGC-GACTGAGGAACAT
073FOedulisLasGalletasTener       TATAACTTAGGTTTATGAATAATCTTTAGTTGGGGC-GACTGAGGAACAT
080FOedulisLosGigantesTener       TATAACTTAGGTTTATGAATAATCTTTAGTTGGGGC-GACTGAGGAACAT
044FOedulisTifnitMoroc            TATAACTTAGGTTTATGAATAATCTTTAGTTGGGGC-GACTGAGGAACAT
074FOedulisLasGalletasTener       TATAACTTAGGTTTATGAATAATCTTTAGTTGGGGC-GACTGAGGAACAT
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294OedulisLanz1                   TATAACTTAGGTTTATGAATAATCTTTAGTTGGGGC-GACTGAGGAACAT
325FOedulisLanz4                  TATAACTTAGGTTTATGAATAATCTTTAGTTGGGGC-GACTGAGGAACAT
370fOedulisGC5                    TATAACTTAGGTTTATGAATAATCTTTAGTTGGGGC-GACTGAGGAACAT
076FOedulisLosGigantesTener       TATAACTTAGGTTTATGAATAATCTTTAGTTGGGGC-GACTGAGGAACAT
71FOedulisLasGalletasTener        TATAACTTAGGTTTATGAATAATCTTTAGTTGGGGC-GACTGAGGAACAT
282FOedulisLanz2                  TATAACTTAGGTTTATGAATAATCTTTAGTTGGGGC-GACTGAGGAACAT
225fOedulisBasqueCoastline        TATAACTTAGGTTTATGAATAATCTTTAGTTGGGGC-GACTGAGGAACAT
017FoedulisMoroc4                 TATAACTTAGGTTTATGAATAATCTTTAGTTGGGGC-GACTGAGGAACAT
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023FOunknownImouraneMoroc         TATAACTTAGGTTTATGAATAATCTTTAGTTGGGGC-GACTGAGGAACAT
324FOedulisLanz4                  TATAACTTAGGTTTATGAATAATCTTTAGTTGGGGC-GACTGAGGAACAT
062FOedulisLaRamblaTener          TATAACTTAGGTTTATGAATAATCTTTAGTTGGGGC-GACTGAGGAACAT
226fOedulisBasqueCoastline        TATAACTTAGGTTTATGAATAATCTTTAGTTGGGGC-GACTGAGGAACAT
285FOedulisLanz2                  TATAACTTAGGTTTATGAATAATCTTTAGTTGGGGC-GACTGAGGAACAT
196fOedulisPontadoSolMadeira      TATAACTTAGGTTTATGAATAATCTTTAGTTGGGGC-GACTGAGGAACAT
020FoedulisMoroc4                 TATAACTTAGGTTTATGAATAATCTTTAGTTGGGGC-GACTGAGGAACAT
065FOedulisLaRamblaTener          TATAACTTAGGTTTATGAATAATCTTTAGTTGGGGC-GACTGAGGAACAT
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191fOedulisPrainhaBeachMadeira    TATAACTTAGGTTTATGAATAATCTTTAGTTGGGGC-GACTGAGGAACAT
096FOturbinatusTorreCadiz         TATAACATAGGTTTATAGGTAATCTTTAGTTGGGGC-GACTGAGGAACAC
256fOturbinatusGreece             TATAACATAGGTTTATAGGTAATCTTTAGTTGGGGC-GACTGAGGAACAC
198ofOkotschyi                    TATATTGATTATTTGTAGGGAATCTTTAGTTGGGGC-GACTAAGGAACAT
Okotschyi202of                    TATATTGATTATTTGTAGTGAATCTTTAGTTGGGGC-GACTAAGGAACAT
400nfOkotschyi                    TATATTGATTATTTGTAGGGAATCTTTAGTTGGGGC-GACTAAGGAACAT
401nfOkotschyi                    TATATTGATTATTTGTAGGGAATCTTTAGTTGGGGC-GACTAAGGAACAT
Gumbilicalis128F                  TATAATAAAAGTTTGCAGATAATCTTTAGTTGGGGC-GACTGAGGAACAT
Gumbilicalis129F                  CATAATAAAAGTTTGCAGATAATCTTTAGTTGGGGC-GACTGAGGAACAT
245fGibbulasppBasque              TATAATAAAAGTTTGCAGATAATCTTTAGTTGGGGC-GACTGAGGAACAT
406fGibbulasppBasque              TATAATAAAAGTTTGCAGATAATCTTTAGTTGGGGC-GACTGAGGAACAT
374fGibbulasppGC5                 TATAATAAAAGTTTGCAGATAATCTTTAGTTGGGGC-GACTGAGGAACAT
338FO.edulis?juvLanz4             TATAATAAAAGTTTGCAGATAATCTTTAGTTGGGGC-GACTGAGGAACAT
412fGibbulasppBasque              TATAATAAAAGTTTGCAGATAATCTTTAGTTGGGGC-GACTGAGGAACAT
145fGumbilicalisLymeRegis         TATAATAAAAGTTTGCAGATAATCTTTAGTTGGGGC-GACTGAGGAACAT
131fGumbilicalisPorthguerra       TATAATAAAAGTTTGCAGATAATCTTTAGTTGGGGC-GACTGAGGAACAT
                                     *       ***      *** ************ **** *******

001FOlineatusMoroc4               GGAAAGCTTGCTTGAATTTAAAAGCATGTTGGGC-AATTAATTTGTTGAT
008FOlineatusMoroc4               GGAAAGCTTCCTTGAATTTAAAAGCATGTTGGGC-AATTAATTTGTTGAT
046FOlineatusCapGhirMoroc         GGAAAGCTTCCTTGAATTTAAAAGCATGTTGGGC-AATTAATTTGTTGAT
047FOlineatusCapGhirMoroc         GGAAAGCTTCCTTGAATTTAAAAGCATGTTGGGC-AATTAATTTGTTGAT
005FOlineatusMoroc4               GGAAAGCTTCCTTGAATTTAAAAGCATGTTGGGC-AATTAATTCGTTGAT
039FOlineatusTifnitMoroc          GGAAAGCTTCCTTGAATTTAAAAGCATGTTGGGC-AATTAATTTGTTGAT
038FOlineatusTifnitMoroc          GGAAAGCTTCCTTGAATTTAAAAGCATGTTGGGC-AATTAATTTGTTGAT
024FOunknownImouraneMoroc         GGAAAGCTTCCTTGAATTTAAAAGCATGTTGGGC-AATTAATTTGTTGAT
040FOlineatusTifnitMoroc          GGAAAGCTTCCTTGAATTTAAAAGCATGTTGGGC-AATTAATTTGTTGAT
027FOlineatusImouraneMoro         GGAAAGCTTCCTTGAATTTAAAAGCATGTTGGGC-AATTAATTTGTTGAT
048FOlineatusCapGhirMoroc         GGAAAGCTTCCTTGAATTTAAAAGCATGTTGGGC-AATTAATTTGTTGAT
028FOlineatusImouraneMoro         GGAAAGCTTCCTTGAATTTAAAAGCATGTTGGGC-AATTAATTTGTTGAT
036FOlineatusTifnitMoroc          GGAAAGCTTCCTTGAATTTAAAAGCATGTTGGGC-AATTAATTTGTTGAT
049FOlineatusCapGhirMoroc         GGAAAGCTTCCTTGAATTTAAAAGCATGTTGGGC-AATTAATTTGTTGAT
037FOlineatusTifnitMoroc          GGAAAGCTTCCTTGAATTTAAAAGCATGTTGGGC-AATTAATTTGTTGAT
026FOlineatusImouraneMoroc        GGAAAGCTTCCTTGAATTTAAAAGCATGTTGGGC-AATTAATTTGTTGAT
236fOlineatusBasqueCoastline      GAAAAGCTTCCTTGAATTTAAAAGCATGTTGGGC-AATTAATTTGTTGAT
029FOlineatusImouraneMoro         GGAAAGCTTCCTTGAATTTAAAAGCATGTTGGGC-AATTGATTTGTTGAT
045FOedulisTifnitMoroc            GGAAAGCTTCCTTGAGTTTAAAAGCATGTTGGGC-AATTAATTTGTTGAT
139fOlineatusPorthguerra          GGAAAGCTTCCTTGAATTTAAAAGCATGTTGGGC-AATTAATTTGTTGAT
70FOatratusLasGalletasTener       GAAAAGCTTCCTTGAACTTAAGAGTGTGTTAAAC-AATTAGTTTATTGAT
385fOatratusGC7                   GAAAAGCTTCCTTGAACTTAAGAGTGTGTTAAAC-AATTAGTTTATTGAT
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066FOatratusLasGalletasTener        GAAAAGCTTCCTTGAACTTAAGAGTGTGTTAAAC-AATTAGTTTATTGAT
307FOatratusFuerte5                 GAAAAGCTTCCTTGAACTTAAGAGTGTGTTAAAC-AATTAGTTTATTGAT
384OatratusGC7                      GAAAAGCTTCCTTGAACTTAAGAGTGTGTTAAAC-AGTTAGTTTATTGAT
364fOatratusGC5                     GAAAAGCTTCCTTGAACTTAAGAGTGTGTTAAAC-AATTAGTTTATTGAT
068FOatratusLasGalletasTener        GAAAAGCTTCCTTGAACTTAAGAGTGTGTTAAAC-AATTAGTTTATTGAT
277FOatratusLanz2                   GAAAAGCTTCCTTGAACTTAAGAGTGTGTTAAAC-AATTAGTTTATTGAT
268fOatratusFuerte4                 GAAAAGCTTCCTTGAACTTAAGAGTGTGTTAAAC-AATTAGTTTATTGAT
297FOatratusFuerte3                 GAAAAGCTTCCTTGAACTTAAGAGTGTGTTAAAC-AATTAGTTTATTGAT
067FOatratusLasGalletasTener        GAAAAGCTTCCTTGAACTTAAGAGTGTGTTAAAC-AATTAGTTTATTGAT
108FOarticulatusTorreCadiz          TAAAAGCTTCCTTGAATTTAGTAATAAGTTAAATTATTTAATTTTTTGAT
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093FOarticulatusCalahondaMalag      TAAAAGCTTCCTTGAATTTAGTAATAAGTTAAATTATTTAATTTTTTGAT
106FOarticulatusTorreCadiz          TAAAAGCTTCCTTGAATTTAGTAATAAGTTAAATTATTTAATTTTTTGAT
064FOedulisLaRamblaTener            ACAAAGCTTCCTTGAGTTCGAAGGCGTGTTGGCCTGGCTGATTTATTGAT
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303FOedulisFuerte5                  ACAAAGCTTCCTTGAGTTCGAAGGCGTATTGGCCTGGCTGATTTATTGAT
013FoedulisMoroc4                   ACAAAGCTTCCTTGAGTTCGAAGGCGTGTTGGCCTGGCTGATTTATTGAT
302FOedulisFuerte5                  ACAAAGCTTCCTTGAGTTCGAAGGCGTGTTGGCCTGGCTGATTTATTGAT
360foedulisGC4                      ACAAAGCTTCCTTGAGTTCGAAGGCGTGTTGGCCTGGCTGATTTATTGAT
407fOedulisBasque                   ACAAAGCTTCCTTGAGTTCGAAGGCGTGTTGGCCTGGCTGATTTATTGAT
061FOedulisLaRamblaTener            ACAAAGCTTCCTTGAGTTCGAAGGCGTGTTGGCCTGGCTGATTTATTGAT
346fOedulisGC1                      ACAAAGCTTCCTTGAGTTCGAAGGCGTGTTGGCCTGGCTGATTTATTGAT
060FOedulisElMedanoTener            ACAAAGCTTCCTTGAGTTCGAAGGCGTGTTGGCCTGGCTGATTTATTGAT
075FOedulisLasGalletasTener         ACAAAGCTTCCTTGAGTTCGAAGGCGTGTTGGCCTGGCTGATTTATTGAT
