UNIVERSITY OF SOUTHAMPTON
School of Ocean and Earth Sciences

POPULATION GENETICS AND EVOLUTIONARY HISTORY OF SOME DEEP-SEA DEMERSAL FISHES FROM THE AZORES - NORTH ATLANTIC

by

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DECLARATION OF AUTHORSHIP

I, **Maria Ana Couto Pinto Aboim**, declare that the thesis entitled

Population genetics and evolutionary history of some deep-sea demersal fishes from the Azores – North Atlantic

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Signed: ……..

Date:…….

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ABSTRACT

MtDNA sequences and microsatellite loci were used as independent molecular markers to reveal the genetic population structure in three deep-sea demersal fish species from the North Atlantic. In the Azores archipelago, the demersal fishery is the second most important after tuna and *Helicolenus dactylopterus*, *Beryx splendens* and *Beryx decadactylus* are among the most captured species.
Partial sequences for the mitochondrial control region and cytochrome *b* gene were obtained for the three species using universal primers. The diversity encountered for these markers is consistent with diversity found on other marine fishes, except for *B.decadactylus* in which the d-loop and cyt *b* diversity was lower than expected.

Haplotype data indicated a strong genetic differentiation between *Helicolenus dactylopterus* NW, Cape Verde and NE Atlantic populations suggesting long distance colonisation processes by jump dispersal events along major oceanic currents. Eight microsatellite loci were developed for *H.dactylopterus* in order to resolve population structure at a finer intraregional scale (within Portuguese waters). Significant deviations from allelic frequencies expected under Hardy-Weinberg equilibrium were detected at several loci. Analysis of F_{IS} revealed significant differences from zero as a result of heterozygote deficiency. Estimates of F_{ST}, R_{ST} and AMOVA were also significant, suggesting that the population structure of this species within Portuguese waters was not homogeneous. Pairwise comparisons of F_{ST}, R_{ST} and genetic distances (D_{SW} and $(\sigma)^2$) between populations revealed a significant separation of the Azores and Peniche (continental Portugal) populations as well as a moderate differentiation among subpopulations of the Azores archipelago.

Beryx splendens and *Beryx decadactylus* are two congeneric species with many similarities in known biology. Analysis of haplotype data revealed striking differences in structure and history of the populations of these two species.
MtDNA sequences confirmed that *Beryx splendens* is constituted by one panmitic population within the Northeast Atlantic as has been previously hypothesised by other authors.
Surprisingly, indices of genetic diversity were lower in the closely-related *B.decadactylus* and there was a strong genetic differentiation between Cape Verde and the rest of the NE Atlantic populations when analysed for the same molecular markers. Differences found are discussed based on the limited knowledge of these species especially with respect to life-history.

Population structure results are discussed in relation to historical and on-going hydro-geographic events. Evidence for the strong influence of several events previous to the last glacial maximum (LGM) on the population demographic history and evolution of deep-sea demersal fish species in the North Atlantic was found.

"Animals living in…the sea waters… are protected from the destruction of theirs species by man. Their multiplication is so rapid and their means of evading pursuit or traps are so great, that there is no likelihood of his being able to destroy the entire species of any of these animals."

Lamarck (19th century)

"…Probably all the great sea-fisheries, are inexhaustible; that is to say nothing we do seriously affects the number of fish. And any attempt to regulate these fisheries seems consequently… to be useless."

Thomas Huxley

"70% of world's fishery resources are in deep trouble and world's fisheries cannot be sustained for much more without severe appliance of new management laws. In spite of some management policies have been put in practice since 1993, 35% of stocks show declining yields, 25% are flat and 40% are still developing and there's no space for growth in landings."

FAO (1997)

" It's difficult to reach the deep-sea and follow the organisms that live there. So, we went to the deep of the cells to find the place where life since the beginning is recorded."

Anonymous

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INTRODUCTION AND GENERAL BACKGROUND

1.1 The Deep-sea

The oceans occupy approximately 70% of the earth's surface and the deep-sea represents 90% of those water masses being the largest habitat of our planet (Angel, 1997).

The deep sea is normally characterized by depths of >1000m where the biotic and abiotic factors seem to vary very little. The light is scarce or inexistent, the pressure is very high, temperature low (-1ºC to +4ºC), the salinity constant, oxygen concentration near saturation and the food input very small (Gage & Tyler, 1991). However, most scientists consider the beginning of this habitat to be around 200m on the transition from the continental shelves to the continental slope considering it the cross between shallow-water fauna and deep-sea fauna (Tyler, 2003). This is also the definition that will be addressed and used throughout this study.

It is considered here that the deep-sea starts on the "shelf break" at 200m deep, and spreads trough the continental slope (200-2000m), the oceanic rise (2000-4000m) and the abyssal plains (4000-6000m) reaching depths of >11,000 m in trenches of the Pacific Ocean (Fig.1.1; Hopper, 1995).

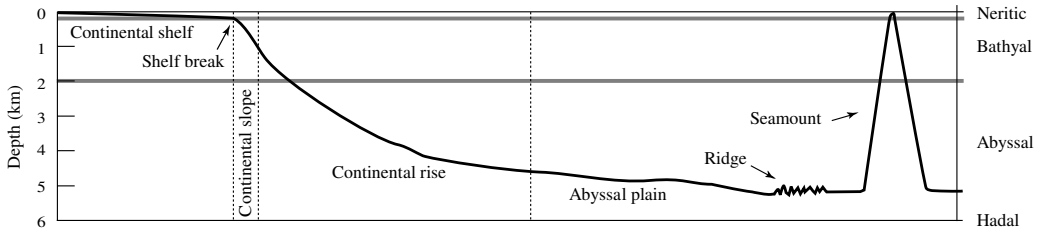


Fig.1.1 – Schematic representation of a cross section of the ocean showing the major depth zones and physiographic features (modified from Gage & Tyler, 2001).

In the nineteenth century it was common knowledge that no form of life could exist in the darkness of the depths and that the deep-sea was a desert. Only with the contribution of several workers as Thomson (1873), Cook (1882), Sanders (1967) and others more recently, it was possible to overturn this misleading theory. It was only in the 60's with new technology introductions, that an impression of the scale of biodiversity of the deep-sea was obtained and in the 70's and 80's that the dynamics of this environment started to be properly understood (Gage & Tyler, 1991; Sumich, 1999).

The remoteness and inhospitability of the deep-sea environment together with the vast vertical dimension have always been the main reasons for the delay on its exploitation. Men and instruments have to be protected from the high pressure and other advert factors in order to explore the deepness of the oceans. Nowadays, some of these problems have been solved by new technologies and the introduction of submersibles, ROV's and other instruments that permit scientists to physically reach the deepest parts of the sea (Gage & Tyler, 1991).

Today, the idea that the deep-sea is a desert with a monotonous landscape has been completely overcome. It became apparent that species diversity is much greater than that revealed by contemporary sampling methods (e.g. Sanders *et al.,* 1965; Hessler & Sanders, 1967; Rice *et al.*, 1982). It is also known that the homogeneous and stable soft sediment floor is interrupted by dynamic zones with heterogenous substrates, variable temperature and in some cases primary production independent from sunlight – the hydrothermal vents, cold seeps and mid-Atlantic ridges (Tyler, 2003).
Other complex, dynamic and rich parts of the deep-sea are the continental slopes and associated seamounts, which include around 9% of the ocean bottom. It is in these areas that the majority of deep-sea fishes are found and consequently where the largest deep-water fishing grounds have developed (Gordon *et al.*, 1995).

At the end of the smooth Continental shelf, at around 200m depths, the gradient of the seabed steepens to become the Continental slope.
The steep Continental slope is the boundary between the continental masses and the ocean basins and plunges in a relatively short geographic distance to 2,000m (Sumich, 1999). Here, demersal fishes are abundant and diversity high. Physical parameters are quite stable as in the rest of the deep-sea apart from some exceptions including: extreme oxygen minimum zones found at 400-800m, higher temperatures in areas of the Mediterranean and Red seas, hydrothermal vents and other chemosynthetic ecosystems including hydrocarbon and brine seeps. Although the changes in physical parameters between the slope and the rise are not dramatic, fish species diversity abruptly decline in the transition from the bathyal to the abyssal zones (Merrett & Haedrich, 1997). Small-scale disturbances in the Continental slope may contribute to increased species richness and diversity at a local or regional scale.

Seamounts are undersea volcanic mountains that rise abruptly from the deep-sea floor to near the sea surface, with circular, elliptical or less regular forms, functioning as islands of continental slope-like habitats surrounded by abyssal depths (Keating *et al.*, 1987). These

topographic features together with oceanic islands have strong effects on ocean circulation producing deflections of currents, formation of trapped waves or generating phenomena such as Taylor columns (reviewed in Rogers, 1994). Specific hydrographical conditions permit retention of plankton and other prey organisms and may increase primary production (Creasey & Rogers, 1999; Coutis & Middleton, 2002). This may lead to larvae retention and the concentration of valuable fish species around seamounts although the exact explanations for this are unclear and may vary between localities (for e.g. Rogers, 1994; Mullineaux & Mills, 1997; Swearer *et al.*, 1999).

1.2 Deep-sea Resources

1.2.1 Deep-sea biodiversity

Recent exploration has greatly modified the assumption of environmental stability and poor biodiversity in the deep-sea. The benthic landscape is now known to be a "topographically complex patchwork" of distinctive habitats and the diversity and composition of deep-sea species is known to vary at local, regional and global scales (Tyler, 2003).

The deep-sea benthic communities are similar to that found in shallower habitats, and are composed by: the meiofauna dominated by nematodes and foraminiferans; the macrofauna comprising numerous metazoan taxa, polychaetes, crustaceans and bivalve molluscs; the megafauna mainly dominated by sessile poriferans, anthozoans and crinoids as well as errant echinoderms, decapod crustaceans and fish (e.g. Hessler & Sanders, 1967; Gage, 1986; Carey *et al.,* 1990; Gage & Tyler, 1991; Grassle & Maciolek, 1992; Lambshead, 1993; Creasey, 1998).

Around 10-15% of the 25,000 world marine fish species live in the deep-sea (Haedrich, 1997). Most deep-sea fish species are demersal - benthopelagic - (22 orders against 13 pelagic ones) (Merret & Haedrich, 1997), i.e., they live in some way associated with the seabed on continental slopes, seamounts or slopes of oceanic islands.

Species diversity and abundance has been recognized to change along bathymetric and latitudinal related gradients. Intraspecific phenotypic (e.g. Rex & Etter, 1998) and genetic changes have also been identified for deep-sea species (reviewed in Creasey & Rogers, 1999 and Rogers, 2003).

Abundance and biomass decrease exponentially with depth because of the decrease in nutrient input from the surface. However, dominance by single species tends to be lower in

the continental slope fish community and species diversity is high. Species diversity generally appears to show a unimodal parabolic distribution with depth, reaching a highest at around 2,000 - 3,000 m. This pattern has been explained by production, predation and competition based hypotheses (Rex, 1981).
Horizontal species distribution and large-scale patterns of distribution in the deep-sea are less understood but believed to be shaped by ecological and historical factors. Biotic and abiotic environmental causes are undoubtedly responsible for geographic variation in deep-sea species diversity; however, historical and hypothetical evolutionary scenarios have been recently pointed out as equally inductive causes (Tyler, 2003).

Deep-sea demersal fish species normally exhibit "k-strategy" life histories that include extreme longevity, late maturity, slow growth and low fecundity as well as other physiological, biological and behavioural adaptations to the deep-sea (Randall & Farrell, 1997; Koslow *et al.*, 2000). These can be adaptations to the patchy environments (seamounts, island and continental slopes) to which bentho and benthopelagic species, living at depth ranges between 200-1000m, are associated.
The deep-sea biogeography, community structure and population dynamics processes are not well understood yet and need to be studied further to have a full description of the deep-sea biodiversity and its distribution.

1.2.1 Deep-sea fisheries

Although some deep-sea fish species such as the black scabbard fish (*Aphanopus carbo*) in Madeira (Martins & Ferreira, 1995) or *Ruvettus* sp. in the South Pacific (Merret & Haedrich, 1997; Koslow *et al.*, 2000) have been traditionally exploited for centuries, it is only very recently that deep-sea resources have become interesting targets for large-scale commercial exploitation.
It was during the 60s and 70s when the first continental shelf fisheries started to show signs of over-fishing (when the maximum sustainable yield is diminished on a continual basis), that governments were forced to seek a solution for the decrease in world landings (Iudicello *et al*., 1999; Haedrich *et al.*, 2001). They faced a choice of either promoting the fishing of the until then "under-utilised" species or motivate and invest in the exploitation of new habitats. The deep-sea was the largest unexploited area remaining and therefore exploratory fishing of this new habitat began.

World War II provided new technologies that were subsequently utilised by fishery fleets and allowed the exploitation of less accessible populations and grounds further away from the coast with very irregular bathymetry (Gordon *et al.*, 1995). These technologies included satellite positioning systems, track plotters, radar, sonar and LORAN (Long-Range Navigation). These enabled boats to go further, orientate better and track fish schools beneath the sea (Dunlap, 1972). The Russians were the first to find deep-water habitats with large aggregations of large deep-sea fishes. The firm white flesh of these species, suitable for filleting and processing, and their abundance made them suitable for exploitation (Troyanovsky & Lisovsky, 1995). Several other countries followed suit and intensive fishing on deep-sea fish assemblages began, causing their economic value to rise enormously. Novel fisheries comprised among others, those on roundnose grenadier (North Atlantic), pelagic armourhead (North Pacific) or bluenose (South Pacific) (FAO, 1997; see Creasey & Rogers, 1999). These fisheries developed very rapidly during the 70s, reached their peaks in the 80s and nowadays they continue to be hit hard with fishing effort increasing exponentially. However, this is not solving the world fisheries crises, as like for many shallow water stocks, most deep-sea fisheries are already viewed as depleted or outside of safe biological limits (Koslow *et al.*, 2000). FAO declared that 70% of world's fishery resources are in decline and in danger of depletion. The world's fisheries cannot be sustained for much without the application of new fisheries management regulations based on full knowledge of biological life cycles of species and intraspecific dynamics (Haedrich *et al.*, 2001).

Deepwater fisheries exploit completely different groups of fishes than the continental shelf fisheries (Koslow *et al.*, 2000). Biological and life-history features of deep-sea fishes are likely to be very limiting for long-term sustainable fisheries. This may especially be the case on seamounts, where these features are more predominant amongst fish species. Fishes in these areas tend to be robust and deep-bodied to be able to swim in strong currents, making them good targets for exploitation. However their slow growth makes them sensitive to over-fishing because of their long life cycle and low levels of recruitment (Rogers, 1994; Koslow *et al.*, 2000).