316FOedulisFuerte1                  ACAAAGCTTCCTTGAGTTCGAAGGCGTGTTGGCCTGGCTGATTTATTGAT
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033FOedulisImouraneMoro             ACAAAGCTTCCTTGAGTTCGAAGGCGTGTTGGCCCGGCTGATTTATTGAT
018FoedulisMoroc4                   ACAAAGCTTCCTTGAGTTCGAAGGCGTGTTGGCCTGGCTGATTTATTGAT
295FOedulisLanz1                    ATAAAGCTTCCTTGAGTTCGAAGGCGTGTTGGCCTGGCTGATTTATTGAT
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073FOedulisLasGalletasTener         ATAAAGCTTCCTTGAGTTCTAAGGCGTGTTGGCCTGGCTGATTTATTGAT
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282FOedulisLanz2                    ACAAAGCTTCCTTGAGTTCTAAGGCGTGTTGGCCTGGCTGATTTATTGAT
225fOedulisBasqueCoastline          ACAAAGCTTCCTTGAGTTCTAAGGCGTGTTGGCCTGGCTGATTTATTGAT
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023FOunknownImouraneMoroc           ACAAAGCTTCCTTGAGTTCTAAGGCGTGTTGGCCTGGCTGATTTATTGAT
324FOedulisLanz4                    ACAAAGCTTCCTTGAGTTCTAAGGCGTGTTGGCCTGGCTGATTTATTGAT
062FOedulisLaRamblaTener            ACAAAGCTTCCTTGAGTTCTAAGGCGTGTTGGCCTGGCTGATTTATTGAT
226fOedulisBasqueCoastline          ACAAAGCTTCCTTGAGTTCAAAGGCGTGTTGGCCTGGCTGATTTATTGAT
285FOedulisLanz2                    ACAAAGCTTCCTTGAGTTAGAAGGCGTGTTGGCCTGGCTGATTTATTGAT
196fOedulisPontadoSolMadeira        ACAAAGCTTCCTTGAGTTTGAAGGCGTGTTGGCCTGGCTGATTTATTGAT
020FoedulisMoroc4                   ACAAAGCTTCCTTGAGTTTGAAGGCGTGTTGGCCTGGCTGATTTATTGAT
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183fOedulisSaoVincentMadeira        ACAAAGCTTCCTTGAGTTTGAAGGCGTGTTGGCCTGGTTGATTTATTGAT
191fOedulisPrainhaBeachMadeira      ACAAAGCTTCCTTGAGTTTGAAGGCGTGTTGGCCTGGTTGATTTATTGAT
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256fOturbinatusGreece               ATAAAGCTTCCTTGAATTTAAGAGCGTGTTAAGCAATTTAGTTTACTGAT
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Okotschyi202of                      ATAAAGCTTCCTTTAATGAGTTGGTTGATTTAGT-AACTAGTATTTTGAA
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401nfOkotschyi                      ATAAAGCTTCCTTTAATGAGTTGGTTGATTTAGT-AACTAGTATTTTGA-
Gumbilicalis128F                    AAAAAGCTTCCTTGTATATAAA---TTATTTAAGTAATTGAATAAATTAA
Gumbilicalis129F                    ACAAAGCTTCCCTGTATATAAA---TTATTTAAGTAATTGAATAAATTAA
245fGibbulasppBasque                AAAAAGCTTCCTTGTATATAAA---TTATTTAAGTAATTGAATAAATTAA
406fGibbulasppBasque                AAAAAGCTTCCTTGTATATAAA---TTATTTAAGTAATTGAATAAATTAA
374fGibbulasppGC5                   AAAAAGCTTCCTTGTATATAAA---TTATTTAAGTAATTGAATAAATTAA
338FO.edulis?juvLanz4               AAAAAGCTTCCTTGTATATAAA---TTATTTAAGTAATTGAATAAATTAA
412fGibbulasppBasque                AAAAAGCTTCCTTGTATATAAA---CTATTTAAGTAATTGAATAAATTAA
145fGumbilicalisLymeRegis           AAAAAGCTTCCTTGTATATAAA---TTATTTAAGTAATTGAATAAATTAA
131fGumbilicalisPorthguerra         AAAAAGCTTCCTTGTATATAAA---TTATTTAAGTAATTGAATAAATTAA
                                      ** **** * *              **        *       * *

001FOlineatusMoroc4                 GCATTCTGCGTTAATTTTGATCCAATCTATTTTGATTAAGAGAAAAAGTT
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008FOlineatusMoroc4                GCATT-TGCGTTAATTTTGATCCAATTTATTTTGATTAAGAGAAAAAGTT
046FOlineatusCapGhirMoroc          GCATT-TGCGTTAATTTTGATCCAATTTATTTTGATTAAGAGAAAAAGTT
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005FOlineatusMoroc4                GCATT-TGCGTTAATTTTGATCCAATTTATTTTGATTAAGAGAAAAAGTT
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268fOatratusFuerte4                  GGG-TTTGCGACCTCG-ATGTTGGACTAA
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407fOedulisBasque                 GGG-TTTGCGACCTCG-ATGTTGGACTAA
061FOedulisLaRamblaTener          GGG-TTTGCGACCTCG-ATGTTGGACTAA
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075FOedulisLasGalletasTener       GGG-TTTGCGACCTCG-ATGTTGGACTAA
316FOedulisFuerte1                GGG-TTTGCGACCTCC-ATGTTGGACTAA
057FOedulisElMedanoTener          GGG-TTTGCGACCTC--ATGTTGGACTAA
033FOedulisImouraneMoro           GGG-TTTGCGACCTCG-ATGTTGGACTAA
018FoedulisMoroc4                 GGG-TTTGCGACCTCG-ATGTTGGACTAA
295FOedulisLanz1                  -GGGTTTGCGACCTCG-ATGTTGGACTAA
012FoedulisMoroc4                 AGGGTTTGCGACCTCG-ATGTTGGACTAA
310FOedulis                       -GGGTTTGCGACCTCG-ATGTTGGACTAA
073FOedulisLasGalletasTener       -GGGTTTGCGACCTCG-ATGTTGGACTAA
080FOedulisLosGigantesTener       -GGGTTTGCGACCTCC-ATGTTGGACTAA
044FOedulisTifnitMoroc            -GGGTTTGCGACCTCG-ATGTTGGACTAA
074FOedulisLasGalletasTener       GGGTTTGCCGACCTCG-ATGTTGGACTAA
079FOedulisLosGigantesTener       GGGGTTGCC-ACCTCG-ATGTTGGACTAA
034FOedulisImouraneMoro           GGGTTTGCG-ACCTCG-ATGTTGGACTAA
294OedulisLanz1                   GGGTTTGCG-ACCTCG-ATGTTGGACTAA
325FOedulisLanz4                  GGGTTTGCG-ACCTCG-ATGTTGGACTAA
370fOedulisGC5                    GGG-TTTGCGACCTCG-ATGTTGGACTAA
076FOedulisLosGigantesTener       GGGTTTGCGACCTCGG-ATGTTGGACTAA
71FOedulisLasGalletasTener        GGG-TTTGCGACCTCG-ATGTTGGACTAA
282FOedulisLanz2                  GGGTTTGCG-ACCTCG-ATGTTGGACTAA
225fOedulisBasqueCoastline        GGGTTTGCG-ACCTCG-ATGTTGGACTAA
017FoedulisMoroc4                 GGGTTTGCG-ACCTCG-ATGTTGGACTAA
077FOedulisLosGigantesTener       GGGTTTGCG-ACCTCG-ATGTTGGACTAA
023FOunknownImouraneMoroc         GGGTTTGCG-ACCTCG-ATGTTGGACTAA
324FOedulisLanz4                  GGGTTTGCG-ACCTCG-ATGTTGGACTAA
062FOedulisLaRamblaTener          GGGTTTGCG--ACCTC-ATGTTGGACTAA
226fOedulisBasqueCoastline        GGGTTTGCG-ACCTCG-ATGTTGGACTAA
285FOedulisLanz2                  GGG-TTTGCGACCTCG-ATGTTGGACTAA
196fOedulisPontadoSolMadeira      GGG-TTTGCGACCTCG-ATGTTGGACTAA
020FoedulisMoroc4                 GGG-TTTGCGACCTCG-ATGTTGGACTAA
065FOedulisLaRamblaTener          GGG-TTTGCGACCTCG-ATGTTGGACTAA
182fOedulisSeixalMadeira          AGG-TTTGCGACCTCG-ATGTTGGACTAA
197FOedulisPontaSolMadeira        GGG-TTTGCGACCTCG-ATGTTGGACTAA
183fOedulisSaoVincentMadeira      GGG-TTTGCGACCTCG-ATGTTGGACTAA
191fOedulisPrainhaBeachMadeira    GGG-TTTGCGACCTCG-ATGTTGGACTAA
096FOturbinatusTorreCadiz         GGG-TTTGCGACCTCG-ATGTTGGACTAA
256fOturbinatusGreece             GGG-TTTGCGACCTCG-ATGTTGGACTAA
198ofOkotschyi                    GGG-TTTGCGACCTCG-ATGTTGGACTAA
Okotschyi202of                    GGG-TTTGCGACCTCG-ATGTTGGACTAA
400nfOkotschyi                    GGG-TTTGCGACCTCG-ATGTTGGACTAA
401nfOkotschyi                    GGG-TTTGCGACCTCG-ATGTTGGACTAA
Gumbilicalis128F                  GGG-TTTGCGACCTCG-ATGTTGGACTAA
Gumbilicalis129F                  GGG-TTTGCGACCTCG-ATGTTGGACTAA
245fGibbulasppBasque              GGG-TTTGCGACCTCG-ATGTTGGACTAA
406fGibbulasppBasque              GGG-TTTGCGACCTCG-ATGTTGGACTAA
374fGibbulasppGC5                 GGG-TTTGCGACCTCG-ATGTTGGACTAA
338FO.edulis?juvLanz4             GGG-TTTGCGACCTCG-ATGTTGGACTAA
412fGibbulasppBasque              GGG-TTTGCGACCTCG-ATGTTGGACTAA
145fGumbilicalisLymeRegis         GGG-TTTGCGACCTCG-ATGTTGGACTAA
131fGumbilicalisPorthguerra       GGG-TTTGCGACCTCG-ATGTTGGACTAA
                                   ** **           ************
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Appendix F

The partial 16S and COI mt DNA sequence alignment file for *Gibbula umbilicalis* and *Osilinus* species used in chapter 6.