1.3 Fisheries Management

Fisheries management consists of trying to achieve an optimum yield for fisheries populations by understanding the biological principles and characteristics of the species

(Allendorf *et al*., 1985). Its goal is to ensure that fisheries do not exceed the sustainability of a population, while maximizing its harvest (Grant *et al.*, 1999).

Since the beginning of deep-sea exploration, studies have been more concerned with the biological and ecological features of this newly accessible habitat in relation to ecosystem function. Relatively little research has been carried out on the response of fished populations to exploitation and now, research work cannot follow the rapid evolution of deep-sea fishing (Haedrich *et al.*, 2001). However, one thing is certain, deep-water fisheries cannot be expected to support the levels of exploitation applied to shelf populations (Gordon *et al.*, 1995).

Fisheries management is mainly based on the assumption that a unit stock has definable patterns of recruitment and mortality and hence a sustainable yield (Carvalho & Hauser, 1995). It is known that many marine species have high dispersal potential, as there are few physical barriers in open sea compared to terrestrial habitats. It is therefore hard to believe in species subdivision, population structure or even stock units in oceanic species. Nevertheless, there are several factors that can contribute to isolation of populations, such as the capacity for larval dispersal, oceanographic circulation, historic barriers or even reproductive barriers (Palumbi, 1996).

Without knowledge of the exact number of interbreeding populations of an exploited species, management policies may not achieve long-term conservation goals. All points of view have to be taken in to consideration and one should always have in mind that stock, population or species are hierarchical classifications of nature implemented by man (Avise & Walker, 2000).

The more common gears used in demersal deep-sea fisheries nowadays are trawls and long-lines which are not very specific or selective gears. Deep-water fisheries cannot be expected to support the levels of exploitation applied to shelf populations because deep-sea fisheries are characterized by high species diversity, low dominance by single-species and low production rates (Gordon *et al.*, 1995; Haedrich *et al.*, 2001). Management and conservation strategies have been developed and applied on these key resources more and more in the recent years.

In most parts of the world's oceans, fisheries management has run into serious problems especially with the implementation of new management systems based on common fisheries policies, as it is the case of the European Community (Symes, 1999). These policies are based on Total Allowable Catch (TAC's), quotas and delimitation of exclusive fishing zones

that try to manage the fishing resources at an international scale. To be able to achieve a sustainable development of fisheries resources at such a global scale it is necessary that an accurate knowledge on the species ecology and biology exists. It is important to understand the evolution and the population dynamics of species in space and time, to predict the impact of fishing exploitation and to forecast the efficiency of management measures, especially in terms of the biological mean of the imposed artificial delimitations.
As a matter of fact, the accurate delineation of stocks and their boundaries has become an essential part of fishery management (Grant *et al.* 1999).

Until now the most successful management policies are regional ones such as that of Nova Scotia or those within EEZs of Iceland and Norway. The bigger problem is with international waters, which correspond to most of the deep-sea environment. Here, all countries are allowed to fish and few specific fisheries regulations exist. The exceptions to this are where Regional Fisheries Management Organizations (RFMO's) have implemented regulations for specific fisheries such as the whaling hunt and drift nets ban in most worldwide waters.
Very recently the first specific deep-sea regulation at an international scale has been implemented by NEAFC by closing down 5 seamount areas to deep-sea bottom trawling in the North Atlantic.

1.4 Population genetics and exploited species conservation

Genetics have contributed to the general globalisation of the term "stock" by creating concrete boundaries and offering a more detailed analysis of the history and evolution of populations. Nowadays, the stock concept implies that genetic diversity and population structure must be considered and preserved in order to optimise the resource use (Bernatchez, 1994).
It is difficult to identify populations and migration between them in marine species using traditional ecological methods (Moritz & Lavery, 1996). Censuring and observation methods are quite difficult to perform on marine populations, especially deep-sea ones, and that is one of the main reasons why genetic information has become such an important tool for fisheries management (Slatkin, 1994). Other biologically important characteristics of populations, such as size and reproductive efficiency can also be assessed by determining the historical establishment of gene pools (Althukov, 1981). Molecular genetics brought an all-new range of questions that were not taken in consideration for lack of information on genotypes (Lincoln, 1995).

By analysing genetic variation one can discriminate among fishes at the species, population and even individual levels; identify hybrids; establish species and population phylogeny and phylogeographic history; discriminate different stocks, analyse their migration patterns and estimate their effective size; assess individual stock contribution to mixed stock fisheries and evaluate the response of stocks to fishing exploitation (Wirgin & Waldman, 1994; Ferguson *et al.*, 1995). Maintenance of stock viability is greatly promoted by the conservation of their gene pools, preserving genetic variability and hence, the possibility of future adaptation (Milligan *et al.*, 1994).

Fisheries biologists were the first to introduce the population concept with the publications of F. Heincke and J. Hjort in the beginning of the century. They observed that fish species presented sub-specific forms "like local races characteristic from different regions" or "varieties like geographic races or polymorphs" (Wright, 1978; Sinclair, 1988). This innovative perspective led to a new and probably one of the most employed concepts of species, the "biological species concept" by Mayr (1942) who defined a species as "groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups" (Rieseberg & Ungerer, 2001).
Therefore the "population" is one of the widest concepts used as a subspecific category and is commonly defined as a group of conspecific individuals forming a breeding unit sharing a particular habitat at a certain time, although this definition is still controversial.
The structure of a population consists of two parts: the demographic structure which includes processes associated with life history such as, mating system, birth, death and dispersal; and the genetic structure that is determined by the actual population structure, but also by mutation, selection and evolution (Slatkin, 1994).
Population genetics is concerned with the analysis of demographic and evolutionary factors affecting the genetic composition of a population (Hartl, 2000; Ewens, 2001). Assuming the focus of this study is population genetics there are some mechanisms and assumptions that have to be taken into consideration before proceeding into a more detailed discussion of fish genetics.

1.4.1 Principles of Population Genetics

It is easy to think of a marine species as a large panmictic population, i.e., a large random mating population, where no evolution occurs, and the gene pool of the population maintains its composition. But these theoretical populations do not exist in nature, they do undergo

evolution that can be driven by several main forces: mutation, genetic drift, gene flow and/or natural selection (Hoelzel & Dover, 1991).
Mutations are changes that occur within the DNA and that can produce new allelic forms and contribute to population differentiation. They can be a change at the single nucleotide level (transitions, transversions) or they can involve deletion, or the addition of one or more nucleotides or processes such as transposition, unequal crossing-over, slippage, gene conversion and duplication. Mutations can be silent or non-synonymous if they specify for the same amino acid or if they change it, respectively (Hillis *et al.*, 1996).

Allelic frequencies of a population can also change by chance, and this is known as genetic drift. This can cause reproductively isolated populations to randomly diverge in terms of genotype frequencies. The effect of this phenomenon on populations depends strongly on their effective population size (number of adults contributing to subsequent generations), the most important component of genetic drift, and its variation in time. In small populations genetic drift can cause strong population divergence and drastic changes in genotype frequencies from generation to generation. If the effective population size of a population is drastically reduced then genetic drift can increase and diversity of the population decrease (bottleneck effect; Russel, 1998). A similar effect can be detected when a population is established by a small number of breeding individuals (the founder effect; Hoelzel & Dover, 1991). In populations with large effective population size the effects of genetic drift are weak and insignificant compared to other evolutionary forces.

Genetically effective migration or gene flow occurs when individuals migrate from a population to another and introduce new alleles changing allelic frequencies within the recipient population. Actually, we can consider migration as a stabilizer of genetic divergence as it increases effective population sizes and reduces genetic drift. Theoretically, 1 to 10 individual migrants per generation maybe sufficient to prevent divergence (Slatkin, 1985).

The main evolutionary force, the natural selection, as introduced by Darwin is the capacity for genotypes to be selected by environmental factors and passed on to following generations. This is probably one of the main forces of evolution and is responsible for maintaining much of the phenotypic variation in nature. Natural selection can act in different directions and it can eliminate genetic variation or maintain it.

In order to understand all the structuring of populations in nature we have to take in to consideration all these aspects and analyse them by looking at variation of distinct alleles at defined loci known as molecular or genetic markers (Allendorf *et al.*, 1985; Gavrilets, 2001).

1.4.1 Molecular Markers

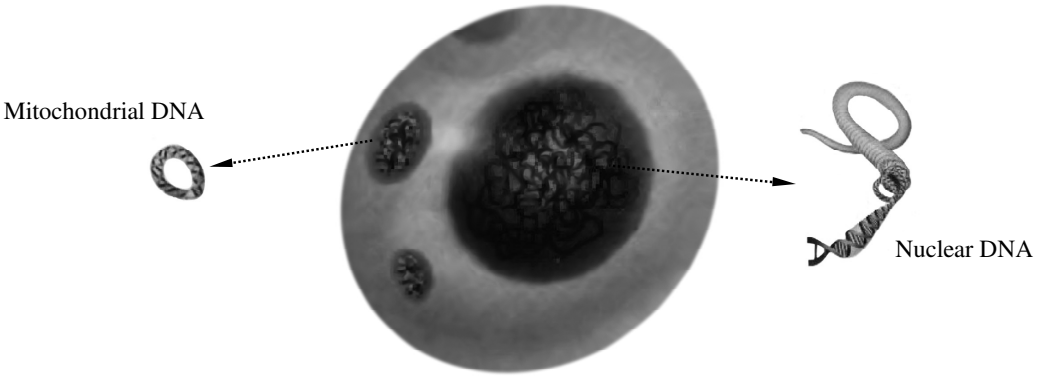


Fig.1.2 – Schematic diagram of DNA nature in cells.

There are many types of genetic markers available to describe population structure (Park & Moran, 1995), and all approaches are based on the premise that migration and mating patterns among populations determine the extent of a common gene pool and therefore their integrity (Carvalho & Hauser, 1998). All techniques provide interesting approaches to problems that need to be addressed and no molecular marker is inherently superior to another (Table 1.1). It is important and worthwhile spending some time analysing which technique or techniques are best suited to approach a certain problem (Hillis *et al.*, 1996).

Method	Types of information			Applications					
	Genotype frequency	Allele frequency	Allele geneology	Parentage	Relatedness	Hybridisation	Population structure: current	Population structure: historic	Population size (historical)
Alloenzymes	√	√	X	*	**	***	**	*	*
RFLP's	√	√	√	*	*	***	***	**	**
RAPD's	X	√	X	*	*	**	**	?	?
Minisatellites	X	X	X	***	**	X	*	*	**
Microsatellites	√	√	X	***	***	*	***	*	**
MtDNA Sequences	X	√	√	*	X	*	**	***	**

Table 1.1. Applications of available molecular markers; √ -Yes; X – No; * to *** - increasing effectiveness. Modified from Moritz & Lavery (1996).

Depending on the problem addressed, on the available resources, time and costing, some molecular markers can be more appropriate than others for studying a given problem.

For example, DNA sequencing provides a resolution appropriate to phylogenetic and population-level studies but microsatellites are more appropriate to studies of parentage and mating systems and sometimes may not be sufficiently conserved for population-level comparisons (Creasey & Rogers, 1999). Some times only by using a combination of different molecular markers can a problem be resolved.

It is not the scope of this section to serve as a manual of all existent molecular markers, but a little explanation on the markers chosen and used throughout this study will be presented.

MtDNA sequencing

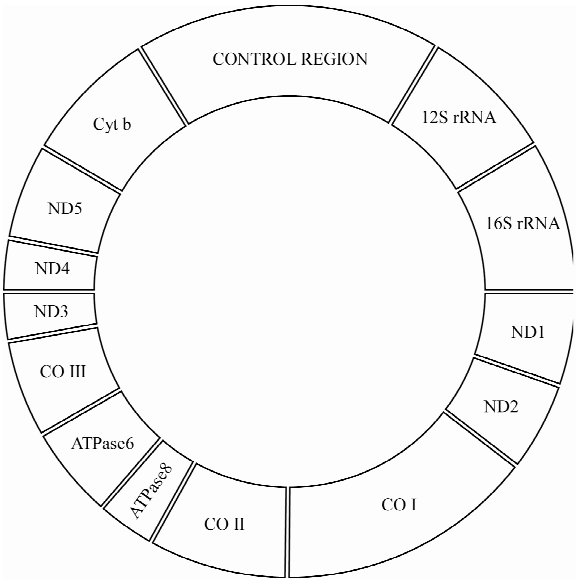


Fig.1.3. Schematic representation of the mitochondrial DNA molecule

Mitochondrial DNA sequences are one of the most widely used tools to assess phylogenetic relationships between similar species, populations of the same species or even between individuals (Taberlet, 1996). Mt-DNA is maternally inherited without recombination, representing only ¼ of the effective population size of nuclear markers, which makes it more sensitive to detect reductions in genetic variation (Ferguson & Danzmann, 1998). The mt-DNA molecule exists in a high copy number in the mitochondria of cells and has a circular structure (Fig.1.3). It is composed of 20,000 base pairs (bp) coding for 40 genes responsible for 2 ribosomal RNAs, 22 transfer RNAs, and 13 proteins essential in respiration (Ferris & Berg, 1987; Hartl & Clark, 1997). It also has a non-coding region (+ 1000bp) responsible for replication, known as the "control region" or "d-loop", that evolves 4 -5 times faster than the entire mt-DNA molecule which itself evolves 10 times faster than nuclear DNA (Brown *et al.*, 1979). It also has a large capacity to accumulate mutations that give it a faster rate of evolution than nuclear DNA in higher animals (Brown *et al.*, 1979). The whole molecule may be regarded as a single locus with multiple alleles (Park & Moran, 1995).

All these, and other technical advantages, such as requirement of only small amounts of fresh, frozen or alcohol-stored tissue, make mt-DNA a very practical genetic tool. Together with the fact that it is one of the most well studied parts of the animal genome, mt-DNA sequencing has become the molecular marker of choice when studying cospecific

populations (e.g.Gold *et al.*, 1993; Norman, *et al.*, 1994; Park & Moran, 1995; Bakke *et al.*, 1996; Taberlet, 1996; Stepien & Faber, 1998; Stepien, 1999; Stamatis *et al.*, 2004).

On the other hand, there are some disadvantages that must not be forgotten. For example, maternal inheritance does not provide information about males in populations, which may display different dispersal behaviour to females.

The haploid nature of this marker is also an issue as no inferences about the neutrality and equilibrium of populations, as well as other aspects based on allelic frequencies can be addressed.

Microsatellites

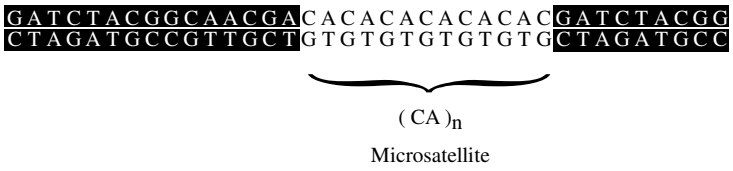


Fig.1.4. Schematic representation of a microsatellite tandem repeat.

Microsatellites are presently one of the most commonly used molecular markers. They consist of tandem repeats of short sequence motifs, from di- to hexanucleotides (2-6bp) that can reach a length of up to 150bp (Fig.1.4; Ashley & Dow, 1994; Schlötterer & Pemberton, 1994; Wright & Bentzen, 1994; Koreth *et al.*, 1996). There are several different types of microsatellite including uninterrupted, interrupted or compound microsatellites, as e.g. $(AG)_n$,, $(GC)_n$ $AT(GC)_n$ or $(GC)_n$ $(AT)_n$ $(GT)_n$ respectively (Estoup & Angers, 1998).

Microsatellites have been detected within the genomes of every organism ever analyzed (Hancock, 1999). They are very abundant in eukaryotes and are spread throughout the entire genome at $7\text{-}10^{-100}$ kilobase intervals (Schlötterer & Pemberton, 1994; Wright & Bentzen, 1994). The mean density of microsatellites within species of different taxonomic groups varies widely (Estoup & Angers, 1998).

What makes microsatellite loci very useful as molecular markers for genetic studies is their instability. The mutation rates of these sequence repeats are higher than other loci in the same genomes (Eisen, 1998) and are thought to be caused by slipped strand mispairing during replication, causing the insertion or deletion of repeat units (Ashley & Dow, 1994). Unequal crossing-over or gene conversions during recombination are other possible phenomena that cause mutations in microsatellites (Hancock, 1999). The high mutation rate

at these loci leads to extensive allelic variation and high levels of heterozygosity (Wright & Bentzen, 1994). Microsatellites are inherited in a simple Mendelian fashion, show no obvious functional role and are often selectively neutral (Ashley & Dow, 1994; Estoup & Angers, 1998).

Since the 1980's microsatellites have become one of the most popular molecular markers used in population genetic studies. Their main advantages over other markers are: high levels of polymorphism, high heterozygosity (sometimes above 50%), codominant single-locus inheritance, abundance in the genome and selective neutrality (Estoup & Angers, 1998). Microsatellite analysis can be applied to numerous ecological and evolutionary studies as population analysis (genetic structure, effective population sizes and sociobiological analysis), assessment of gene flow and hybridization zones, construction of pedigrees, mating systems and migration and inference of colonization patterns; besides forensic studies, linkage or genome mapping, and paternity testing (Ashley & Dow, 1994; Goldstein & Schlötterer, 1999).

Microsatellites also present some disadvantage features which make them unsuitable for phylogenetic and systematic studies. The technically demanding, time consuming and expensive development stage may also limit their potential use as genetic markers (Estoup & Angers, 1998).

Some of the attributes exhibited by microsatellites make them very suitable markers for fisheries research: (1) since they are very abundant, sufficient markers can be promptly developed using a single strategy; (2) their high allelic variation makes them attractive for several research topics including, species that present low levels of variation for other markers such as populations that have suffered severe bottlenecks, or recently derived or geographically proximate populations with limited genetic variation; (3) their codominant inheritance in a Mendelian fashion makes them more informative than other markers (mtDNA, RFLP's) for population structure studies; (4) as they are assayed by PCR only minute amounts of samples are required; and (5) although their development is expensive and time consuming, at a later stage in studies, screening of multiple loci can be efficient (Wright & Bentzen, 1994).

1.4.2 Bases for molecular data analysis

Independently of the molecular marker used, data from DNA is easy comparable and permits to search for the relatedness among organisms at any level, from sibling individuals to species of different kingdoms (Avise, 1994).

Population geneticists normally start by describing the genetic variation found in the molecular markers used. The most informative molecular tool available is DNA sequencing, given information on mutations at the single nucleotide level. This permits to test different models of sequence evolution against various alternative phylogenies (Swofford *et al.*, 1996). These are the principles of phylogenetics the base of all DNA sequences analysis. Phylogenetic methods try to compare and find the correct evolutionary path that connects different sequences, which may belong to individuals from the same species or from different families (Pages & Holmes, 1998). The alignment of sequences is very important and can be based in different methods and models. Maximum parsimony (MP) and maximum likelihood (ML) are the two main alternatives for phylogenetic reconstruction based on discrete characters. MP presupposes that the best tree (diagram which illustrates sequences' historical relationship) is the one that requires the least number of nucleotide changes along the branches. On another hand, ML is based on a certain model of sequence evolution to find the tree with higher probability. Several sequence mutational models exist in the literature based on base frequencies and substitution rates.

Alternatively, comparison of sequences can be made from distance data using clustering algorithm or optimal criterions adopted by methods such as UPGMA (Unweighted Pair Group Method with Aritmetic means) or Neighbour-joining. These methods tend to be used more often as they implicate less computational time and effort; however, they normally cause loss of genetic information.

Population genetics can be the next step, to determine if the genetic variation found in the molecular markers changes through generations or is portioned between populations. If this is the case, the main forces responsible and how they act to change allele frequencies should be determined.

Besides the existence of several molecular methods for studies of population genetics, there are also several analytical approaches by which one can extract biological information from molecular data (Weir, 1996a).

The level of information that can be obtained depends directly on the origin and type of molecular marker being used, especially if it is of a haploid (mitochondrial DNA) or diploid (nuclear DNA) nature. Genetic markers can be codominant, when we can distinguish the heterozygotes from homozygotes or dominant, when hetero and homozygotes are indistinguishable and both present the same pattern. Microsatellites and alloenzymes are codominant markers and allow analysis based on allelic frequencies and Mendelian

inheritance and therefore are the only ones that allow to test for fits to Hardy-Weinberg equilibrium, the basis of all genetic analysis.

Hardy and Weinberg independently worked out the mathematical basis of population genetics in 1908, showing that if the population is very large, random mating is taking place, there are no mutations and migrations affecting the allele frequencies in the population and genotypes have equal fitness, i.e., there is no selection; allele frequencies remain unchanged (or in equilibrium) over generations unless other factors intervene.

$p^2 + 2pq + q^2 = 1$ p=allele A frequency

AA Aa aa q=allele a frequency

If the observed frequencies do not show a significant difference from these expected frequencies, the population is said to be in Hardy-Weinberg equilibrium (HWE). If not, there is a violation of one of the assumptions of the formula and the population is not in HWE. This may indicate, small population size, assortative mating, inbreeding, high mutation rate, massive migration, selection or unequal transmission ratio of one or more genes. All these phenomena change allelic or genotype frequencies by increasing/decreasing homozygosity/heterozygosity. Nuclear markers, such as RAPD's are dominant, and to calculate allelic frequencies Hardy-Weinberg equilibrium has to be assumed. Mitochondrial markers behave as a single locus, and no heterozygotes or homozygotes exist making, H-W equilibrium impossible to be calculated. Genetic distances between sequences have to be calculated instead of allelic frequencies.

The representativity and method of sampling as well as the type of data used are also important factors to take in consideration on the analysis of molecular data (Weir, 1996). Several types of data can be extracted from molecular markers. For instance, band patterned based techniques such as allozymes, RFLP's or RAPD's produce data that can be transformed into presence/absence matrixes. Equally, the sampling strategy cannot be neglected because it has direct influence on the analysis of the data. Different samples from a population will show different levels of genetic differentiation because they will have different sets of individuals, which makes the randomness of sampling also an important factor (Weir, 1996b).

The analysis of molecular data must be based on theoretical or biological models (Weir, 1996a). In population genetics we must contend and understand the combined effects of numerous factors such as population size, patterns of mating, geographical distribution, mutational mechanisms, migration, natural selection and others. But factors are so many and interact in such a complex manner that they cannot be analyzed all at once. To simplify these situations, models have to be created where an essential factor(s) can be considered more important and others neglected, eliminating external superfluous detail (Hartl, 2000).
Several models have been proposed over the years but their description is far beyond the scope of this chapter and only a small overview will be given on them.
The first to emerge was the "classical model", which proposed an ideal population, infinite in size, with random mating and no disturbing forces as mutation, migration or selection. This model is completely hypothetical, as no such populations exist in nature. Little by little several steps were taken in order to introduce more realism to this model, by supposing that natural populations were finite or introducing specified forces of selection, mutation or migration.

Understanding of mutational mechanisms and rates of different molecular markers is also essential for an accurate estimation of population parameters.
Theories on sequence evolution have also been proposed and developed by several authors. Darwin (1859) was the first to propose a theory of evolution based on natural selection, far before the knowledge of the structure of DNA and mechanisms of inheritence. With Mendel's discovery that genetic variation was generated by mutation a new theory – Neo-Darwinism – emerged, in which mutation is recognized as the ultimate source of variation but the dominant role in shaping the gene-pool of a population was given to natural selection. Nowadays, evolutionary studies use the Neutral theory of evolution (Kimura, 1983) as the null hypothesis. This hypothesis states that genetic variability is caused by random genetic drift of selective neutral mutant alleles and if other evolutionary forces intensity offsets the influence of chance effects, the hypothesis is rejected.
Several sequence mutational models have been proposed based on base frequencies and substitution rates. The simplest one is the Jukes & Cantor (1969), which considers equal frequencies for the four bases and equal substitution rates. Others allow for unequal nucleotide frequencies and/or different substitution rates between transitions and transversions: Kimura 2-parameters (Kimura, 1980) takes into account different substitution rates between transitions and transversions calculated from the data; Tajima & Nei (Tajima& Nei, 1984) considers unequal nucleotide frequencies; Tamura & Nei (Tamura & Nei, 1994)

allows a distinction between purines and pyrimidines transition rates; and others more recent and complex such as the codon model or the secondary structure model continue to be developed to find the more realistic mutational model.

Several models of mutation have also been proposed by geneticists to describe the accumulation of mutations in microsatellites specifically: the SMM (Stepwise mutation Model; Kimura & Otha, 1978) - mutations increase or decrease allele sizes by single units; and the IAM (Infinite Allele Model; Kimura & Crow, 1964) – each mutation produces a new allele not present in the population; were the first. The KAM (K-allele Model; Crow & Kimura, 1970) – k different alleles are allowed and any allele has a constant probability of mutating towards any other (k-1) allelic states; and the TPM (Two-phased Model; Di Rienzo *et al.*, 1994)- in which mutations introduce a gain or loss of X nucleotides; derived from the other two.

Based on these assumptions, analysis of molecular data can be performed in different ways and be used to address different hypothesis or questions.

CHAPTER 2
CURRENT STUDY DETAILS

2.1. Characterization of the study area (North Atlantic)

The North Atlantic is the most completely studied of all oceans, and yet still resists thorough description and rationalization.

It is the youngest of all the oceans formed only 175-90 MY ago with the separation of the Pangea continent. The distance between its two margins is relatively small which means that margins strongly influence surface and deep-water phenomena (Longhurst, 1999).

Surface and intermediate waters derived from other ocean's move northward through the South into the North Atlantic. These warm waters suffer intense cooling in the Norwegian Sea and sink by vertical convection forming the Northeast Atlantic Deep Water. This water mass moves into the Northwest Atlantic basin where it mixes with other water masses forming the North Atlantic Deep Water which by its turn flows southward into the Southern Ocean and other oceans thereafter (Schmitz & McCartney, 1993). The warm and saline Mediterranean water is also important for the Mid-water (1000-1500m) circulation pattern.

Surface and intermediate waters of the North Atlantic are influenced by the strong cyclonic gyre provoked by the Gulf Stream current (Fig.2.1). When this reaches the middle of the ocean in its western-eastern movement, breaks into two branches: the North Atlantic drift (flows in direction of Northern Europe) and the Azores drift that passes just south of the archipelago with the same name (Onken, 1993). The Azores current breaks in two directions flowing through the Gulf of Cadiz and Canary Islands down the African coast (Johnson & Stevens, 2000).

One of the most important geographic features is the Mid Atlantic Ridge, which divides the North Atlantic in two halves and whose topography has a major influence on the near-bottom water circulation and other processes (Levin & Gooday, 2003).

Other topographic features that can influence hydrographic events but at local or regional scales are oceanic islands and seamounts. Most North Atlantic seamounts are distributed along the Mid-Atlantic Ridge, however, several others are spread throughout the entire basin apparently in sort of bunches (Epp & Smoot, 1989). Some archipelagos are also found within the North Atlantic basin, such as the Antilles, the Bermudas, Cape Verde and the Macaronesian archipelagos: Azores, Madeira and Canaries. The term seamount is generally defined as a submarine volcanic mountain that rises several kilometres from the sea bottom,

with circular or elliptical forms, to sea level (Epp & Smoot, 1989). Seamounts are places with high productivity where a lot of valuable fish species concentrate (Rogers, 1994) because they work as favourable patchy habitats for bathyal benthic and benthopelagic species separated by vast distances of open water. They have already been pointed out as possible intervenients in the distribution of species, working as stepping stone places for oceanic colonization processes or in migration patterns.

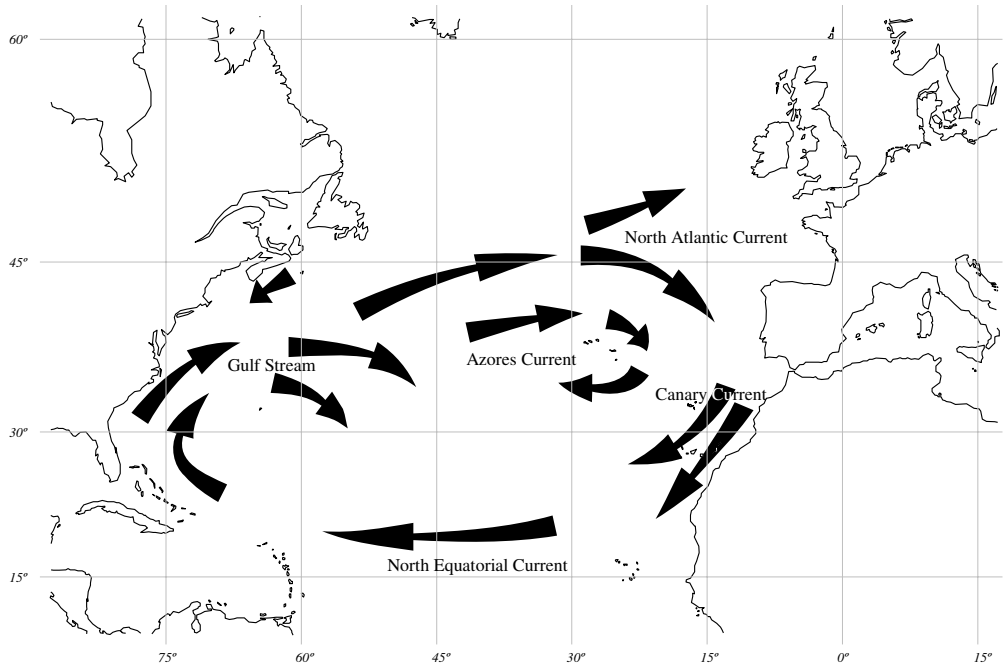


Fig.2.1 Simplified schematic representation of surface circulation in the North Atlantic.