```
133FOlineatusUK                    TTAATTCGTGCTGAGTTAGGACAACCCGGTGCTCTTCTCGGAGATGACCA
47FOlineatusCapGhirMorocco         TTAATTCGTGCTGAGTTAGGACAACCCGGTGCTCTTCTCGGAGATGACCA
235FOlineatusBasque                TTAATTCGTGCTGAGTTAGGACAACCCGGTGCTCTTCTCGGAGATGACCA
139FOlineatusUK                    TTAATTCGTGCTGAGTTAGGACAACCCGGTGCTCTTCTCGGAGATGACCA
251FOlineatusGreece                TTAATTCGTGCTGAGTTAGGACAACCCGGTGCTCTTCTCGGAGATGACCA
29FOlineatusImouraneMorocco        TTAATTCGTGCTGAGTTAGGACAACCCGGTGCTCTTCTCGGAGATGACCA
37FOlineatusTifnitMorocco          TTAATTCGTGCTGAGTTAGGACAACCCGGTGCTCTTCTCGGAGATGACCA
321FoatratusFuerte1                TTAATTCGTGCTGAGCTAGGGCAACCTGGTGCTCTTCTTGGAGATGACCA
69FOatratusLasGalletasTenerife     TTAATTCGTGCTGAGCTAGGGCAACCTGGTGCTCTTCTTGGAGATGACCA
277FoatratusLanz2                  TTAATTCGTGCTGAGCTAGGGCAACCTGGTGCTCTTCTTGGAGATGACCA
31FOedulisImouraneMorocco          TTAATTCGTGCTGAGCTAGGACAACCTGGTGCTCTTTTAGGAGACGACCA
354FoedulisGC3                     TTAATTCGTGCTGAGCTAGGACAACCTGGTGCTCTTTTAGGAGACGACCA
264FoedulisLanz3                   TTAATTCGTGCTGAGCTAGGACAACCTGGTGCTCTTTTAGGAGACGACCA
76FOedulisLosGigantesTenerife      TTAATTCGTGCTGAGCTAGGACAACCTGGTGCTCTTTTAGGAGACGACCA
182FOedulisMadeira                 TTAATTCGTGCTGAGCTAGGGCAACCTGGTGCTCTTTTAGGAGACGACCA
54FOedulisCapGhirMorocco           TTAATTCGTGCTGAGCTAGGGCAACCTGGTGCTCTTTTAGGAGACGACCA
183FOedulisMadeira                 TTAATTCGTCCTGAGCTAGGGCAACCTGGTGCTCTTTTAGGAGACGACCA
192FOedulisMadeira                 TTAATTCGTGCTGAGCTAGGGCAACCTGGTGCTCTTTTAGGAGACGACCA
102FOturbinatusMalaga              TTAATTCGTGCTGAACTAGGACAACCCGGCGCTCTTCTCGGGGATGACCA
112FOturbinatusMalaga              TTAATTCGTGCTGAACAAGGACAACCCGGCGCTCTTCTCGGGGATGACCA
256FOturbinatusGreece              TTAATTCCTGCAGAACTAAGACAACCCATCGCTCTTCTCGGGGATGACCA
246FGibbulasppBasque               CTAATTCGTGCTGAATTAGGACAGCCAGGAGCTCTCCTCGGCGATGACCA
338FoedjuvLanz4                    CTAATTCGTGCTGAATTAGGACAGCCAGGAGCTCTCCTCGGCGATGACCA
93FOarticulatusMalaga              TTGATTCGTGCTGAGCTGGGGCAACCCGGTGCTCTTCTTGGAGACGACCA
                                    * **** * * **     * ** **    *****  * ** ** *****

133FOlineatusUK                    ACTATACAATGTAATTGTTACAGCCCACGCTTTTGTAATAATTTTCTTTT
47FOlineatusCapGhirMorocco         ACTATACAATGTAATTGTTACAGCCCACGCTTTTGTAATAATTTTCTTTT
235FOlineatusBasque                ACTATACAATGTAATTGTTACAGCTCACGCTTTTGTAATAATTTTCTTTT
139FOlineatusUK                    ACTATACAATGTAATTGTTACAGCTCACGCTTTTGTAATAATTTTCTTTT
251FOlineatusGreece                ACTATACAATGTAATTGTTACAGCCCACGCTTTTGTAATAATTTTCTTTT
29FOlineatusImouraneMorocco        ACTATACAATGTAATTGTTACAGCCCACGCTTTTGTAATAATTTTCTTTT
37FOlineatusTifnitMorocco          ACTATACAATGTAATTGTTACAGCCCACGCTTTTGTAATAATTTTCTTTT
321FoatratusFuerte1                ACTATATAATGTAATTGTTACAGCCCACGCTTTTGTAATGATTTTCTTTT
69FOatratusLasGalletasTenerife     ACTATATAATGTAATTGTTACAGCCCACGCTTTTGTAATGATTTTCTTTT
277FoatratusLanz2                  ACTATATAATGTAATTGTTACAGCCCACGCTTTTGTAATGATTTTCTTTT
31FOedulisImouraneMorocco          ACTATATAATGTAATTGTTACAGCTCATGCGTTCGTAATAATTTTCTTTT
354FoedulisGC3                     ACTATATAATGTAATTGTTACAGCTCATGCGTTCGTAATAATTTTCTTTT
264FoedulisLanz3                   ACTATATAACGTAATTGTTACAGCTCATGCGTTCGTAATAATTTTCTTTT
76FOedulisLosGigantesTenerife      ACTATATAATGTAATTGTTACAGCTCATGCGTTCGTAATAATTTTCTTTT
182FOedulisMadeira                 ACTATATAATGTAATTGTTACAGCTCATGCGTTCGTAATAATTTTCTTTC
54FOedulisCapGhirMorocco           ACTATATAATGTAATTGTTACAGCTCATGCGTTCGTAATAATTTTCTTTC
183FOedulisMadeira                 ACTATATAATGTAATTGTTACAGCTCATGCGTTCGTAATAATTTTCTTTC
192FOedulisMadeira                 ACTATATAATGTAATTGTTACAGCTCATGCGTTCGTAATAATTTTCTTTC
102FOturbinatusMalaga              ATTATATAATGTAATTGTTACAGCTCACGCATTCGTAATAATTTTCTTTT
112FOturbinatusMalaga              ATTATATAATGTAATTGTTACAGCTCACGCATTCGTAATAATTTTCTTTT
256FOturbinatusGreece              ATTATATAATGTAATTGTTACAGCTCACGCATTCGTAATAATTTTCTTTT
246FGibbulasppBasque               ACTATATAATGTAATTGTAACAGCTCATGCTTTTGTAATAATTTTCTTTT
338FoedjuvLanz4                    ACTATATAATGTAATTGTAACAGCTCATGCTTTTGTAATAATTTTCTTTT
93FOarticulatusMalaga              ACTATATAATGTAATTGTTACAGCTCACGCTTTTGTAATAATTTTTTTCT
                                   * **** ** ******** ***** ** ** ** ***** ***** **

133FOlineatusUK                    TAGTAATACCTTTAATGATTGGTGGGTTCGGTAACTGACTAATCCCTTTA
47FOlineatusCapGhirMorocco         TAGTAATACCTTTAATGATTGGTGGGTTCGGTAACTGACTAATCCCTTTA
235FOlineatusBasque                TAGTAATACCTTTAATGATTGGTGGGTTCGGTAACTGACTAATCCCTTTA
139FOlineatusUK                    TAGTAATACCTTTAATGATTGGTGGGTTCGGTAACTGACTAATCCCTTTA
251FOlineatusGreece                TAGTAATACCCTTAATGATTGGTGGATTCGGTAACTGACTAATCCCTTTA
29FOlineatusImouraneMorocco        TAGTAATACCCTTAATGATTGGTGGATTCGGTAACTGACTAATCCCTTTA
37FOlineatusTifnitMorocco          TAGTAATACCTTTAATGATTGGTGGATTCGGTAACTGACTAATCCCTTTA
321FoatratusFuerte1                TAGTGATACCTTTAATAATTGGTGGGTTCGGTAACTGGCTAATTCCCCTA
69FOatratusLasGalletasTenerife     TAGTGATACCTTTAATAATTGGTGGGTTCGGTAACTGGCTAATTCCCCTA
277FoatratusLanz2                  TAGTGATACCTTTAATAATTGGTGGGTTCGGTAACTGGCTAATTCCCCTA
31FOedulisImouraneMorocco          TAGTAATGCCTTTAATAATTGGTGGATTTGGTAATTGATTAATTCCCCTA
354FoedulisGC3                     TAGTAATGCCTTTAATAATTGGTGGATTTGGTAATTGATTAATTCCCCTA
264FoedulisLanz3                   TAGTAATGCCTTTAATAATTGGTGGATTTGGTAATTGATTAATTCCCCTA
76FOedulisLosGigantesTenerife      TAGTAATGCCTTTAATAATTGGTGGATTTGGTAATTGATTAATTCCCCTA
182FOedulisMadeira                 TAGTAATGCCTTTAATAATTGGTGGATTTGGTAATTGATTAATTCCCCTA
54FOedulisCapGhirMorocco           TAGTAATGCCTTTAATAATTGGTGGATTTGGTAATTGATTAATTCCCCTA
183FOedulisMadeira                 TAGTAATGCCTTTAATAATTGGTGGATTTGGTAACTGATTAATTCCCCTA
192FOedulisMadeira                 TAGTAATGCCTTTAATAATTGGTGGATTTGGTAACTGATTAATTCCCCTA
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102FOturbinatusMalaga              TAGTGATACCTTTAATAATTGGTGGATTTGGTAACTGATTAATTCCTTTA
112FOturbinatusMalaga              TAGTTATACCTTTAATAATTGGTGGATTTGGTAACTGATTAATTCCTTTA
256FOturbinatusGreece              TAGTGATACCTTTAATAATTGGTGGATTTGGTAACTGATTAATTCCTTTA
246FGibbulasppBasque               TAGTTATACCCCTAATAATTGGAGGATTTGGTAACTGACTAATTCCTTTA
338FoedjuvLanz4                    TAGTTATACCCCTAATAATTGGAGGATTTGGTAACTGACTAATTCCTTTA
93FOarticulatusMalaga              TAGTTATACCTCTGATAATTGGCGGATTTGGTAACTGATTAATTCCCCTG
                                   **** ** **  * ** ***** ** ** ***** **  **** **  *

133FOlineatusUK                    ATACTAGGGGCACCCGATATAGCGTTCCCCCGACTAAATAATATAAGTTT
47FOlineatusCapGhirMorocco         ATACTAGGGGCACCCGATATAGCGTTCCCCCGACTAAATAATATAAGTTT
235FOlineatusBasque                ATACTAGGGGCACCCGATATAGCGTTCCCCCGACTAAATAATATAAGTTT
139FOlineatusUK                    ATACTAGGGGCACCCGATATAGCGTTCCCCCGACTAAATAATATAAGTTT
251FOlineatusGreece                ATACTAGGGGCACCCGATATAGCGTTTCCCCGACTAAATAATATAAGTTT
29FOlineatusImouraneMorocco        ATACTAGGGGCACCCGATATAGCGTTTCCCCGACTAAATAATATAAGTTT
37FOlineatusTifnitMorocco          ATACTAGGGGCACCCGATATAGCGTTCCCCCGACTAAATAATATAAGTTT
321FoatratusFuerte1                ATACTAGGGGCACCTGATATAGCGTTTCCGCGACTTAATAATATAAGTTT
69FOatratusLasGalletasTenerife     ATACTAGGGGCACCTGATATAGCGTTTCCGCGACTTAATAATATAAGTTT