2.1.1 The Azores archipelago

The Azores archipelago is situated in the middle of the Northern Atlantic Ocean, on the top of the mid Atlantic Ridge at the junction of 3 tectonic plates: North American plate, Eurasian plate and African plate (Fig. 2.2; Buforn *et al.*, 1988).

It's a group of 9 volcanic islands distributed between 37º-40ºN and 25º-31ºW that have risen from the ocean basin 4000m below. The weather is extremely variable in this area because it lies within a temperate zone of the northern hemisphere that can experience polar low-pressure fronts all year around (Morton *et al.*, 1998). Sea temperature changes between 15ºC

to 23ºC and the surface isotherms place Azores in a boundary between tropical and temperate regions (Gorshov, 1978).

Azores is situated in a very particular position in respect to sea circulation (Martins, 1987). The archipelago is in the middle of the North Atlantic basin and respective current gyre suffering direct influence of the Gulf Stream (Fig.2.1). This movement can be in the origin of colonization of the islands by some New World fauna and flora (Morton *et al.*, 1998). As we go deeper into the slope water region (1.8º-4ºC) the current pattern changes a lot being more influenced by the Northeast Deep-Water mass formation formed by vertical convection near the Norwegian Sea and that will find its way into the Northwest Atlantic basin forming the North Atlantic Deep-Water (see page 20 and Levin & Gooday, 2003).

The Azores archipelago is located in the ICES fishing area X. Is characterized by a variable topography, rocky bottoms and because big valleys separate the islands no continental platform is formed (Martins, 1987).

The coastal fishing areas are few and narrow constituting only 1,1% of the EEZ (Economic Exclusive Zone), considering depths <1000m (Isidro, 1996). This value rises to 3% if we include the several seamounts that exist around the islands of the archipelago, and are also productive places that permit fishing activity at shallower depths (Martins, 1987 and Isidro, 1996). Several seamounts make part of the Azores territory being the most important fishing areas in the archipelago.

Due to all these features, Azores is one of the most exceptional areas to fish and study of the Atlantic Ocean and should be carefully considered in global management proposals especially due to its small area of fishing grounds compared with most of the countries in which, continental shelf constitutes 50% of their EEZ (Martins, 1987).

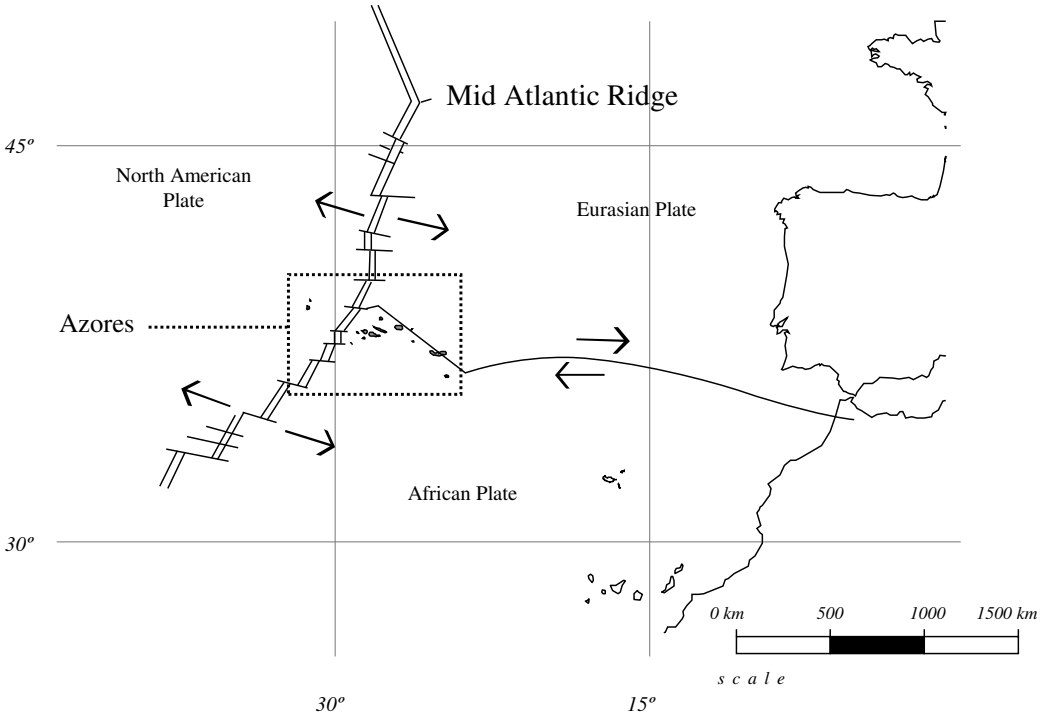


Fig. 2.2 The Azores archipelago.

2.2 Demersal fishes and fisheries in the studied area

The deep-sea demersal fish community is best known in the North Atlantic than in any other ocean in the world. A wide rate of investigations on the abundance, faunal composition, vertical and horizontal distribution, biogeographical and temporal patterns of deep-sea demersal fish species have been compiled by Thiel (1983), Haedrich & Merrett (1988), Vinck *et al.* (1994) and Haedrich (1997) for example.

There are around 505 species (belonging to 72 families) of deep-sea demersal fish species on the slope of the North Atlantic, 77.4% of which are endemic to this topographic area.

Alefocephalidae (slick heads), Macroridae (grenadiers) and Ophidiidae (cusk eels) are the dominant families in this order (Merrett & Haedrich, 1997 and Levin & Gooday, 2003)

Nevertheless, an accurate description of the deep-water fishery in the North Atlantic is a difficult task, as some of these fisheries tend to develop very quickly and decline equally rapidly. Simultaneously, it is often difficult to determine the target and by-catch species from landing data and consequently, to obtain accurate catch and effort data.

Some deep-water fisheries are well established, hence well described, and have been exploited for almost a century now, such as the long-line fishery for black scabbard fish (*Aphannopus carbo*) in Madeira and the semi-pelagic trawl fisheries for blue whithing (*Microsmesistius poutassou*) and argentine (*Argentina silus*) in the Rockall Trough. Others are more recent but also long-established fisheries like redfish (*Sebastes spp.*) and Greenland halibut (*Reinhardtius hippoglossoides*) in the Norwegian Sea, crustaceans and *Pagellus bogaraveo* off the Iberian Peninsula, and roundnose grenadier (*Coryophaenoides rupestris*) in the Northwest Atlantic. Trawl fisheries to the West of Scotland and Ireland are probably the more important and mediatic fisheries nowadays (Koslow *et al.*, 2000; Gordon, 2001; Haedrich *et al.*, 2001).

In the Azores, the demersal fishery is the most important resource after the tuna fishery, in terms of capture quantities (around 30% of total landings) and commercial value (around 50%) (Menezes & MdaSilva, 1997; Lotaçor, 2003).
Is mainly directed to *Pagellus bogaraveo*, but it's considered a multispecific fishery as other demersal fish species are equally captured in significant quantities: bluemouth (*Helicolenus dactylopterus*), alfonsino (*Beryx splendens*), imperador (*Beryx decadactylus*), conger (*Conger conger*), forkbeard (*Phycis phycis*), wreckfish (*Polyprion americanus*), white scabbardfish (*Lepidopus caudatus*) and others (Menezes *et al.*, 1997).
In terms of fisheries conservation, the North Atlantic fisheries management is mainly divided into five international organizations which are responsible for the management of fisheries and conservation of the resources: the International Council for the Exploitation of the Sea, the Northeast Atlantic Fisheries Comission, Northwest Atlantic Fisheries Organization, the Western Central Atlantic Fishery Comission, Fishery Committee for the Estaern Central Atlantic and a regional organization the Sub-regional Comission on Fisheries responsible for the Cape Verde area (Fig.2.3).

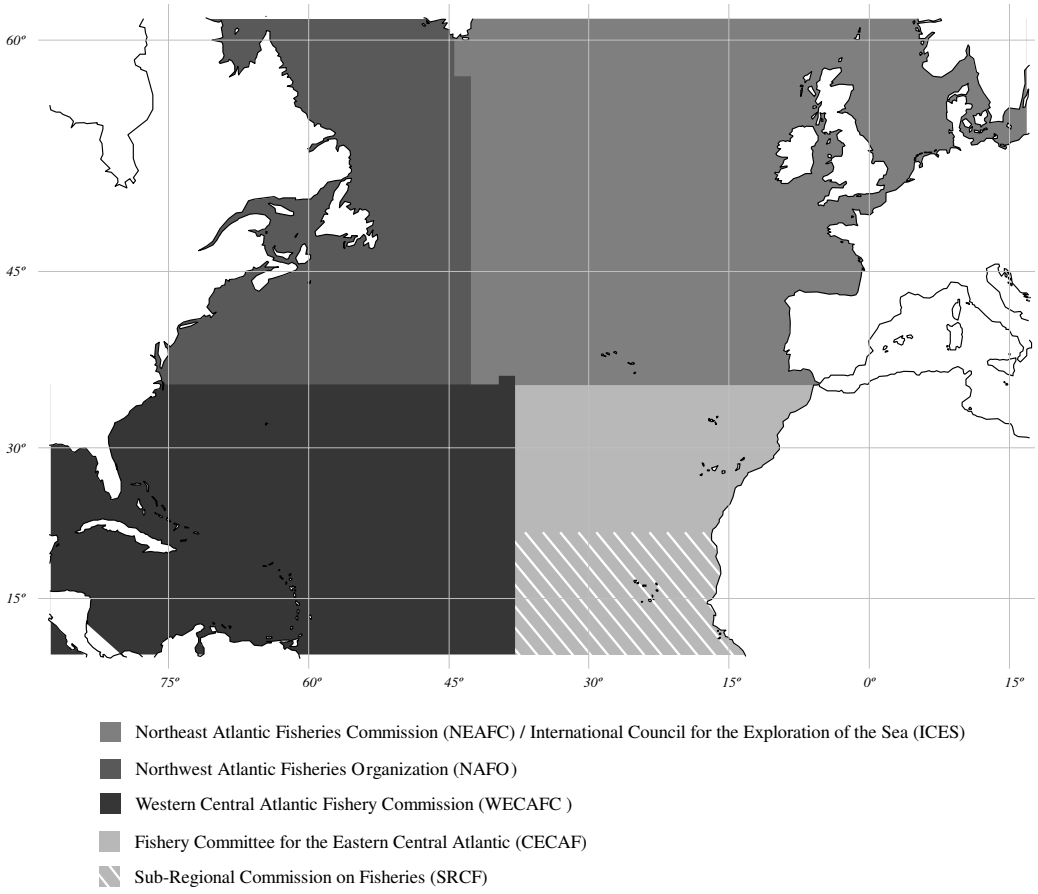


Fig. 2.3 North Atlantic Fisheries Management Organizations map

2.3 Characterization of the studied species

2.3.1. *Helicolenus dactylopterus* (Dela Roche 1809) - BLUEMOUTH

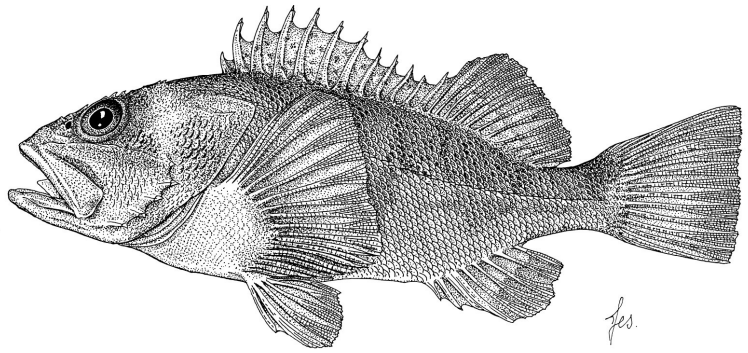


Fig.2.4

Morphology and Taxonomy

Helicolenus dactylopterus (Dela Roche 1809) is a demersal fish of the Order Scorpaeniformes and belongs to the Family Sebastidae. Is commonly known as bluemouth (English), rascasse du fond (French) or boca-negra and cantarilho (Portuguese). *H.dactylopterus* possesses a compressed body, head with ridges and spiny fins. As indicated by its name, it can be easily recognized by the large mouth with a darkly colored trachea. It has a variable colour, normally reddish, along the dorsal ridge and flanks, with a pink to white ventral surface coloration. The flanks maybe patterned with bars of darker color.

The morphological differences between the populations of this species along with their geographical and ecological separation have been little studied. For this reason the phylogenetic position of the genus has not yet been defined. Some authors have included it in the family Scorpaenidae and the sub-family Sebastinae (Echmeyer, 1969; Nelson, 1984 and Hureau & Litvinenko, 1986) while others place it in the family Sebastidae and the sub-family Sebastinae (Eschmeyer, 1998; Froese & Pauly, 2005). The genus *Helicolenus* contains 9 species including *H.dactylopterus*, *H.alporti*, *H.avius, H.barathi, H.fedorofi, H.higeldorfi, H.legerichi, H.mouchezi* and *H.percoides*. *Helicolenus dactylopterus* has been divided into 4 different populations corresponding to the areas of South Africa, the Golf of

Guinea, Northeast Atlantic (from Norway to North Africa) and Northwest Atlantic (Nova Scotia to Venezuela) (Echmeyer, 1969). This was prior to the work of Barsukov (1980) that proposed the subdivision of the species *Helicolenus dactylopterus* into several subspecies of which *Helicolenus dactylopterus dactylopterus* corresponds to the northeast Atlantic and Mediterranean zones that include our study area (Azores).

Distribution

Like many fish in this family (Sebastidae) bluemouth is typical of high-energy continental and oceanic island slopes and seamounts. Is commonly found between 400-600m depths along the edge of the continental shelf and upper continental slope of the eastern Atlantic (from Norway to the Gulf of Guinea), the Mediterranean (Massutí *et al.*, 2001) the western Atlantic (from Canada to Brazil), and off the west coast of South Africa. It is also distributed on the slopes of the Macaronesian islands (Azores, Madeira, Canaries and Cape Verde), on seamounts of the Mid-Atlantic Ridge (Hureau & Litvinenko, 1986) and non-axial seamounts such as the Josephine Bank (Maul, 1976).

Biology and Ecology

Helicolenus dactylopterus is a representative long-lived deep-sea fish with conservative reproductive traits. It is known that adult fish lead a very sedentary life style according to tagging experiments and seabed observations. Around the Azores archipelago many tagged specimens have been recaptured, after more than one year, exactly in the same places as they were originally caught and tagged (Menezes unpubl. data).
In the Bay of Biscay several individuals of this species were observed remaining in place at the bottom even in the proximity of a submersible. Bluemouth can be characterized as a typical sit-and-wait ambush predator, attacking prey as they pass (Uiblein *et al.*, 2003). Is considered a selective carnivorous feeding mainly on benthic crustaceans (decapods), fishes and gelatinous plankton (pyrosomes) and accidentally some cephalopods and polychaetes (Nouar & Maurin, 2000).