277FoatratusLanz2                  ATACTAGGGGCACCTGATATAGCGTTTCCGCGACTTAATAATATAAGTTT
31FOedulisImouraneMorocco          ATACTAGGAGCACCTGATATAGCATTCCCTCGACTTAATAATATAAGTTT
354FoedulisGC3                     ATACTAGGAGCACCTGATATAGCATTCCCTCGACTTAATAATATAAGTTT
264FoedulisLanz3                   ATACTAGGAGCACCTGATATAGCATTCCCTCGACTTAATAATATAAGTTT
76FOedulisLosGigantesTenerife      ATACTAGGAGCACCTGATATAGCATTCCCTCGACTTAATAACATAAGTTT
182FOedulisMadeira                 ATACTAGGAGCACCCGATATGGCATTCCCTCGACTTAATAATATAAGTTT
54FOedulisCapGhirMorocco           ATACTAGGAGCACCCGATATGGCATTCCCTCGACTTAATAATATAAGTTT
183FOedulisMadeira                 ATACTAGGAGCACCTGATATGGCATTCCCTCGACTTAATAATATAAGTTT
192FOedulisMadeira                 ATACTAGGAGCACCTGATATGGCATTCCCTCGACTTAATAATATAAGTTT
102FOturbinatusMalaga              ATACTAGGAGCACCAGATATAGCATTTCCCCGACTTAATAATATAAGTTT
112FOturbinatusMalaga              ATACTAGGAGCACCAGATATAGCATTTCCCCGACTTAATAATATAAGTTT
256FOturbinatusGreece              ATACTAGGAGCACCAGATATAGCATTTCCCCGACTTAATAATATAAGTTT
246FGibbulasppBasque               ATGCTAGGAGCACCTGATATAGCTTTCCCTCGACTTAATAACATAAGTTT
338FoedjuvLanz4                    ATGCTAGGAGCACCTGATATAGCTTTCCCTCGACTTAATAACATAAGTTT
93FOarticulatusMalaga              ATGCTAGGAGCGCCCGACATGGCGTTTCCGCGACTAAACAATATAAGTTT
                                   ** ***** ** ** ** ** ** ** ** ***** ** ** ********

133FOlineatusUK                    CTGACTTCTCCCTCCTTCTTTAACTCTTTTATTAGCCTC-TTCTGCCGTA
47FOlineatusCapGhirMorocco         CTGACTTCTCCCTCCTTCTTTAACCCTTTTATTAGCCTC-TTCTGCCGTA
235FOlineatusBasque                CTGACTTCTCCCTCCTTCTTTAACCCTTTTATTAGCCTC-TTCTGCCGTA
139FOlineatusUK                    CTGACTTCTCCCTCCTTCTTTAACCCTTTTATTAGCCTC-TTCTGCCGTA
251FOlineatusGreece                CTGACTTCTCCCTCCTTCTTTAACCCTTTTATTAGCCTC-TTCTGCCGTA
29FOlineatusImouraneMorocco        CTGACTTCTCCCTCCTTCTTTAACCCTTTTATTAGCCTC-TTCTGCCGTA
37FOlineatusTifnitMorocco          CTGACTTCTCCCTCCTTCTTTAACCCTTTTATTAGCCTC-TTCTGCCGTA
321FoatratusFuerte1                CTGACTTCTTCCCCCTTCTTTAACTCTCTTACTAGCTTCCTTCCGCTGTA
69FOatratusLasGalletasTenerife     CTGACTTCTTCCCCCTTCTTTAACTCTCTTACTAGCTTC-TTCCGCTGTA
277FoatratusLanz2                  CTGACTTCTTCCCCCTTCTTTAACTCTCTTACTAGCTTC-TTCCGCTGTA
31FOedulisImouraneMorocco          CTGACTTCTACCTCCTTCTTTAACCCTTCTATTAGCATC-TTCCGCTGTA
354FoedulisGC3                     CTGACTTCTACCTCCTTCTTTAACCCTTCTATTAGCATC-TTCCGCTGTA
264FoedulisLanz3                   CTGACTTCTACCTCCTTCCTTAACCCTTCTATTAGCATC-TTCCGCTGTA
76FOedulisLosGigantesTenerife      CTGACTTCTACCTCCTTCTTTAACCCTTCTATTAGCATC-TTCCGCTGTA
182FOedulisMadeira                 CTGACTTCTACCTCCTTCTTTAACCCTTCTATTAGCATC-TTCCGCTGTA
54FOedulisCapGhirMorocco           CTGACTTCTACCTCCTTCTTTAACCCTTTTATTAGCATC-TTCCGCTGTA
183FOedulisMadeira                 CTGACTTCTACCTCCTTCTTTAACCCTTCTATTAGCATC-TTCCGCTGTA
192FOedulisMadeira                 CTGACTTCTACCTCCTTCTTTAACCCTTCTATTAGCATC-TTCCGCTGTA
102FOturbinatusMalaga              CTGGCTTCTTCCTCCTTCTTTAACTCTTTTATTAGCGTC-TTCCGCTGTA
112FOturbinatusMalaga              CTGGCTTCTTCCTCCTTCTTTAACTCTTTTATTAGCCTC-TTCCGCTGTA
256FOturbinatusGreece              CTGGCTTCTTCCTCCTTCTTTAACTCTTTTATTAGCGTC-TTCCGCTGTA
246FGibbulasppBasque               TTGGCTACTTCCCCCTTCTTTAACACTTTTATTAGCTTC-TTCAGCAGTA
338FoedjuvLanz4                    TTGGCTACTTCCCCCTTCTTTAACACTTTTATTAGCTTC-TTCAGCAGTA
93FOarticulatusMalaga              CTGGCTCCTCCCTCCGTCTTTGACCCTTTTATTAGCCTC-TTCTGCTGTA
                                    ** ** ** ** ** ** ** ** **  ** **** ** *** ** ***

133FOlineatusUK                    GAAAGCGGAGTTGGGACAGGATGAACTGTATATCCTCCTTTATCGGGAAA
47FOlineatusCapGhirMorocco         GAAAGCGGAGTTGGGACAGGATGAACTGTATATCCTCCTTTATCGGGAAA
235FOlineatusBasque                GAAAGCGGAGTCGGGACAGGATGAACTGTATATCCTCCTTTATCGGGAAA
139FOlineatusUK                    GAAAGCGGAGTTGGGACAGGATGAACTGTATATCCTCCTTTATCGGGAAA
251FOlineatusGreece                GAAAGCGGAGTTGGGACAGGATGAACTGTATATCCTCCTTTATCGGGAAA
29FOlineatusImouraneMorocco        GAAAGCGGAGTTGGGACAGGATGAACTGTATATCCTCCTTTATCGGGAAA
37FOlineatusTifnitMorocco          GAAAGCGGAGTTGGGACAGGATGAACTGTATATCCCCCTTTATCGGGAAA
321FoatratusFuerte1                GAAAGTGGAGTTGGTACCGGATGAACTGTGTATCCCCCTTTATCCGGAAA
69FOatratusLasGalletasTenerife     GAAAGTGGAGTTGGTACCGGATGAACTGTGTATCCCCCTTTATCCGGAAA
277FoatratusLanz2                  GAAAGTGGAGTTGGTACCGGATGAACTGTGTATCCCCCTTTATCCGGAAA
31FOedulisImouraneMorocco          GAAAGGGGAGTTGGAACAGGGTGAACTGTGTATCCCCCTTTATCGGGAAA
354FoedulisGC3                     GAAAGGGGAGTTGGAACAGGGTGAACTGTGTATCCCCCTTTATCGGGAAA
264FoedulisLanz3                   GAAAGGGGAGTTGGAACAGGGTGAACCGTGTATCCCCCTTTATCGGGAAA
76FOedulisLosGigantesTenerife      GAAAGAGGAGTTGGAACAGGATGAACCGTGTATCCCCCTTTATCGGGAAA
182FOedulisMadeira                 GAAAGGGGAGTTGGAACAGGGTGAACCGTGTATCCCCCTTTATCGGGAAA
54FOedulisCapGhirMorocco           GAAAGGGGAGTTGGAACAGGGTGAACCGTGTATCCCCCCTTTATCGGGAAA
183FOedulisMadeira                 GAAAGGGGAGTTGGAACAGGATGAACCGTGTATCCCCCTTTATCGGGAAA
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192FOedulisMadeira                GAAAGGGGAGTTGGAACAGGATGAACCGTGTATCCCCCTTTATCGGGAAA
102FOturbinatusMalaga             GAAAGAGGAGTGGGAACAGGGTGAACTGTATACCCCCCTTTATCAGGAAA
112FOturbinatusMalaga             GAGAGAGGAGTAGGAACAGGGTGAACTGTATACCCCCCTTTATCAGGAAA
256FOturbinatusGreece             GAGAGAGGAGTGGGGACAGGGTGAACTGTATACCCCCCTTTATCAGGAAA
246FGibbulasppBasque              GAAAGAGGAGTTGGTACGGGATGAACAGTTTACCCTCCGCTTTCAGGTAA
338FoedjuvLanz4                   GAAAGAGGAGCTGGTACGGGATGAACAGTTTACCCTCCGCTTTCAGGTAA
93FOarticulatusMalaga             GAAAGCGGAGTAGGAACAGGATGAACTGTTTATCCCCCTTTGTCTGGGAA
                                  ** ** ****  ** ** ** ***** ** ** ** **  * ** ** **

133FOlineatusUK                   TTTAGCTCACGCCGGTGCCTCTGTTGATCTAGCTATTTTTTCTCTTCACT
47FOlineatusCapGhirMorocco        TTTAGCTCACGCCGGTGCCTCTGTTGATCTAGCTATTTTTTCTCTTCACT
235FOlineatusBasque               TTTAGCTCACGCCGGTGCCTCTGTTGATCTAGCTATTTTTTCTCTTCACT
139FOlineatusUK                   TTTAGCTCACGCCGGTGCCTCTGTTGATCTAGCTATTTTTTCTCTTCACT
251FOlineatusGreece               TTTAGCTCACGCCGGTGCCTCTGTTGATCTAGCTATTTTTTCTCTTCACT
29FOlineatusImouraneMorocco       TTTAGCTCACGCCGGTGCCTCTGTTGATCTAGCTATTTTTTCCCTTCACT
37FOlineatusTifnitMorocco         TTTAGCTCACGCCGGTGCCTCTGTTGATCTAGCTATTTTTTCTCTTCACT
321FoatratusFuerte1               TCTAGCTCACGCCGGTGCGTCCGTTGACCTAGCCATTTTTTCTCTTCACT
69FOatratusLasGalletasTenerife    TCTAGCTCACGCCGGTGCGTCCGTTGACCTAGCCATTTTTTCTCTTCACT
277FoatratusLanz2                 TCTAGCTCACGCCGGTGCGTCCGTTGACCTAGCCATTTTTTCTCTTCACT
31FOedulisImouraneMorocco         CTTAGCTCACGCCGGTGCATCAGTTGACCTAGCTATTTTTTCTCTCCACT
354FoedulisGC3                    CTTAGCTCACGCCGGTGCATCAGTTGACCTAGCTATTTTTTCTCTCCACT
264FoedulisLanz3                  CTTAGCTCACGCCGGTGCATCAGTTGACCTAGCTATTTTTTCTCTCCACT
76FOedulisLosGigantesTenerife     CTTAGCTCACGCCGGTGCATCAGTTGACCTAGCTATTTTTTCTCTCCACT
182FOedulisMadeira                CTTAGCTCACGCCGGTGCATCAGTTGACCTAGCTATTTTTTCTCTCCACT
54FOedulisCapGhirMorocco          CTTAGCTCACGCCGGTGCATCAGTTGACCTAGCTATTTTTTCTCTCCACT
183FOedulisMadeira                CTTAGCTCACGCCGGTGCATCAGTTGACCTAGCTATTTTTTCTCTCCACT
192FOedulisMadeira                CTTAGCTCACGCCGGTGCATCAGTTGACCTAGCTATTTTTTCTCTCCACT
102FOturbinatusMalaga             CCTGGCTCACGCCGGTGCATCAGTTGACTTGGCTATTTTTTCTCTTCACT
112FOturbinatusMalaga             CCTGGCTCACGCCGGTGCATCAGTTGACTTGGCTATTTTTTCTCTTCACT
256FOturbinatusGreece             CCTGGCTCACGCCGGTGCATCAGTTGACTTGGCTATTTTTTCTCTTCACT
246FGibbulasppBasque              CTTGGCTCATGCAGGTGCTTCTGTTGACTTAGCTATTTTTTCTTTACACT
338FoedjuvLanz4                   CTTGGCTCATGCAGGTGCTTCTGTTGACTTAGCTATTTTTTCTTTACACT
93FOarticulatusMalaga             TTTAGCTCACGCCGGTGCTTCTGTTGATTTGGCTATTTTCTCTCTTCACT
                                    * ***** ** ***** ** *****  * ** ***** **  * ****

133FOlineatusUK                   TAGCCGGTGTCTCTTCTATTTTAGGGGCGGCTAATTTCATTACAACAGTA
47FOlineatusCapGhirMorocco        TAGCCGGTGTCTCTTCTATTTTAGGGGCTGCTAATTTCATTACAACAGTA
235FOlineatusBasque               TAGCCGGTGTCTCTTCTATTTTAGGGGCTGCTAATTTCATTACAACAGTA
139FOlineatusUK                   TAGCCGGTGTCTCTTCTATTTTAGGG-CTGCTAATTTCATTACAACAGTA
251FOlineatusGreece               TAGCCGGTGTCTCTTCTATTTTAGGGGCTGCTAATTTCATTACAACAGTA
29FOlineatusImouraneMorocco       TAGCCGGTGTCTCTTCTATTTTAGGGGCCGCTAATTTCATTACAACAGTA
37FOlineatusTifnitMorocco         TAGCCGGTGTCTCTTCTATTTTAGGGGCTGCTAATTTTATTACAACAGTA
321FoatratusFuerte1               TAGCTGGTGTCTCCTCTATCTTAGGGGCTGCTAACTTCATTACAACGGTA
69FOatratusLasGalletasTenerife    TAGCTGGTGTCTCTTCTATTTTAGGGGCTGCTAACTTCATTACAACGGTA
277FoatratusLanz2                 TAGCTGGTGTCTCTTCTATTTTAGGGGCTGCTAACTTCATTACAACGGTA
31FOedulisImouraneMorocco         TAGCTGGTGTTTCATCTATTTTAGGGGCCGCTAACTTTATCACAACAGTG
354FoedulisGC3                    TAGCTGGTGTTTCATCTATTTTAGGGGCCGCTAACTTTATCACAACAGTG
264FoedulisLanz3                  TAGCTGGTGTTTCATCTATTTTAGGGGCCGCTAACTTTATCACAACAGTG
76FOedulisLosGigantesTenerife     TAGCTGGTGTTTCATCTATTTTAGGGGCCGCTAACTTTATCACAACAGTG
182FOedulisMadeira                TAGCTGGTGTTTCATCTATTTTAGGAGCCGCTAACTTTATCACAACAGTG
54FOedulisCapGhirMorocco          TAGCTGGTGTTTCATCTATTTTAGGGGCCGCTAACTTTATCACAACAGTG
183FOedulisMadeira                TAGCTGGTGTTTCATCTATTTTAGGGGCCGCTAACTTTATCACAACAGTG
192FOedulisMadeira                TAGCTGGTGTTTCATCTATTTTAGGGGCCGCTAACTTTATCACAACAGTG
102FOturbinatusMalaga             TAGCCGGTGTTTCTTCTATTTTAGGGGCTGCTAATTTCATTACAACAGTA
112FOturbinatusMalaga             TAGCCGGTGTTTCTTCTATTTTAGGGGCTGCTAATTTCATTACAACAGTA
256FOturbinatusGreece             TAGCCGGTGTTTCTTCTATTTTAGGAGCTGCTAATTTCATTACAACAGTA
246FGibbulasppBasque              TAGCTGGTGTTTCTTCTATTCTAGGGGCTGCAAATTTTATTACCACAGTA
338FoedjuvLanz4                   TAGCTGGTGTTTCTTCTATTCTAGGGGCTGCAAATTTTATTACCACAGTA
93FOarticulatusMalaga             TGGCTGGTGTTTCTTCTATTTTAGGGGCTGCTAATTTTATTACAACAGTA
                                  * ** ***** ** *****  ****  * ** ** ** ** ** ** **

133FOlineatusUK                   ATTAACATACGATGACAAGGAATAAAATTTGAACGACTACCTTTATTTGT
47FOlineatusCapGhirMorocco        ATTAACATACGATGACAAGGAATAAAATTTGAACGACTACCTTTATTTGT
235FOlineatusBasque               ATTAACATACGATGACAAGGAATAAAATTTGAACGACTACCTTTATTTGT
139FOlineatusUK                   ATTAACATACGATGACAAGGAATAAAATTTGAACGACTACCTTTATTTGT
251FOlineatusGreece               ATTAACATACGATGACAAGGAATAAAATTTGAACGACTACCTTTATTTGT
29FOlineatusImouraneMorocco       ATTAACATACGATGACAAGGAATAAAATTTGAACGACTACCTTTATTTGT
37FOlineatusTifnitMorocco         ATTAACATACGATGACAAGGAATAAAATTTGAACGACTACCTTTATTTGT
321FoatratusFuerte1               ATTAACATACGGTGACAAGGAATAAAATTTGAACGACTACCTTTATTTGT
69FOatratusLasGalletasTenerife    ATTAACATACGGTGACAAGGAATAAAATTTGAACGACTACCTTTATTTGT
277FoatratusLanz2                 ATTAACATACGGTGACAAGGAATAAAATTTGAACGACTACCTTTATTTGT
31FOedulisImouraneMorocco         ATTAATATACGATGACAAGGAATGAAGTTTGAACGACTACCTTTATTTGT
354FoedulisGC3                    ATTAACATACGATGACAAGGAATGAAGTTTGAACGACTACCTTTATTTGT
264FoedulisLanz3                  ATTAATATACGATGACAAGGAATGAAGTTTGAACGACTACCTTTATTTGT
76FOedulisLosGigantesTenerife     ATTAATATACGATGACAAGGAATGAAGTTTGAACGACTACCTTTATTTGT
182FOedulisMadeira                ATTAATATACGATGACAAGGAATGAAGTTTGAACGACTACCTTTATTTGT
54FOedulisCapGhirMorocco          ATTAATATACGATGACAAGGAATGAAGTTTGAACGACTACCTTTATTTGT
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183FOedulisMadeira                ATTAATATACGATGACAAGGAATGAAGTTTGAACGACTACCTTTATTTGT
192FOedulisMadeira                ATTAATATACGATGACAAGGAATGAAGTTTGAACGACTACCTTTATTTGT
102FOturbinatusMalaga             ATTAATATACGGTGACAAGGAATAAAATTTGAACGATTGCCTTTATTTGT
112FOturbinatusMalaga             ATTAATATACGGTGACAAGGAATAAAATTTGAACGATTGCCTTTATTTGT
256FOturbinatusGreece             ATTAATATACGGTGACAAGGAATAAAATTTGAACGATTACCTTTATTTGT
246FGibbulasppBasque              ATTAATATACGATGACAAGGAATGAAATTTGAGCGGTTACCTCTTTTTGT
338FoedjuvLanz4                   ATTAATATACGATGACAAGGAATGAAATTTGAGCGGTTACCTCTTTTTGT
93FOarticulatusMalaga             ATTAATATGCGGTGGCAGGGCATGAAATTTGAACGACTACCTTTATTTGT
                                  ***** ** ** ** ** ** ** ** ***** **  * *** * *****

133FOlineatusUK                   TTGATCAGTAAAAATCA---CAGCTATCTTACTCCTACTTTTATTACCAG
47FOlineatusCapGhirMorocco        TTGATCAGTAAAAATCA---CAGCTATCTTACTCCTACTTTCATTGCCAG
235FOlineatusBasque               TTGATCAGTAAAAATCA---CAGCTATCTTACTCCTACTTTCATTACCAG
139FOlineatusUK                   TTGATCAG-AAAAATCA---CAGCTATCTTACTCCTACTTTCATTACCAG
251FOlineatusGreece               TTGGTCAGTAAAAATCA---CAGCTATCTTACTCCTACTTTCATTACCAG
29FOlineatusImouraneMorocco       TTGGTCAGTAAAAATCA---CAGCTATCTTACTCCTACTTTCATTACCAG
37FOlineatusTifnitMorocco         TTGATCAGTAAAAATCA---CAGCTATCTTACTCCTACTTTCATTACCAG
321FoatratusFuerte1               TTGGTCAGTAAAGATTA---CAGCTATTCTACTTTTACTTTCACTGCCAG
69FOatratusLasGalletasTenerife    TTGGTCAGTAAAGATTA---CAGCTATTCTACTTTTACTTTCACTGGCAG
277FoatratusLanz2                 TTGGTCAGTAAAGATTA---CAGCTATTCTACTTTTACTTTCACTGCCAG
31FOedulisImouraneMorocco         CTGATCAGTAAAAATCA---CAGCTATTTTACTTCTACTTTCATTACCAG
354FoedulisGC3                    CTGATCAGTAAAAATCA---CAGCTATTTTACTTCTACTTTCATTACCAG
264FoedulisLanz3                  CTGATCAGTAAAAATCA---CAGCTATTTTACTTCTACTTTCATTACCAG
76FOedulisLosGigantesTenerife     CTGATCAGTAAAAATCA---CAGCTATTTTACTTCTACTTTCATTACCAG
182FOedulisMadeira                CTGATCAGTAAAAATCA---CAGCTATTTTACTTCTGCTTTCATTACCAG
54FOedulisCapGhirMorocco          CTGATCAGTAAAAATCA---CAGCTATTTTACTTCTACTTTCATTACCAG
183FOedulisMadeira                CTGATCAGTAAAAATCA---CAGCTATTTTACTTCTACTTTCATTACCAG
192FOedulisMadeira                CTGATCAGTAAAAATCA---CAGCTATTTTACTTCTACTTTCATTACCAG
102FOturbinatusMalaga             CTGATCCGTAAAAATCA---CAGCTATTTTGCTTTTACTTTCATTACCAG
112FOturbinatusMalaga             CTGATCCGTAAAAATCA---CAGCTATTTTGCTTTTACTTTCATTACCAG
256FOturbinatusGreece             CTGATCCGTAAAAATCA---CAGCTATTTTGCTTTTGCTTTTATTACCAG
246FGibbulasppBasque              TTGATCAGTAAAAATTA---CTGCCATCTTACTTTTATTATCATTACCAG
338FoedjuvLanz4                   TTGATCAGTAAAAAGTCTATCTGCCATCTTACTTTTATTATCATTACCAG
93FOarticulatusMalaga             CTGGTCAGTAAAAATTA---CAGCCATTTTACTTTTACTTTCACTACCAG
                                   ** ** * *** *      * ** **  * **  *  * * * *  ***

133FOlineatusUK                   -TTTTAGC-TGGGGCTATTACTATGCT-TTTA-ACAGAAAACGGCC-GCG
47FOlineatusCapGhirMorocco        -TTTTAGC-TGGGGCTATTACTATGCT-TTTA-ACAGAAAACGGCC-GCG