Studies indicate this species has a relatively slow growth rate, considerable longevity and sexual dimorphism (Isidro, 1989; Kelly *et al.*, 1999).
A maximum age of 43 years old and length of 47cm have been recorded for males, which are more abundant in the larger length classes (Estácio *et al.*, 2001). The mean length of females

is significantly smaller than males (White *et al.*, 1998) but they can also reach lengths of 46cm (Estácio *et al.*, 2001).

In terms of growth rate, dimorphism is also found; male growth is fastest during the first few years while females exhibit growth acceleration between 7 and 11 years (Esteves et al., 1997).

Very little is known about the life cycle of this species, especially its reproductive strategies, and until now, information was quite contradictory on this subject (Krefft, 1961). The order Scorpaenidae is mainly oviparous with some confirmed cases of viviparity only in the family Sebastinae (Indian Ocean). However, the reproductive mode of *Helicolenus* appears to lie between that of *Scorpaena* and *Sebastes* in being intermediate between the viviparous and oviparous condition (Krefft, 1961). This species is known to have internal fertilization with brooding of eggs until the early stage of development (early embryo) consistent with zygoparity (Muñoz *et al.,* 1999; Sequeira *et al.*, 2003). Spawning occurs multiple times in a single season and fecundity is relatively high for a scorpaenid (11,000 to 87,000 eggs) (Muñoz & Casadevall, 2002). This leads to the assumption that this species may have a more limited dispersal than fish species with broadcast spawning. Curiously, asynchrony has been found between the annual reproductive cycles of the two sexes. Females reach maturity between January and May, while males are mature during the period from June to October. Fertilization takes place during this time and females store the sperm in the ovaries for periods up to 6 months (Isidro, 1989; Estácio et al., 2001). Females mature at 3 years of age while males mature later at 5 (Krug *et al.*, 1998). Other studies have confirmed this life cycle for *Helicolenus dactylopterus* in other parts of the world (Petrakis *et al.*, 1998; White *et al.*, 1998; Kelly *et al.*, 1999 and Muñoz *et al.*, 1999).

Specific fisheries data

The fishing grounds of bluemouth are spread throughout the entire North Atlantic. It does not have a specific directional fishery but is caught by long-line or trawls that operate at various depths.

European catches have fluctuated and increased a lot in the last decade (Fig.2.5). In 1998 production rates of bluemouth have tripled reaching the highest peak of 0.6 MT in two years. Since then it has decreased considerably but has not yet reached the catch levels recorded in the beginning of the 90's.

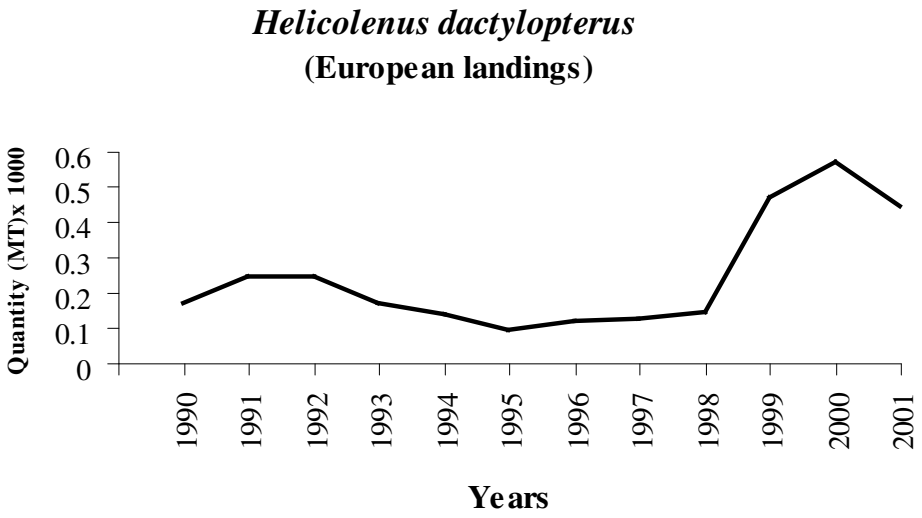


Fig.2.5 Annual landing rates of *Helicolenus dactylopterus* in Europe (FAO, 2004)

In the Azores bluemouth is caught by long-line fishery directed at *Pagellus bogaraveo*, and represents an significant proportion of the total landings and commercial value of the demersal fishery. The actual annual landings are approximately 350-ton a year, which represents half of the quantity presented in 1994 the most productive year for demersal fisheries in the archipelago (Fig.2.6; Lotacor, 2003). In the Azores, bluemouth is commonly caught with total lengths between 14 and 47 cm, which for male individuals correspond to an age of 3 to 14 years old and for females, 3 to 12 years old.

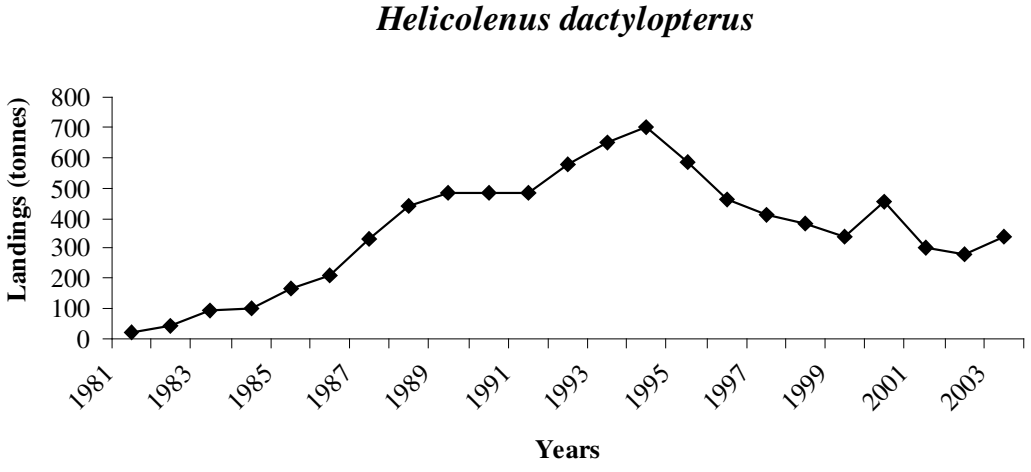


Fig.2.6. Annual landings of *Helicolenus dactylopterus* in Azores (Lotaçor, 2003)

2.3.2 *Beryx splendens* (Lowe 1834) – ALFONSINO

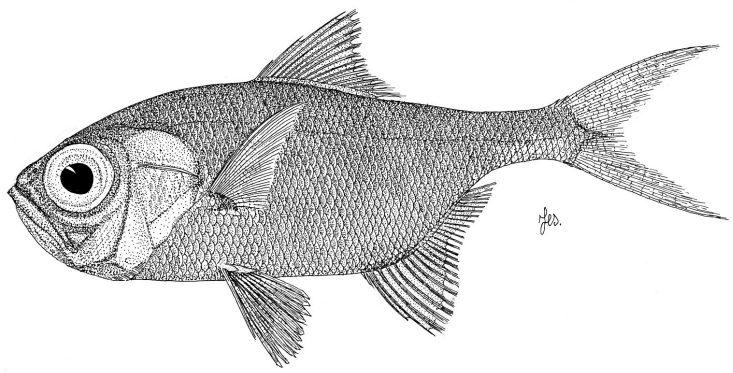


Fig.2.7

Morphology and taxonomy

Beryx splendens (Lowe 1834) is a demersal fish species of the family Berycidae commonly known as alfonsino or golden eye perch (english), béryx long (French) or alfonsim and salongo (Portuguese) (Bougis, 1945). This species is characterized by its slightly elongated body shape, bright red colour, big eyes and robust strong-swimmer type body.
The Berycidae family is constituted by the Centroberyx and Beryx genera and this last one includes three species: *B. splendens*, *B. decadactylus* and *B. mollis* (endemic of Osaka Bay).

Distribution

Beryx splendens has a worldwide distribution from tropical to temperate waters (Bougis, 1945 and Busakhin, 1982). It inhabits rocky areas of the slope or seamounts at a wide range of depths (25-1240m) but is normally found between 400-600m (Maul, 1986; Ivanin, 1987 and Relini *et al.*, 1995). This species has a wide spread distribution in waters of the eastern Atlantic (Portugal, Spain, Morocco, Madeira, Azores and Canaries Islands and as far south as South Africa), western Atlantic (Gulf of Maine to the Gulf of Mexico), Pacific (Japan and Australia) (Ikenouye, 1969; Bushakin, 1982; Maul, 1986; Ivanin, 1987; Lehodey *et al*., 1994 e 1997; Lehodey & Grandperrin, 1996a e 1996b) and it has also been recently recorded in the Mediterranean sea (Relini *et al.*, 1995).

Biology and Ecology

Very little is known about biology and life cycle of *B.splendens* and most existent works refer to age and growth (Ikenouye 1969; Massey & Horn 1990; Rico *et al.*, 2001), reproduction (Lehodey *et al.*1997; González *et al.*, 2003), development (Mundy, 1990), systematics and distribution (Lehodey *et al.*, 1994) in areas where this species is an economically important fishery resource such as off Japan, New Zealand and Russia.

Alfonsin larvae and juveniles are epipelagic for several months, apparently moving deeper and settling on the bottom as they grow (Mundy, 1990). Actually, the hypothesis of two differentiated parts of the life history has been proposed, known as the vegetative zones, constituted by larvae and juveniles in a growing phase and the reproductive zones, represented by the mature individuals ready to spawn (Lehodey & Grandperrin, 1996a).

Adults are benthopelagic and representative of the typical strong swimming, robust, deep-bodied fish typical of the demersal habitat of seamounts and continental slopes (Koslow *et al.*, 2000). They are normally found in groups 5-20m of the bottom of the upper slope, or even in schools 10-50m above seamounts (Maul, 1981,1990).

It is presumed that individuals have a continual feeding activity and are known to migrate vertically over night for feeding on fish and crustaceans on the slope (Galaktionov, 1985; Dubochkin & Kotlyar, 1989). The diet of *B.splendens* consists of small meso- or benthopelagic fishes and decapods or species with diurnal vertical migration (Dürr & González, 2002).

Alfonsinos can live until 20 years of age and reach lengths greater than 50cm. Median life expectancy is 12 years for females (≅ 43,5cm) and 9 years for males (≅ 36 cm). Like many other fishes, individuals grow throughout their entire life with a higher rate during first year (18-19cm) during which they reach 50% of their total length and progressively decreasing until reaching an annual rate of 2 to 5 cm at around 8 years of age (Lehodey & Grandperrin, 1996a and Anibal *et al.*, 1998). It is not evidently clear but it can be said that females grow faster than males and medium size increases with depth (Lehodey *et al.*, 1994; Rico *et al.*, 2001).

The first sexual maturation occurs around the second year of life but L_{50} is only reached at 7 years old by males and a little bit earlier by females, at 6 years old. In both hemispheres maturation occurs in late Spring and spawning during the Summer months, followed by a pos- laying period in the autumn and rest until next spring (Ikenouey, 1969; Mundy, 1990; Lehodey *et al.*, 1994 and 1997; Lehodey & Grandperrin, 1996a). But there is also the theory that spawning season of this species varies with climatic zones (González *et al.*, 2003).

B.splendens is gonochoric with no evidences of sexual dimorphism (González *et al.*, 2003). Fecundity is high (270 000-700 000 eggs per spawn) typical of a K-type reproduction strategy (Lehodey *et al.,* 1997).

Specific Fisheries Data

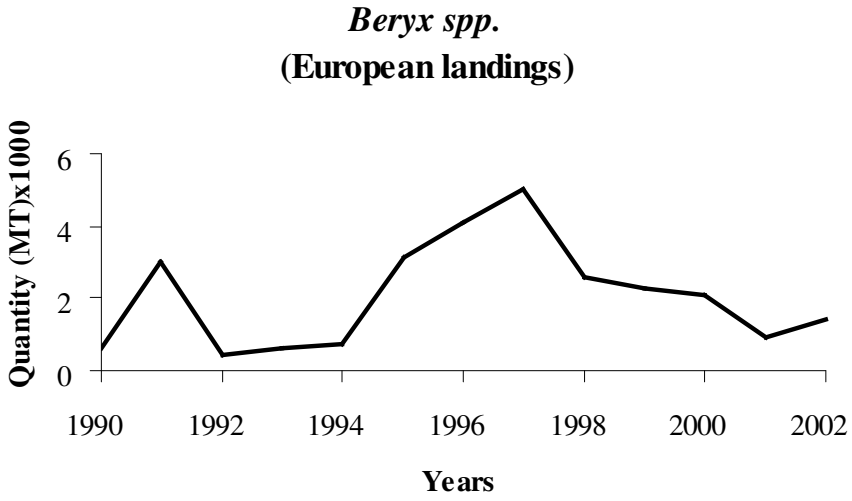


Fig.2.8. Annual landing rates of *Beryx spp.*in Europe (FAO, 2004).

Commercial fisheries for alfonsino exist where they are abundant. It is normally caught as by-catch by bottom long-line or trolling gears which operate in deep areas of the slope or seamounts (Galaktinov, 1985; Maul, 1986; Ivanin, 1987and Relini *et al.*, 1995). In some places, is the main target species of small-scale demersal fisheries performed with handlines and bottom drop lines (Rico *et al.*, 2001).

Catches are usually recorded for *Beryx spp.* as a whole (Fig.2.8) except for rare occasions when specific statistics are presented for specific regions, such as in Azores (Fig.2.9; Lotaçor, 2003).

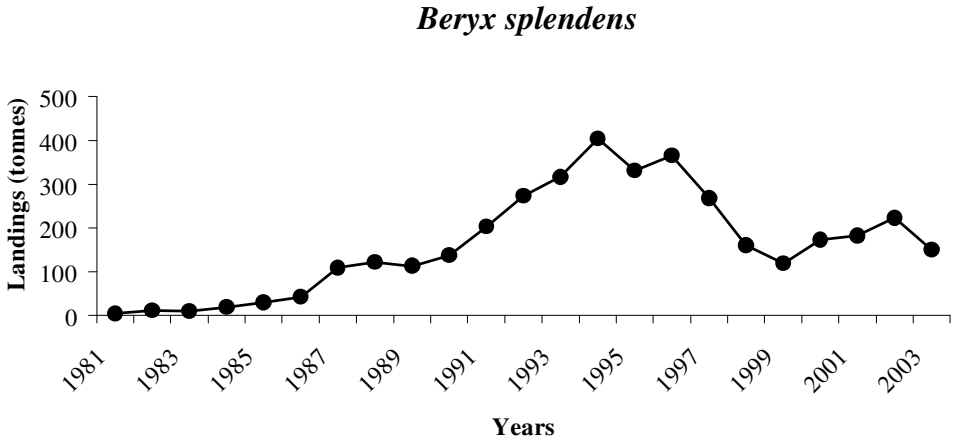


Fig. 2.9. Annual landings of *Beryx splendens* in Azores (Lotaçor, 2003)

2.3.3. *Beryx decadactylus* (Cuvier 1829) – IMPERADOR

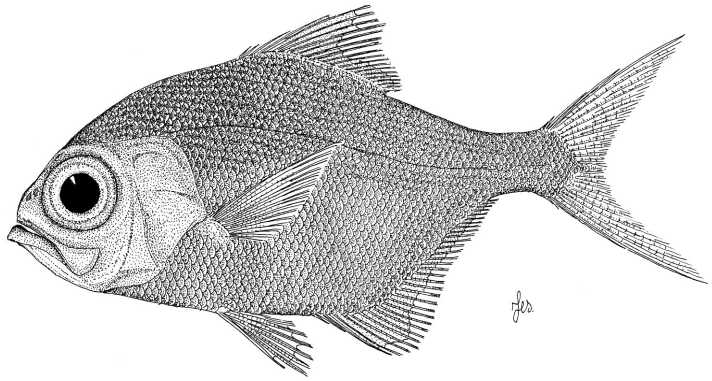


Fig.2.10

Morphology and taxonomy

Beryx decadactylus (Cuvier, 1829), commonly known as imperador (Portugal and UK) in several places is the second representative species of the Berycidae family in the Atlantic. It is quite difficult to differentiate between this species and *Beryx splendens*. Both species are sympatric apparently with similar biology and morphology, differentiating from one another only in body shape. *B.decadactylus* has a rounder and wider body than *B.splendens*, showing

a head length smaller than body height. In early life stages it is almost impossible to differentiate between individuals from the two species based on morphology alone (Mundy, 1990).

Distribution

Beryx decadactylus is found in the Atlantic, Mediterranean, southern Indian Ocean, western Pacific and Hawaii; around seamounts and continental slope at 400-800 m depth.

Biology and Ecology

No studies exist on the biology, behavior or ecology of *Beryx decadactylus* alone. Most information about this species is extrapolated from *Beryx splendens* works because of the similarity between the two species. There is some comparative work on the two species focusing on diet (Dürr & González, 2002) and larval morphology (Mundy, 1990).

Specific Fisheries Data

As stated earlier, landing statistics are normally presented for *Beryx spp.* as a whole (Fig 2.7), except for some small fisheries areas, e.g.Azores (Fig.2.12). However, with this information, it can be said that *Beryx decadactylus* is caught in smaller numbers at greater depths than alfonsino.

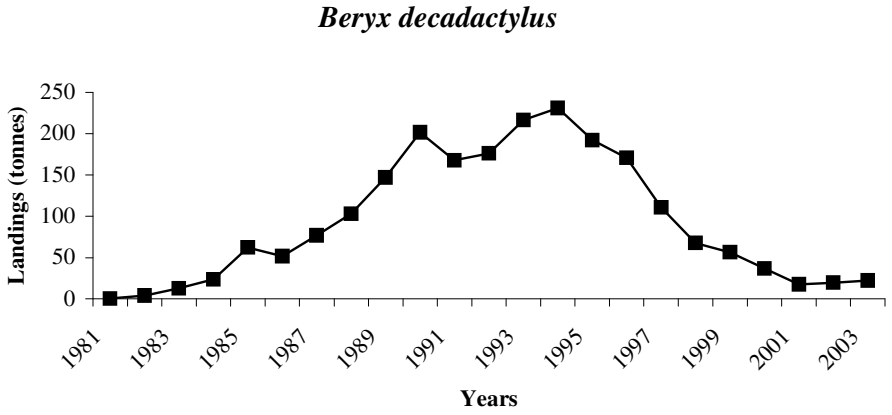


Fig.2.11 – Annual landings of *Beryx decadactylus* in Azores (Lotaçor, 2003).

2.3 Actual state of work done on the subject

Since genetics has become an important tool in marine species conservation, several studies of this matter have been conducted on deep-sea fish species, especially on those subject to exploitation (reviewed in Creasey & Rogers, 1999 and Rogers, 2003).

Because of the extreme environmental conditions of the deep-sea it is very difficult to discern interactions of target species at the population and community level either by *in site* observation or indirect approaches. Molecular techniques can provide valuable data about phylogeny, structure and endemicity of target species. Together with morphometric and hydrographical data can be used to infer population structure, gene flow, recruitment, reproductive strategies and behaviour of deep-sea species.

Over the last quarter of century numerous genetic studies have been undertaken to determine variation and population structure of deep-sea organisms using a variety of molecular techniques.

Until the mid-ninety's, allozyme electrophoresis was widely used to study many deep-sea species of invertebrates and vertebrates including crustaceans (France, 1994; Creasey, 1998), molluscs (Gooch & Schopf, 1972), echinoderms (Ayala *et al.*, 1975) and fish (Johnson & Utter, 1976; Lowry *et al.*, 1996). With progress, molecular techniques like RFLP's, RAPD's and mtDNA sequencing were quickly adopted and applied on deep-sea studies in order to explain genetic diversity (e.g. Miya & Nishida, 1999), phylogeography (e.g. Kojima *et al.*, 2001), demographic history and phylogeny of deep-sea organisms. Hydrothermal vents organisms' phylogeny and population structuring have also been approached using these techniques (e.g. Creasey *et al.*, 1997; Autem *et al.,* 1985). More recently, microsatellites have also been undertaken and used to try to solve finer-regional scale population differentiation on organisms like corals (Le Goff-Vitry *et al.*, 2004), toothfish (Smith & McVeagh, 2000), and redfish (Roques *et al.*, 1999a and 2001) for example.

However, contrary to the big number of studies on the biodiversity of deep-sea species in the North Atlantic, genetic studies are quite more rare. Most of molecular approaches have been made to deep-sea species from the Pacific Ocean (reviewed in Rogers, 2003).

In the North Atlantic deep-sea demersal fish species' population genetic studies exist on goraz (Stockley, 2005), wreckfish (Sedberry *et al.*, 1996), redfish (Roques *et al.*, 2001), cod (Ruzzante *et al.*, 1996), and some others, but lots of work still needs to be done, to obtain an accurate vision of the general population structure and biology of these species.

Since 1995, a project for monitoring the Azorean demersal fishery commenced in order to obtain the basis for an optimised management strategy of the resource (Menezes *et al*., 1998). Seasonal research cruises have been annually conducted to obtain information on the ecological and biological features of the demersal fish species, and several studies on age, growth, reproduction and feeding have been published (Esteves *et al.*, 1997; Morato *et al.*, 1999, 2001; Estácio *et al.*, 2001; Rico *et al.*, 2001; González *et al.*, 2003).

A genetic approach for characterisation of the life history of commercial species has been carried out for a few species. Some very preliminary work was done using allozyme electrophoresis and only recently some work on DNA has been done on some of the most captured demersal species in the archipelago such as *Pagellus bogaraveo*, *Beryx splendens* and *Polyprion americanus* (Sedberry *et al.*, 1999; Stockley, 2001; Stockley *et al.*, 2005).

2.4 Aims and objectives

With increased focus upon the deep-sea environment and the exploitation of deep-sea resources, it is apparent that understanding of the degree of population/stock structuring and evolutionary story is essential, in order to comprehend the impact (direct /indirect) of fishing and the future of the species.

In the present study, DNA sequencing and microsatellites were used to investigate the level of genetic differentiation of North Atlantic populations within three deep-sea demersal fish species commercially exploited in the Azores archipelago by a bottom long-line fishery directed at *Pagellus bogaraveo*.

Helicolenus dactylopterus (Sebastidae), *Beryx decadactylus* and *Beryx splendens* (Berycidae) are significant representatives in terms of quantity and commercial value of the demersal fishery.

MtDNA sequences and microsatellites can provide information on the degree of stock separation and population structure between different areas at an oceanic and regional geographic scale. An idea about life-history, reproductive strategy and behaviour can also be obtained for each species. Aspects of evolutionary and demographic history can equally be inferred.

Most of all, the three different species can be compared in terms of all these different aspects and an overall comprehension can be extrapolated for North Atlantic deep-sea demersal fishes in general.

This thesis provides the first examination of the population genetics of these selected deep-sea Azorean fish and North Atlantic species exploited by demersal fisheries. To deeply understand the local population dynamics of the studied species, a wider geographic scale

has to be analysed, and the geographical area of interest to this study was whenever possible expanded to the North Atlantic scale.

Section II refer to all the material and methods used throughout this study and include Chapter 4, a detailed explanation of the methodology used to develop and design microsatellite markers for *Helicolenus dactylopterus*.

In Chapter 5 the population structure of *Helicolenus dactylopterus* is analysed by sequences of two mtDNA regions, a non-coding (control region) and a coding region (cyt *b*). Evidences for historical influences on the genetic population structure are also given.

Chapter 6 gives an account of the population structure of *Helicolenus dactylopterus*, but at a finer regional geographic scale (Northeast Atlantic and within island groups of the Azores archipelago) using more sensitive molecular markers, the microsatellites developed in Chapter 4.

Chapter 7 refers to other two deep-sea demersal species *Beryx splendens* and *Beryx decadactylus*. The population structure in the Northeast Atlantic of these two close related species is described by analysis of mtDNA sequences. A comparative approach and possible explanations for differences in population structure and biological aspects are presented.

Chapter 8 discusses the results of the other chapters in a general context of evolution, conservation and relevance of this study to the knowledge of deep-sea demersal fish species population genetics as well as for the fisheries management of these resources.

PART II
MATERIALS AND METHODS

CHAPTER 3
GENERAL METHODOLOGY

The aim of this section is to give a general view of the techniques used throughout this study. General methodologies, from laboratory procedures to statistical analyses, are presented here and explained. Specific methods and conditions will be described in further detail in the appropriate chapters.

It seemed appropriate to dedicate here an entire chapter to microsatellite development for *Helicolenus dactylopterus*. Specific primers for amplification of microsatellite loci for this species did not exist before and were isolated for the present study. The method described here is not new but a modification of some existing protocols; nevertheless the objective of the description is to permit whoever reads this thesis to replicate this microsatellite isolation procedure.

3.1.Sampling

Since 1997 the Departamento de Oceanografia e Pescas da Universidade dos Açores has been conducting annual and seasonal surveys as part of different EU-funded projects regarding the demersal fishery in the Azores archipelago (Menezes *et al*., 1998).

Several sampling cruises have been undertaken around the Azorean islands and adjacent seamounts every spring (ARQDAÇOP97 to ARQDAÇOP03) and, sporadically, around the Madeira and Cape Verde archipelagos (ARQMADP97 and ARQCABO00) on board of the research vessel "Arquipélago" (Fig.3.1). The fishing gear used in sampling surveys is identical to the one used in the commercial demersal fishery in the Azores. This is a bottom long line "stone-buoy" type of gear composed of a mainline to which several branch-lines with hooks are attached. Longlines are set in skates, each divided in 4 skate-sides with around 30 hooks. A floating buoy is set every two skate-sides alternating with stones, which results in a semi-buoying gear that permits effective fishing of some bentho-pelagic species in addition to others that are more associated with the seabed (Menezes *et al.*, 1998; Fig.3.2).

Fig.3.1- The r/v "Arquipélago".

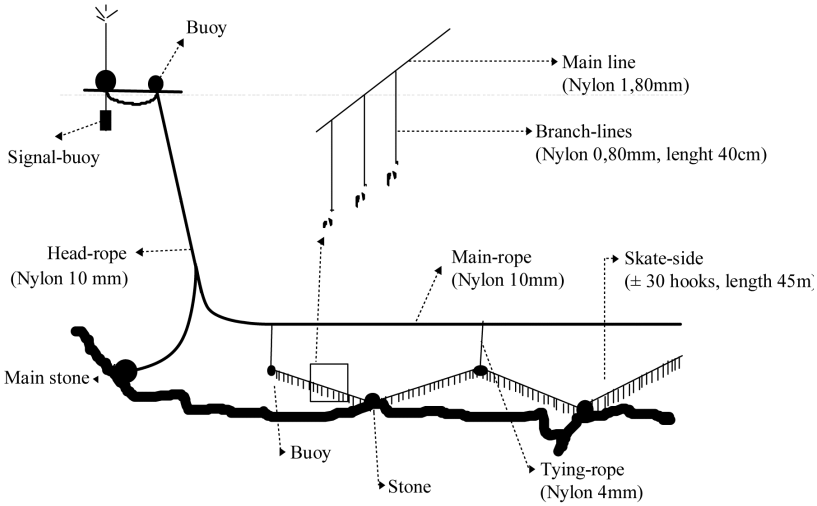


Fig.3.2- A schematic representation of the fishing gear used in sampling surveys (bottom long-line stone-buoy type of gear).

The aim of these surveys was to collect the maximum information in order to understand the biology, ecology and the impact of fisheries on exploited demersal fish species in this region. Each sampled individual suffered a complete biological sampling for future studies on growth, age, maturation and life cycle. In addition, a piece of muscle and/or liver was taken from each fresh individual and stored in 95% ethanol, for genetic analysis.

Several deep-sea demersal fish species including the ones chosen for this study; *Helicolenus dactylopterus* (bluemouth), *Beryx decadactylus* (imperador), and *Beryx splendens* (alfonsino); were captured during these cruises.

In order to understand the population dynamics of these species at a regional scale one needs to have a wider perspective, so, whenever possible samples from other places in the North Atlantic were collected. Collecting areas and numbers of individuals per site were sampled considering the molecular markers to be used and available resources and facilities. In some cases, it was possible to acquire samples from mainland Portugal (Peniche) and the Northwest Atlantic (USA coast), in addition to the ones from the Macaronesian archipelagos already described.

Location of sample sites can be found in Fig.3.3. Cruises designations, dates, sample sites and sizes are presented in Table 3.1.