235FOlineatusBasque               -TTTTAGC-TGGGGCTATTACTATGCT-TTTA-ACAGAAAACGGCC-GCG
139FOlineatusUK                   CTTTTAGC-TGGGGCTATTACTATGCT-TTTA-ACAGAAAACGGCC-GCG
251FOlineatusGreece               -TTTTAGC-TGGGGCTATTACTATGCT-TTTA-ACAGAAAACGGCC-GCG
29FOlineatusImouraneMorocco       -TTTTAGC-TGGGGCTATTACTATGCT-TTTA-ACAGAAAACGGCC-GCG
37FOlineatusTifnitMorocco         -TTTTAGC-TGGGGCTATTACTATGCT-TTTA-ACAGAAAACGGCCCGCG
321FoatratusFuerte1               -TTTTAGC-CGGTGCTATTACTATACT-TTTA-ACAGCAAACGGCC-GCG
69FOatratusLasGalletasTenerife    -TTTTAGC-CGGTGCTATTACTATACT-TTTA-ACAGAAAACGGCC-GCG
277FoatratusLanz2                 -TTTTAGC-CGGTGCTATTACTATACT-TTTA-ACAGAAAACGGCC-GCG
31FOedulisImouraneMorocco         -TTTTAGC-TGGAGCTATTACCATACT-TTTA-ACAGAAAACGGCC-GCG
354FoedulisGC3                    -TTTTAGCCTGGAGCTATTACCATACT-TTTA-ACAGTAAACGGCC-GCG
264FoedulisLanz3                  -TTTTAGC-TGGAGCTATTACCATACT-TTTA-ACAGAAAACGGCC-GCG
76FOedulisLosGigantesTenerife     -TTTTAGC-TGGAGCTATTACCATACT-TTTA-ACAGAAAACGGCC-GCG
182FOedulisMadeira                -TTTTAGC-TGGAGCTATTACCATACT-TTTA-ACAGAAAACGGCC-GCG
54FOedulisCapGhirMorocco          -TTTTAGC-TGGAGCTATTACCATACT-TTTA-ACAGAAAACGGCC-GCG
183FOedulisMadeira                -TTTTAGC-TGGAGCTATTACCATACT-TTTA-ACAGAAAACGGCC-GCG
192FOedulisMadeira                -TTTTAGC-TGGAGCTATTACCATACT-TTTA-ACAGAAAACGGCC-GCG
102FOturbinatusMalaga             -TTTTAGC-CGGAGCCATTACTATGCT-TTTA-ACAGAAAACGGCC-GCG
112FOturbinatusMalaga             -TTTTAGC-CGGAGCCATTACTATGCT-TTTA-ACAGAAAACGGCC-GCG
256FOturbinatusGreece             -TTTTAGC-CGGAGCCATTACTATGCT-TTTA-ACAGAAAACGGCC-GCG
246FGibbulasppBasque              -TTTTAGC-CGGTGCCATTACAATACT-ATTA-ACTGAAAACGGCC-GCG
338FoedjuvLanz4                   -TTTTAGC-CGGTGCCATTACAATACTTATTATACTGAAAACGGCC-GCG
93FOarticulatusMalaga             -TTTTAGC-TGGGGCTATTACTATACT-TTTA-ACAGAAAACGGCC-GCG
                                   *******  ** ** ***** ** **  *** ** * ******** ***

133FOlineatusUK                   GTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAGG
47FOlineatusCapGhirMorocco        GTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAGG
235FOlineatusBasque               GTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAGG
139FOlineatusUK                   GTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAGG
251FOlineatusGreece               GTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAGG
29FOlineatusImouraneMorocco       GTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAGG
37FOlineatusTifnitMorocco         GTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAGG
321FoatratusFuerte1               GTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAGG
69FOatratusLasGalletasTenerife    GTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAGG
277FoatratusLanz2                 GTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAGG
31FOedulisImouraneMorocco         GTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAGG
354FoedulisGC3                    GTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAGG
264FoedulisLanz3                  GTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAGG
76FOedulisLosGigantesTenerife     GTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAGG
182FOedulisMadeira                GTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAGG
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54FOedulisCapGhirMorocco          GTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAGG
183FOedulisMadeira                GTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGAAGG
192FOedulisMadeira                GTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAGG
102FOturbinatusMalaga             GTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAGG
112FOturbinatusMalaga             GTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAGG
256FOturbinatusGreece             GTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAGG
246FGibbulasppBasque              GTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAGG
338FoedjuvLanz4                   GTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAGG
93FOarticulatusMalaga             GTACCCTAACCGTGCAAAGGTAGCATAATCATTTGCCTTTTAATTGGAGG
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133FOlineatusUK                   CTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCTTTCGAAATATTTA
47FOlineatusCapGhirMorocco        CTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCTTTCGAAATATTTA
235FOlineatusBasque               CTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCTTTCGAAATATTTA
139FOlineatusUK                   CTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCTTTCGAAATATTTA
251FOlineatusGreece               CTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCTTTCGAAATATTTA
29FOlineatusImouraneMorocco       CTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCTTTCGAAATATTTA
37FOlineatusTifnitMorocco         CTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCTTTCGAAATATTTA
321FoatratusFuerte1               CTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCTTTCGAAATATTTA
69FOatratusLasGalletasTenerife    CTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCTTTCGAAATATTTA
277FoatratusLanz2                 CTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCTTTCGAAATATTTA
31FOedulisImouraneMorocco         CTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCTTTTGGAATATTTA
354FoedulisGC3                    CTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCTTTTGGAATATTTA
264FoedulisLanz3                  CTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCTTTTGGAATATTTA
76FOedulisLosGigantesTenerife     CTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCTTTTGGAATATTTA
182FOedulisMadeira                CTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCTTTTGGAATATTTA
54FOedulisCapGhirMorocco          CTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCTTTTGGAATATTTA
183FOedulisMadeira                CTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCTTTTGGAATATTTA
192FOedulisMadeira                CTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCTTTTGGAATATTTA
102FOturbinatusMalaga             CTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCTTTCGAAATATTTA
112FOturbinatusMalaga             CTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCTTTCGAAATATTTA
256FOturbinatusGreece             CTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCTTTCGAAATATTTA
246FGibbulasppBasque              CTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCTTTTAAAATATTTA
338FoedjuvLanz4                   CTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCTTTTAAAATATTTA
93FOarticulatusMalaga             CTGGTATGAATGGTTTGACGAGAGCTATGCTGTCTCTTTCGAAATATTTA
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133FOlineatusUK                   AAAATTAACTTTTAGGTAAAGAGGCCTAAATTCGTTTAAGGGACAAGAAG
47FOlineatusCapGhirMorocco        AAAATTAACTTTTAGGTAAAGAGGCCTAAATTCGTTTAAGGGACAAGAAG
235FOlineatusBasque               AAAATTAACTTTTAGGTAAAGAGGCCTAAATTCGTTTAAGGGACAAGAAG
139FOlineatusUK                   AAAATTAACTTTTAGGTAAAGAGGCCTAAATTCGTTTAAGGGACAAGAAG
251FOlineatusGreece               AAAATTAACTTTTAGGTAAAGAGGCCTAAATTCGTTTAAGGGACAAGAAG
29FOlineatusImouraneMorocco       AAAATTAACTTTTAGGTAAAGAGGCCTAAATTCGTTTAAGGGACAAGAAG
37FOlineatusTifnitMorocco         AAAATTAACTTTTAGGTAAAGAGGCCTAAATTCGTTTAAGGGACAAGAAG
321FoatratusFuerte1               AAAATTAACTTTTAGGTAAAGAGGCCTAAATTTGTTTAAGGGACAAGAAG
69FOatratusLasGalletasTenerife    AAAATTAACTTTTAGGTAAAGAGGCCTAAATTTGTTTAAGGGACAAGAAG
277FoatratusLanz2                 AAAATTAACTTTTAGGTAAAGAGGCCTAAATTTGTTTAAGGGACAAGAAG
31FOedulisImouraneMorocco         AAAATTAACTTTTAGGTGAAGAGGCCTGAATTTATTTAAGGGACAAGAAG
354FoedulisGC3                    AAAATTAACTTTTAGGTGAAGAGGCCTGAATTTATTTAAGGGACAAGAAG
264FoedulisLanz3                  AAAATTAACTTTTAGGTGAAGAGGCCTGAATTTATTTAAGGGACAAGAAG
76FOedulisLosGigantesTenerife     AAAATTAACTTTTAGGTGAAGAGGCCTGAATTTATTTAAGGGACAAGAAG
182FOedulisMadeira                AAAATTAACTTTTAGGTGAAGAGGCCTGAATTTATTTAAGGGACAAGAAG
54FOedulisCapGhirMorocco          AAAATTAACTTTTAGGTGAAGAGGCCTGAATTTATTTAAGGGACAAGAAG
183FOedulisMadeira                AAAATTAACTTTTAGGTGAAGAGGCCTGAATTTATTTAAGGGACAAGAAG
192FOedulisMadeira                AAAATTAACTTTTAGGTGAAGAGGCCTGAATTTATTTAAGGGACAAGAAG
102FOturbinatusMalaga             AAAATTAACCTTTAGGTGAAGAGGCCTGAATCTATTTAAGGGACAAGAAG
112FOturbinatusMalaga             AAAATTAACCTTTAGGTGAAGAGGCCTGAATCTATTTAAGGGACAAGAAG
256FOturbinatusGreece             AAAATTAACCTTTAGGTGAAGAGGCCTGAATTTATTTAAGGGACAAGAAG
246FGibbulasppBasque              AAAATTAACTTTTAGGTGAAGAGGCCTAAATTTATTTAAGGGACAAGAAG
338FoedjuvLanz4                   AAAATTAACTTTTAGGTGAAGAGGCCTAAATTTATTTAAGGGACAAGAAG
93FOarticulatusMalaga             AAAATTAACTTTTAGGTAAAGAGGCCTAAATTTATTTAAGGGACAAGAAG
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133FOlineatusUK                   ACCCTGTTGAGCTTTAGTGATCCTATAAATTGATGGGCAATTT-ATTGGT
47FOlineatusCapGhirMorocco        ACCCTGTTGAGCTTTAGTGATCCTATAAATTGATGGGCAACTT-ATTGGT
235FOlineatusBasque               ACCCTGTTGAGCTTTAGTGATCCTATAAATTGATGGGCAATTT-ATTGGT
139FOlineatusUK                   ACCCTGTTGAGCTTTAGTGATTCTATAAATTGATGGGCAATTT-ATTGGT
251FOlineatusGreece               ACCCTGTTGAGCTTTAGTGATCCTATAAATTGATGGGCAATTT-ATTGGT
29FOlineatusImouraneMorocco       ACCCTGTTGAGCTTTAGTGATCCTATAAATTGATGGGCAATTT-ATTGGT
37FOlineatusTifnitMorocco         ACCCTGTTGAGCTTTAGTGATCCTATAAATTAATGGGCAATTT-ATTGGT
321FoatratusFuerte1               ACCCTGTTGAGCTTTAGCGATCTTGTAAATTTTTGTGCAGTTT-GCTTAT
69FOatratusLasGalletasTenerife    ACCCTGTTGAGCTTTAGCGATCTTGTAAATTTTTGTGCAGTTT-GCTTAT
277FoatratusLanz2                 ACCCTGTTGAGCTTTAGTGATCTTGTAAATTTTTGTGCAGTTT-GCTTAT
31FOedulisImouraneMorocco         ACCCTGTTGAGCTTTAGTGACTTTATAAATTTATATATAATTT-GTTATT
354FoedulisGC3                    ACCCTGTTGAGCTTTAGTGACTTTATAAATTTATATATAATTT-GTTATT
264FoedulisLanz3                  ACCCTGTTGAGCTTTAGTGACTTTATAAATTTATATATAATTT-GTTATT
76FOedulisLosGigantesTenerife     ACCCTGTTGAGCTTTAGTGACTTTATAAATTTATATATAATTT-GTTATT
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182FOedulisMadeira                ACCCTGTTGAGCTTTAGTGACTTTATAAATTTATATATAATTT-GTTATT
54FOedulisCapGhirMorocco          ACCCTGTTGAGCTTTAGTGACTTTATAAATTTATATATAATTT-GTTATT
183FOedulisMadeira                ACCCTGTTGAGCTTTAGTGACTTTATAAATTTATATATAATTT-GTTATT
192FOedulisMadeira                ACCCTGTTGAGCTTTAGTGACTTTATAAATTTATATATAATTT-GTTATT
102FOturbinatusMalaga             ACCCTGTTGAGCTTTAGTGATTTTATAAATTTACAGGTAATCTAGTTAAT
112FOturbinatusMalaga             ACCCTGTTGAGCTTTAGTGATTTTATAAATTTACAGGTAATCTAGTTAAT
256FOturbinatusGreece             ACCCTGTTGAGCTTTAGTGATTTTATAAATTTACAGGTAATCTAGTTAAT
246FGibbulasppBasque              ACCCTGTTGAGCTTTAGTGATGTTGTAAAC-GATGAAGAATAA-GTTATT
338FoedjuvLanz4                   ACCCTGTTGAGCTTTAGTGATGTTGTAAAC-GATGGAGAATAA-GTTATT
93FOarticulatusMalaga             ACCCTGTTGAGCTTTAGTGATCCTGTAAGTTTACACGCAGCTT-AATAAC
                                  ***************** **   * ***          *       *

133FOlineatusUK                   TGTTGTGTATTTGTGTATTA-TATATAACGTAAGTTTAAAGGTAATCTTT
47FOlineatusCapGhirMorocco        TGTTGTGTATTTGTGTATTA-TATATAACGTAAGTTTAAAGGCAATCTTT
235FOlineatusBasque               TGTTGTGTATTTGTGTATTA-TATATAACGTAAGTTTAAAGGTAATCTTT
139FOlineatusUK                   TGTTGTGTATTTGTGTATTA-TATATAACGTAAGTTTAAAGGTAATCTTT
251FOlineatusGreece               TGTTGTGTATTTGTGTATTA-TATATAACGTAAGTTTAAAGGTAATCTTT
29FOlineatusImouraneMorocco       TGTTGTGTATTTGTGTATTA-TATATAACGTAAGTTTAAAGGTAATCTTT
37FOlineatusTifnitMorocco         TGTTGTGTATTTGTGTATTA-TATATAACGTAAGTTTAAAGGTAATCTTT
321FoatratusFuerte1               ATTTGTATATTTGT--------ATATAACATAAGTTTATAGGTAATCTTT
69FOatratusLasGalletasTenerife    ATTTGTATATTTGT--------ATATAACATAAGTTTATAGGTAATCTTT
277FoatratusLanz2                 ATTTGTATATTTGT--------ATATAACATAAGTTTATAGGTAATCTTT
31FOedulisImouraneMorocco         GATTATATTTTTGTATATTAATGTATAACTTAGGTTTATGAATAATCTTT
354FoedulisGC3                    GATTATATTTTTGTATATTAATGTATAACTTAGGTTTATGAATAATCTTT
264FoedulisLanz3                  GATTATATTTTTGTATATTAATGTATAACTTAGGTTTATGAATAATCTTT
76FOedulisLosGigantesTenerife     GATTATATTTTTGTATATTAATGTATAACTTAGGTTTATGAATAATCTTT
182FOedulisMadeira                GATTATATTTTTGTATATTAATGTATAACTTAGGTTTATGAATAATCTTT
54FOedulisCapGhirMorocco          GATTATATTTTTGTATATTAATGTATAACTTAGGTTTATGAATAATCTTT
183FOedulisMadeira                GATTATATTTTTGTATATTAATGTATAACTTAGGTTTATGAATAATCTTT
192FOedulisMadeira                GATTATATTTTTGTATATTAATGTATAACTTAGGTTTATGAATAATCTTT
102FOturbinatusMalaga             GATTATAGTTCGGTATATTAATATATAACATAGGTTTATAGGTAATCTTT
112FOturbinatusMalaga             GATTATAGTTCGGTATATTAATATATAACATAGGTTTATAGGTAATCTTT
256FOturbinatusGreece             GATTATAGTTCGGTATATTAATATATAACATAGGTTTATAGGTAATCTTT
246FGibbulasppBasque              ATTGAT-TTGTTGTGTATTTAT-TATAATAAAAGTTTGCAGATAATCTTT
338FoedjuvLanz4                   ATTGAT-TTGTTGTGTATTTAT-TATAATAAAAGTTTGCAGATAATCTTT
93FOarticulatusMalaga             TGTTGTATTTTTGTGTATTTATATATAACATAAGTTTGTAGGTAATCTTT
                                    *  *      **         *****   * ****      *******

133FOlineatusUK                   AGTTGGGGC-GACTGAGGAACATGGAAAGCTTCCTTGAATTTAAAAGCAT
47FOlineatusCapGhirMorocco        AGTTGGGGCCGACTGAGGAACATGGAAAGCTTCCTTGAATTTAAAAGCAT
235FOlineatusBasque               AGTTGGGGC-GACTGAGGAACATGGAAAGCTTCCTTGAATTTAAAAGCAT
139FOlineatusUK                   AGTTGGGGC-GACTGAGGAACATGGAAAGCTTCCTTGAATTTAAAAGCAT
251FOlineatusGreece               AGTTGGGGC-GACTGAGGAACATGGAAAGCTTCCTTGAATTTAAAAGCAT
29FOlineatusImouraneMorocco       AGTTGGGGC-GACTGAGGAACATGGAAAGCTTCCTTGAATTTAAAAGCAT
37FOlineatusTifnitMorocco         AGTTGGGGC-GACTGAGGAACATGGAAAGCTTCCTTGAATTTAAAAGCAT
321FoatratusFuerte1               AGTTGGGGC-GACTGAGGAACATGAAAAGCTTCCTTGAACTTAAGAGTGT
69FOatratusLasGalletasTenerife    AGTTGGGGC-GACTGAGGAACATGAAAAGCTTCCTTGAACTTAAGAGTGT
277FoatratusLanz2                 AGTTGGGGC-GACTGAGGAACATGAAAAGCTTCCTTGAACTTAAGAGTGT
31FOedulisImouraneMorocco         AGTTGGGGC-GACTGAGGAACATACAAAGCTTCCTTGAGTTCGAAGGCGT
354FoedulisGC3                    AGTTGGGGC-GACTGAGGAACATACAAAGCTTCCTTGAGTTCGAAGGCGT
264FoedulisLanz3                  AGTTGGGGC-GACTGAGGAACATACAAAGCTTCCTTGAGTTCGAAGGCGT
76FOedulisLosGigantesTenerife     AGTTGGGGC-GACTGAGGAACATACAAAGCTTCCTTGAGTTCTAAGGCGT
182FOedulisMadeira                AGTTGGGGC-GACTGAGGAACATACAAAGCTTCCTTGAGTTTGAAGGCGT