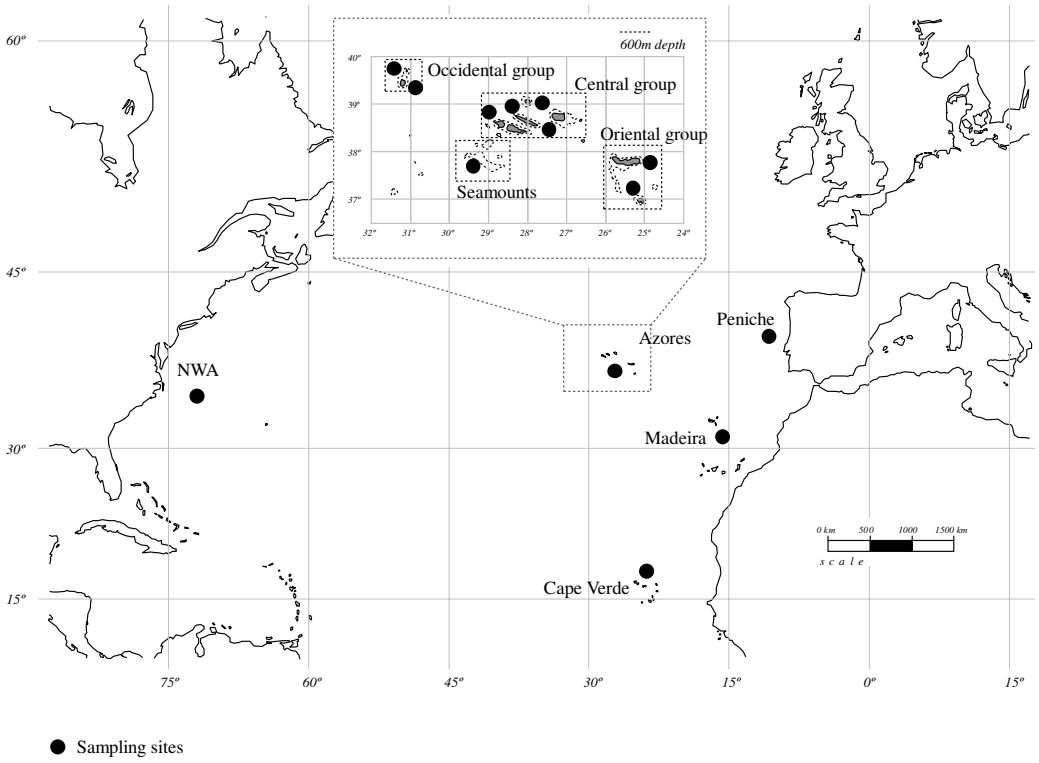


Fig.3.3 – North Atlantic map with sampling sites and Azores archipelago detail.

Individuals from Peniche were acquired in the local fish market during landing of regional fishing boats. Tissue from under the gills (to avoid damage of specimens) was taken and stored in 95% ethanol.

Bluemouth fins from the East Coast of the USA (South Carolina) were collected by Dr.George Sedberry from the South Carolina Department of Natural Resources and stored in a sacorsyl-urea solution.

Species	Location	Area	Cruise	Date	Sample size	Codes
Helicolenus dactylopterus	Azores	Oriental group	ARQDAÇOP00	May2000	88	Hd1/n
		Ocidental group	ARQDAÇOP00	June2000	80	Hd 5/n
		Central group	ARQDAÇOP00	May2000	34	Hd 4/n
		Seamounts	ARQDAÇOP00	May2000	80	Hd 6/n
	Madeira	Madeira	ARQMADP97	Jun1997	26	Hd Mn
	Portugal	Peniche	-	Aug2001	80	Hd Pn-n
	Cape Verde	Cape verde	ARQCABO00	Oct2000	75	Hd CBn-n
	USA	Carolina	-	Mar2002	44	Hd USAn
Beryx decadactylus	Azores	Oriental group	ARQDAÇOP99	Jun1999	30	Bd 6/n
		Ocidental group	ARQDAÇOP99	Jun1999	30	Bd5/n
		Central group	ARQDAÇOP01	Jun2001	32	Bd4/n
		Seamounts	ARQDAÇOP01	Jun2001	24	Bd 1/n
	Madeira	Madeira	ARQMADP97	Jun1997	18	Bd Mn
	Portugal	Peniche	-	Apr2003	28	Bd Pn
	Cape Verde	Cape verde	ARQCABO00	Oct2000	15	Bd CBn-n
Beryx splendens	Azores	Azores	ARQDAÇOP97	1997	35	Bs An
	Madeira	Madeira	ARQMADP97	Jun1997	35	Bs Mn
	Cape Verde	Cape Verde	ARQCABO00	Oct2000	25	Bs CVn

Table 3. 1. List of samples utilized in the present study, including sampling sites and sampling cruises details.

3.2 Laboratory techniques

3.2.1. DNA Extraction

All the molecular markers chosen for this study require genomic DNA to be extracted.

The DNA from a small piece of tissue (around 1-3mg≈ $1mm^3$) from each fish was digested in 410µl of extraction buffer (350µl dH_2O, 40µl 1M Tris-HCl and 20µl 25%SDS) and 10µl of proteinase K (14-22mg/ml in 10mM TrisHCl, pH7.5 - Roche) for 1.5-2 hours at 55ºC. The tissue was then subjected to a phenol/chloroform protocol based on Sambrook *et al.* (1989) and optimised after Hillis & Moritz (1996).

Precipitation of the DNA was then carried out by standing for at least 1 hour at –20ºC, with 350µl 100% ice-cold ethanol and 35µl 3M sodium acetate. Following this, the solution was centrifuged at 0ºC for 15 min to pellet the DNA, which was then washed with 70% ice-cold ethanol.

DNA was resuspended in 50µl elution buffer (buffer AE from Dneasy kits, Qiagen) and stored at -20ºC until further use.

In some cases some RNAse (1µl before digestion) was used to remove excess RNA sometimes present in liver samples.

3.2.2 Electrophoresis and DNA quantification

Electrophoresis gels were used in this study to visualize and analyse DNA using the principle that different sized charged molecules migrate at different rates through the gel matrix when electrical power is applied.

1% agarose gels were used [25ml 1x TAE + 0,25g agarose (Sigma)] stained with ethidium bromide (1µl of 10mg/ml etbr) (Sigma) and a current supply of 70v applied.

Size and quantity of DNA fragments was calculated by comparison with a 1Kb ladder (Life Technologies; Fig.3.4).

Gels were visualised under UV light using an UviDoc 008-XD gel documentation system and images digitised. UviDoc ver.98 software was used to quantify the DNA fragments by comparative analysis of fluorescence intensity.

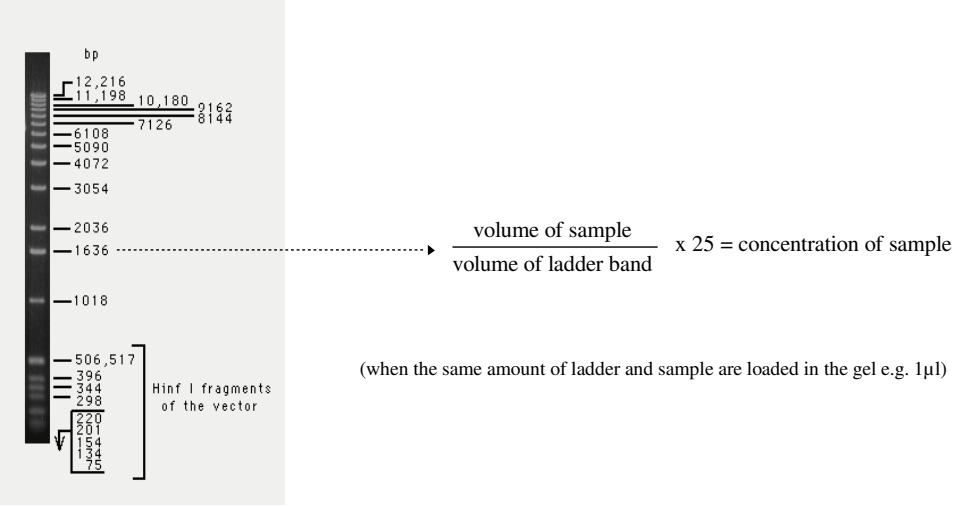
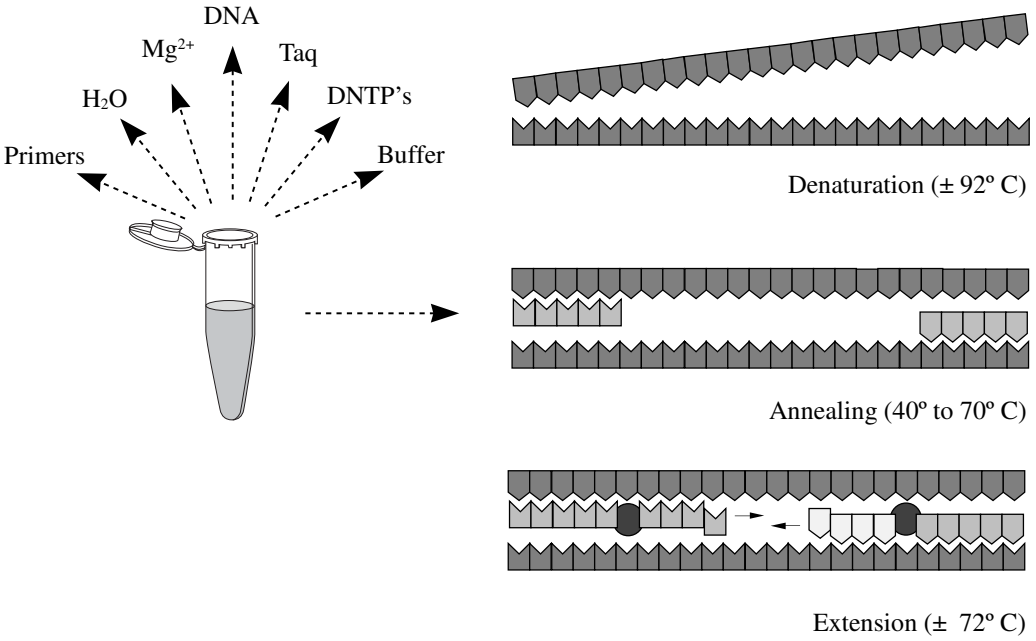


Fig.3.4 – DNA quantification using a 1Kb ladder in a electrophoresis gel.

3.2.3 PCR (Polymerase Chain Reaction)

The polymerase chain reaction (PCR) is one of the key processes in molecular biology. This reaction permits the synthetic amplification of a minute amount of DNA in millions of copies in a few hours (Mullis, 1990).



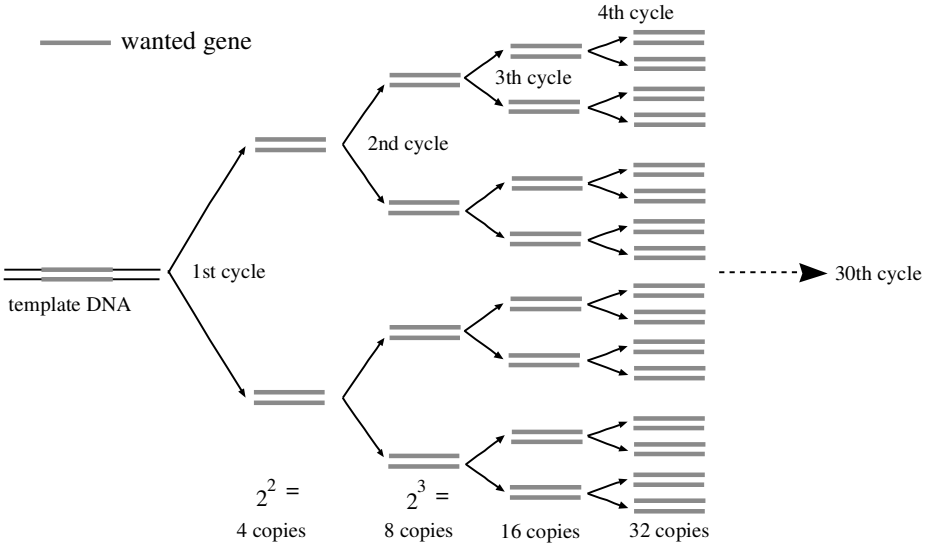


Fig.3.5 – Schematic representation of a PCR reaction

PCR consists of a simple process with three parts: denaturation of the double-stranded target DNA, annealing of open chains with specific primers and extension of the complementary DNA strands (Fig.3.5). The principle of PCR is that it artificially performs the natural

replication of DNA made by cells, using an enzymatic thermal process. Denaturation of target DNA from double to single strands is normally performed by heating the sample to 94ºC. Following this, the temperature is lowered to allow the oligonucleotide primers to bind to the specific template sites and, finally, temperature is set to 72ºC to permit the Taq polymerase enzyme to work and synthesise the complementary DNA segment (Palumbi, 1996a). This cycle is repeated several times (30-40 times), which permits the number of copies to grow exponentially.

In order to work, PCR reactions need a suitable chemical environment usually composed of a buffer solution, the enzyme (Taq), Mg^{2+} (cofactor in the enzymatic catalysis), DNTP's (material for synthesis), primers and templates. These are the basic requirements for any PCR reaction, but for each case, temperatures, timings, and product concentrations have to be chosen in order to optimise the reactions and obtain the required results (Hoelzel & Dover, 1991).

The Polymerase Chain Reaction brought a new range of possibilities in molecular genetic analysis as it permits the selection of a desired DNA fragment from the genome for study and direct sequencing of that segment. Another advantage is the fact that this technique only requires small amounts of DNA, which avoids the sacrifice of individuals and permits work with samples such as saliva, nails, hair, etc. The robustness of this technique eliminates the requirement of high quality DNA, which allows easier sample storage in the field (Bernatchez, 1994).

In this study, PCR was used to specifically amplify different mtDNA regions using universal primers, screen nuclear DNA for microsatellite rich sequences and amplify those same repeat sequences.

3.2.4 Sequencing

Sequences were obtained by a cycle-sequencing reaction based on a dideoxynucleotide chain termination method by Sanger *et al.* (1977).

Purified PCR products (QIAquick PCR kit Qiagen) were used as templates in a cycle sequencing reaction using ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kits (Applied Biosystems) under the following conditions: 10µl reactions – 4µl Dye; 0.16 µl primer; 1µl sample and 4.84 µl H_2O - at 96ºC for 10s, 50ºC for 5s, 60ºC for 4min during 25 cycles and a holding step at 4ºC.

The primer anneals to the complementary DNA strand and extension is performed in the presence of dideoxynucleotides that induce termination of the synthesis. Terminators are

labelled with different coloured fluorescent dyes that will present different emission spectra on an electrophoresis gel illuminated by laser (Fig.3.6). The resulting cycle sequencing fragments were cleaned up using a DyeEx Spin Kit (Qiagen) following the supplier's instructions. Finally, products were visualized using an ABIPrism 377 sequencer by electrophoresis in a polyacrylamide gel and electropherograms read using DNA Sequencing Analysis Software Version 3.3 ABI (Applied Biosystems).