54FOedulisCapGhirMorocco          AGTTGGGGC-GACTGAGGAACATACAAAGCTTCCTTGAGTTTGAAGGCGT
183FOedulisMadeira                AGTTGGGGC-GACTGAGGAACATACAAAGCTTCCTTGAGTTTGAAGGCGT
192FOedulisMadeira                AGTTGGGGC-GACTGAGGAACATACAAAGCTTCCTTGAGTTTGAAGGCGT
102FOturbinatusMalaga             AGTTGGGGC-GACTGAGGAACACATAAAGCTTCCTTGAATTTAAGAGCGT
112FOturbinatusMalaga             AGTTGGGGC-GACTGAGGAACACATAAAGCTTCCTTGAATTTAAGAGCGT
256FOturbinatusGreece             AGTTGGGGC-GACTGAGGAACACATAAAGCTTCCTTGAATTTAAGAGCGT
246FGibbulasppBasque              AGTTGGGGC-GACTGAGGAACATAAAAAGCTTCCTTGTATAT-AAATTAT
338FoedjuvLanz4                   AGTTGGGGC-GACTGAGGAACATAAAAAGCTTCCTTGTATAT-AAATTAT
93FOarticulatusMalaga             AGTTGGGGC-GACTAAGGAACATTAAAAGCTTCCTTGAATTTAGTAATAA
                                  ********* **** *******   ************

133FOlineatusUK                   GTTGGGCAA-TTAATTTGTTGATGCATTTGCGTTAATTTTGATCCAATTT
47FOlineatusCapGhirMorocco        GTTGGGCAA-TTAATTTGTTGATGCATTTGCGTTAATTTTGATCCAATTT
235FOlineatusBasque               GTTGGGCAA-TTAATTTGTTGATGCATTTGCGTTAATTTTGATCCAATTT
139FOlineatusUK                   GTTGGGCAA-TTAATTTGTTGATGCATTTGCGTTAATTTTGATCCAATTT
251FOlineatusGreece               GTTGGGCAA-TTAATTTGTTGATGCATTTGCGTTAATTTTGATCCAATTT
29FOlineatusImouraneMorocco       GTTGGGCAA-TTGATTTGTTGATGCATTTGCGTTAATTTTGATCCAATTT
37FOlineatusTifnitMorocco         GTTGGGCAA-TTAATTTGTTGATGCATCTGCGTTAATTTTGATCCAATTT
321FoatratusFuerte1               GTTAAACAA-TTAGTTTATTGATGCGTTTATGTTAATTTTGATCCAATTT
69FOatratusLasGalletasTenerife    GTTAAACAA-TTAGTTTATTGATGCGTTTATGTTAATTTTGATCCAATTT
277FoatratusLanz2                 GTTAAACAA-TTAGTTTATTGATGCGTTTATGTTAATTTTGATCCAATTT
31FOedulisImouraneMorocco         GTTGGCCTGGCTGATTTATTGATGCGTTTGTGTTAATTTTGATCCAATTT
354FoedulisGC3                    GTTGGCCTGGCTGATTTATTGATGCGTTTGTGTTAATTTTGATCCAATTT
264FoedulisLanz3                  GTTGGCCTGGCTGATTTATTGATGCGTTTGTGTTAATTTTGATCCAATTT
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76FOedulisLosGigantesTenerife      GTTGGCCTGGCTGATTTATTGATGCGTTTGTGTTAATTTTGATCCAATTT
182FOedulisMadeira                 GTTGGCCTGGCTGATTTATTGATGCGTTTGTGTTAATTTTGATCCAATTT
54FOedulisCapGhirMorocco           GTTGGCCTGGCTGATTTATTGATGCGTTTGTGTTAATTTTGATCCAATTT
183FOedulisMadeira                 GTTGGCCTGGTTGATTTATTGATGCGTTTGTGTTAATTTTGATCCAATTT
192FOedulisMadeira                 GTTGGCCTGGCTGATTTATTGATGCGTTTGTGTTAATTTTGATCCAATTT
102FOturbinatusMalaga              GTTAAGCAATTTAGTTTACTGATGCATTTATGTTAATTTTGATCCAATTT
112FOturbinatusMalaga              GTTAAGCAATTTAGTTTACTGATGCATTTATGTTAATTTTGATCCAATTT
256FOturbinatusGreece              GTTAAGCAATTTAGTTTACTGATGCATTTATGTTAATTTTGATCCAATTT
246FGibbulasppBasque               -TTAAGTAATTGAATAAATTAATGCTTATGTGTTA-TTTTGATCCAAATT
338FoedjuvLanz4                    -TTAAGTAATTGAATAAATTAATGCTTATGTGTTA-TTTTGATCCAAATT
93FOarticulatusMalaga              GTTAAATTATTTAATTTTTTGATGCATTTATGTTAATTTTGATCCAATTT
                                    **           *    * **** * *  **** *********** **

133FOlineatusUK                    ATTTTGATTAAGAGAAAAAGTTACCACAGGGATAACAGCGTAATCTTTCT
47FOlineatusCapGhirMorocco         ATTTTGATTAAGAGAAAAAGTTACCACAGGGATAACAGCGTAATCTTTCT
235FOlineatusBasque                ATTTTGATTAAGAGAAAAAGTTACCACAGGGATAACAGCGTAATCTTTCT
139FOlineatusUK                    ATTTTGATTAGGAGAAAAAGTTACCACAGGGATAACAGCGTAATCTTTCT
251FOlineatusGreece                ATTTTGATTAAGAGAAAAAGTTACCACAGGGATAACAGCGTAATCTTTCT
29FOlineatusImouraneMorocco        ATTTTGATTAAGAGAAAAAGTTACCACAGGGATAACAGCGTAATCTTTCT
37FOlineatusTifnitMorocco          ATTTTGATTAAGAGAAAAAGTTACCACAGGGATAACAGCGTAATCTTTCT
321FoatratusFuerte1                ATTTTGATTAAGAGAAAAAGTTACCACAGGGATAACAGCGTAATCTTTCT
69FOatratusLasGalletasTenerife     ATTTTGATTAAGAGAAAAAGTTACCACAGGGATAACAGCGTAATCTTTCT
277FoatratusLanz2                  ATTTTGATTAAGAGAAAAAGTTACCACAGGGATAACAGCGTAATCTTTCT
31FOedulisImouraneMorocco          ATTTTGATTAAGAGAAAAAGTTACCACAGGGATAACAGCGTAATCTTTCT
354FoedulisGC3                     ATTTTGATTAAGAGAAAAAGTTACCACAGGGATAACAGCGTAATCTTTCT
264FoedulisLanz3                   ATTTTGATTAAGAGAAAAAGTTACCACAGGGATAACAGCGTAATCTTTCT
76FOedulisLosGigantesTenerife      ATTTTGATTAAGAGAAAAAGTTACCACAGGGATAACAGCGTAATCTTTCT
182FOedulisMadeira                 ATTTTGATTAAGAGAAAAAGTTACCACAGGGATAACAGCGTAATCTTTCT
54FOedulisCapGhirMorocco           ATTTTGATTAAGAGAAAAAGTTACCACAGGGATAACAGCGTAATCTTTCT
183FOedulisMadeira                 ATTTTGATTAAGAGAAAAAGTTACCACAGGGATAACAGCGTAATCTTTCT
192FOedulisMadeira                 ATTTTGATTAAGAGAAAAAGTTACCACAGGGATAACAGCGTAATCTTTCT
102FOturbinatusMalaga              ATTTTGATTAAGAGAAAAAGTTACCACAGGGATAACAGCGTAATCTTTCT
112FOturbinatusMalaga              ATTTTGATTAAGAGAAAAAGTTACCACAGGGATAACAGCGTAATCTTTCT
256FOturbinatusGreece              ATTTTGATTAAGAGAAAAAGTTACCACAGGGATAACAGCGTAATCTTTCT
246FGibbulasppBasque               ATTTTGATTAAGAGAAAAAGTTACCACAGGGATAACAGCGTAATCTTTCT
338FoedjuvLanz4                    ATTTTGATTAAGAGAAAAAGTTACCACAGGGATAACAGCGTAATCTTTCT
93FOarticulatusMalaga              ATTTTGATTAAGAGAAAAAGTTACCACAGGGATAACAGCGTAATCTTTCT
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133FOlineatusUK                    GGAGAGATCTAATCGAAAGAAGGGTTTGCGACCTCG-ATGTTGGACTAA
47FOlineatusCapGhirMorocco         GGAGAGATCTAATCGAAAGAAGGGTTTGCGACCTCG-ATGTTGGACTAA
235FOlineatusBasque                GGAGAGATCTAATCGAAAGAAGGGTTTGCGACCTCG-ATGTTGGACTAA
139FOlineatusUK                    GGAGAGATCTAATCGAAAGAAGGGTTTGCGACCTCG-ATGTTGGACTAA
251FOlineatusGreece                GGAGAGATCTAATCGAAAGAAGGGTTTGCGACCTCG-ATGTTGGACTAA
29FOlineatusImouraneMorocco        GGAGAGATCTAATCGAAAGAAGGGTTTGCGACCTCG-ATGTTGGACTAA
37FOlineatusTifnitMorocco          GGAGAGGTCTAATCGAAAGAAGGGTTTGCGACCTCG-ATGTTGGACTAA
321FoatratusFuerte1                GGAGAGATCTAATCGAAAGAAGGGTTTGCGACCTCG-ATGTTGGACTAA
69FOatratusLasGalletasTenerife     GGAGAGATCTAATCGAAAGAAGGGTTTGCGACCTCG-ATGTTGGACTAA
277FoatratusLanz2                  GGAGAGATCTAATCGAAAGAAGGGTTTGCGACCTCG-ATGTTGGACTAA
31FOedulisImouraneMorocco          GGAGAGATCTAATCGAAAGGAGGGTTTGCGACCTCG-ATGTTGGACTAA
354FoedulisGC3                     GGAGAGATCTAATCGAAAGGAGGGTTTGCGACCTCG-ATGTTGGACTAA
264FoedulisLanz3                   GGAGAGATCTAATCGAAAGGAGGGTTTGCGACCTCG-ATGTTGGACTAA
76FOedulisLosGigantesTenerife      GGAGAGATCTAATCGAAAGGAGGGTTTGCGACCTCGGATGTTGGACTAA
182FOedulisMadeira                 GGAGAGATCTAATCGAAAGGAAGGTTTGCGACCTCG-ATGTTGGACTAA
54FOedulisCapGhirMorocco           GGAGAGATCTAATCGAAAGGAGGGTTTGCGACCTCG-ATGTTGGACTAA
183FOedulisMadeira                 GGAGAGATCTAATCGAAAGGAGGGTTTGCGACCTCG-ATGTTGGACTAA
192FOedulisMadeira                 GGAGAGATCTAATCGAAAGGAGGGTTTGCGACCTCG-ATGTTGGACTAA
102FOturbinatusMalaga              GGAGAGATCTAATCGAAAGAAGGGTTTGCGACCTCG-ATGTTGGACTAA
112FOturbinatusMalaga              GGAGAGATCTAATCGAAAGAAGGGTTTGCGACCTCG-ATGTTGGACTAA
256FOturbinatusGreece              GGAGAGATCTAATCGAAAGAAGGGTTTGCGACCTCG-ATGTTGGACTAA
246FGibbulasppBasque               GGAGAGATCTTATCGAAAGAAGGGTTTGCGACCTCG-ATGTTGGACTAA
338FoedjuvLanz4                    GGAGAGATCTTATCGAAAGAAGGGTTTGCGACCTCG-ATGTTGGACTAA
93FOarticulatusMalaga              GGAGAGATCTAATCGAAAGAAGGGTTTGCGACCTCG-ATGTTGGACTAA
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