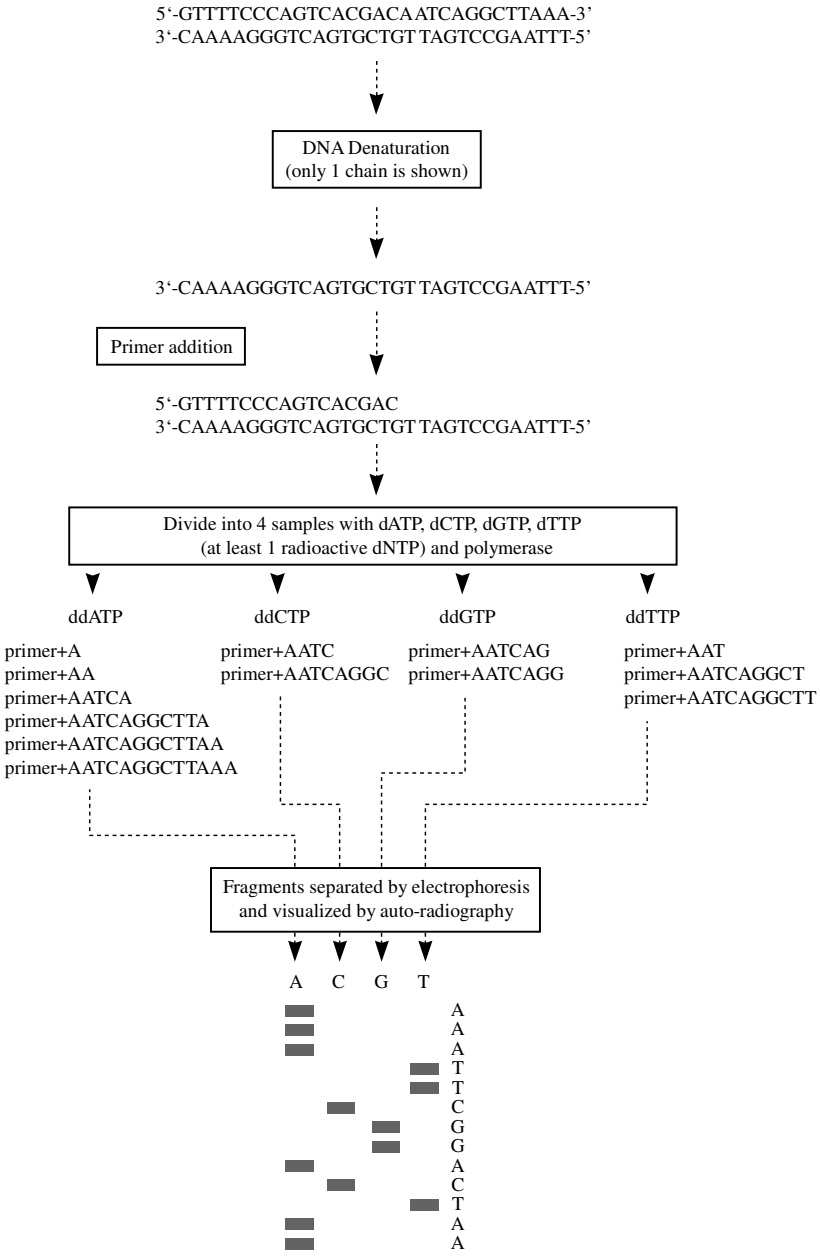


Fig.3.6– Schematic representation of a sequencing procedure

3.2.5 Microsatellites

Methodology and material used to develop and work with microsatellite loci throughout this study will be explained in the next chapter.

3.3 Statistical analysis

The background to statistical analysis has already been explained in the first part of this study. Specific analysis problems and specific software programs will be given in the respective chapters, whenever relevant. Hence, this section will be simply dedicated to the presentation of some models and equations that are traditionally used in population genetic analysis.

3.3.1 Genetic variation

Measurements of genetic variation can be carried out at a number of levels, from allozymes to single nucleotides. Here, variation will only be considered at the nucleotide level as appropriate to mitochondrial sequence and nuclear microsatellite analysis.

Genetic variability can be estimated using a number of parameters including: the mean number of alleles per locus, the percentage of polymorphic loci, number of segregating sites, average number of pairwise nucleotide differences and observed or expected heterozygosities based upon Hardy-Weinberg assumptions, among others.

Gene and nucleotide diversity are the most frequently used diversity indices to compare levels of variation between different populations in terms of allelic frequencies and nucleotide pairwise differences respectively.

Gene diversity or heterozygosity, h (Nei, 1987) is defined, as the probability of two randomly chosen genotypes are different in the sample

$$h = 1 - \sum_{i=1}^{m} \chi_i^2$$

Where m is the number of alleles and χ_i the population frequency of the ith allele at a locus.

Nucleotide diversity, π (Tajima, 1983; Nei, 1987) is defined as the probability that two homologous nucleotides, randomly chosen from the sample, are different.

$$\pi = \sum_{ij} \chi_i \chi_j \pi_{ij}$$

Where χ_i is the population frequency of the *i*th haplotype and π_{ij} is the proportion of different nucleotides between the *i*th and the *j*th haplotypes.

3.3.2 Population variation and comparison

When analysing intra-specific variability one of the most common methodologies used for understanding population genetic structure, is Wright's F-statistics.

Wright (1951) introduced the concept of F-statistics or fixation indices, which measure the reduction of heterozygosity - expected with random mating at any one level of a population hierarchy - relative to another.

Gametes drawn from the same population or sub-population are more likely to have a common ancestor than gametes drawn from different ones. The level of such genetic differentiation may increase by random genetic drift, as mutations accumulate over time, and decrease with migration, as input of new alleles occur.

Wright's F- statistics permits the assessment of this differentiation by describing the hierarchically subdivided populations as "the correlation between random gametes within a population, relative to gametes of the total population" Wright (1965).

Three indices F_{IS}, F_{IT} and F_{ST} (depending on the hierarchical level being compared) are used in F-statistics and are related accordingly to the following equation:

$$F_{ST} = 1 - \frac{1 - F_{IT}}{1 - F_{IS}}$$

F_{ST} is a measure of overall population subdivision and has a value between zero (no differentiation) and one (complete differentiation).

$$F_{ST} = \frac{H_T - H_S}{H_T}$$

Where H_T is the mean expected heterozygosity in the total population and H_S the mean expected heterozygosity across sub-populations. A qualitative guideline for the interpretation of F_{ST} has been suggested by Wright (1978):

- Little genetic differentiation: $0<F_{ST}<0.05$
- Moderate genetic differentiation: $0.05<F_{ST}<0.15$
- Great genetic variation: $0.15<F_{ST}<0.25$
- Very great genetic differentiation: $0.25<F_{ST}$

F_{IS} is the fixation index, which relates subpopulations with regional aggregates, or groups of inbred organisms to the subpopulation to which they belong, measuring the decrease of heterozygosity between these two levels (Nei, 1987).

F_{IS} is often referred to as the inbreeding coefficient as it measures the correlation of genes used to assess random mating or inbreeding between subpopulation within the same sample. The average frequency of homozygotes among different subpopulations is always greater than expected with random mating between them. This is known as the Wahlund effect (Wahlund, 1928) and is one possible cause of high values of F_{IS}.

$$F_{IS} = \frac{H_S - H_I}{H_S}$$

At another level of hierarchy, F_{IT}, measures reduction of heterozygosity of the regional aggregates relative to the total combined population, or in terms of inbreeding, the heteorzygosity of the inbred organisms relative to the total population.

$$F_{IT} = \frac{H_T - H_I}{H_T}$$

For microsatellites, an analogous alternative to F_{ST}, the R_{ST} statistic has been proposed by Slatkin (1995). R_{ST} is based on the assumption that in microsatellites allele size depends on the size of its ancestor, assuming the stepwise mutation model contrary to the Infinite Allele Model assumed in F_{ST}. The major difference is that the index is defined in terms of allele size:

$$R_{ST} = \frac{S - S_W}{S}$$

Where S is the variance of allele size considering the total population and S_W the average variance in allele size within sub-populations.

Debate has always been open about the more useful strategy for microsatellite analysis because of serious doubts about the appropriate model of mutation to apply to these markers (see Balloux & Lugon-Moulin, 2002).

Classically, two models of mutation have been considered for microsatellite loci: the Infinite Alleles Model (IAM; Kimura & Crown, 1964) which states that a mutation always induces a new allele not previously existent in the population; and the Stepwise Mutation Model (SMM; Kimura & Ohta, 1978) which assumes that mutations involve only a single repeat unit (deletion or addition) towards alleles possibly present in the population already. But more recently, some variations have been introduced and several intermediate models arose. Among the most cited ones is the Two-Phase Model (TPM; DiRienzo *et al.*, 1994), a variation of the SMM where the possibility of occasional changes by multiple repeat units has been introduced. Also, a more realistic variation of the IAM, the K-Allele Model (KAM; Crown & Kimura, 1970) has been considered for microsatellite loci. In this model K different alleles are allowed and any allele has a constant probability of mutating towards any of those alleles despite the parental allelic states. Several other intermediate models exist and authors don't seem to reach a common decision on each of the models represent more accurately the mutation process of microsatellite markers. Because of that, both F_{ST} and R_{ST} were applied to microsatellite data in this study.

Fixation indices have also been used to estimate gene flow for a long time (Slatkin, 1985). Only a small number of migrants is required to prevent genetic divergence among populations (estimated for example by F_{ST}). The relation is translated by:

$$F_{ST} = \frac{1}{1 + 4Nm}$$

Where N is the population size, and m the number of migrants per generation.

However, more recently, serious questions have been raised about the credibility of estimation of actual numbers of migrants from F-statistics (see Whitlock & MacCauley, 1999) and it was decided not to use these calculations in this study.

Another useful method of modelling population genetic structure is known as AMOVA (Analysis of Molecular Variance) (Excoffier *et al.*, 1992) and permits a hierarchical analysis by partitioning the total variance into covariance components at the level of individuals within populations, sub-populations or demes within regions and then between populations between geographic regions.

This approach has the advantage of taking into account the number of mutations between alleles/haplotypes rather than simply considering allele/haplotype frequencies as in F_{ST}.

Source of variation	**Degrees of freedom**	**Sum of squares (SSD)**	**Variance components**
Among groups	G-1	SSD(AG)	$n''\sigma_a^2 + n'\sigma_b^2 + \sigma_c^2$
Among populations/ Within groups	P-G	SSD(AP/WG)	$n\sigma_b^2 + \sigma_c^2$
Within populations	(2)N-P	SSD(WP)	σ_c^2
Total	(2)N-1	SSD(T)	σ_T^2

G= number of groups in the structure

P= Total number of populations

N= Total number of individuals (genotypic data x2) or gene copies (haplotypic data)

The variance components are used to infer fixation indices assuming:

$$F_{CT} = \frac{\sigma_a^2}{\sigma_T^2} \qquad F_{SC} = \frac{\sigma_b^2}{\sigma_b^2 + \sigma_c^2} \qquad F_{ST} = \frac{\sigma_a^2 + \sigma_b^2}{\sigma_T^2}$$

Where σ_i^{2}'s are the different variance components with a=group, b=population and c= haplotypes.

3.3.3 Demographic history and evolution

Molecular genetic data can provide information on the relationship among existent populations, but can also reveal information on recent evolutionary history such as past population size (Avise *et al.*, 1988).

Several estimators presented above can reveal, by themselves or when used in conjunction with others, interesting data with respect to demographic processes and population trajectory.

For example, *h* and π can be used to measure genetic diversity of genomes but can also be used to estimate recent historical demographic events in populations of a species (Grant & Bowen, 1998; Benzie *et al.*, 2002; Arnaud-Haond *et al.*, 2003).

Grant and Bowen (1998) suggested that populations of marine species presenting different combinations of haplotype and nucleotide diversity could be categorized into four groups related to influence of historical demographic processes (Fig.3.7).

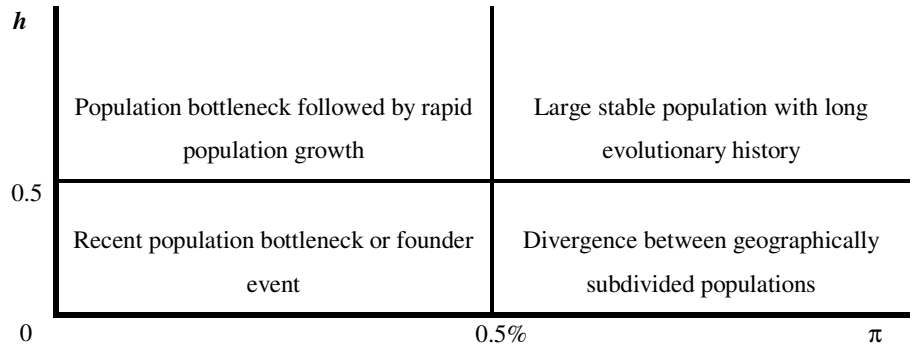


Fig.3.7 - Classification of historical demographic processes in populations (accordinging to Grant & Bowen, 1998).

F_{ST} when used in conjunction with geographic distances in isolation by distance models can also be a source of evidence for historic processes (Slatkin, 1993). It was proposed that species with no evidence of isolation by distance and low levels of F_{ST} may have suffered a recent colonisation process, contrary to evidence of no gene flow when F_{ST} values are high. Mismatch distribution of pairwise nucleotide differences together with neutrality tests can also be a source of data on recent evolutionary history by inferring past population sizes (Rogers & Harpeding, 1992).

Episodes of population growth or decline leave a characteristic signature in the distribution of nucleotide site differences between pairs of individuals. In histograms showing the distribution of relative frequencies and pairwise mismatch distributions the shape of curves will depend on past demographic events.

CHAPTER 4
DEVELOPMENT AND ISOLATION OF POLYMORPHIC MICROSATELLITE MARKERS FOR THE DEMERSAL FISH *Helicolenus dactylopterus* (DELA ROCHE 1809)

4.1 Abstract

In this Chapter the development and isolation of specific microsatellite loci for the demersal fish *Helicolenus dactylopterus* (Sebastidae) from the Azores archipelago is described. A non-radioactive enriched library method was used. Primers were designed for 12 identified loci and 6 were successfully amplified in multiplex optimised PCR reactions. Observed and expected heterozygosities ranged between 0.378 – 0.868 and 0.529 – 0.925 respectively. Departures from the expected Hardy-Weinberg equilibrium were observed in 3 loci and are likely to be a consequence of population structure within the Azores archipelago. Resulting data may be used as tools for the identification of fish stocks and establishment of scientifically based fisheries management.

4.2 Introduction

As previously discussed in Chapter 1, microsatellites have presently become one of the most popular molecular markers used in population genetics, as a result of their high mutation rates, variability, apparent neutrality and abundance throughout many eukaryotic genomes.
Their major drawback is that it is usually necessary to detect and isolate microsatellite loci from most species investigated for the first time. These repetitive sequences are usually found in non-coding regions where the mutation rate is higher than in coding regions. With such a high mutation rate in microsatellite and flanking sequences they are highly unconserved and it is difficult to design primers to universally amplify these loci. However, in some cases, primers designed for one species can be used to amplify homologous loci in studies on other related taxa – the Cross-Priming strategy (Estoup & Angers, 1998). Highly conserved flanking regions have been reported for some microsatellite loci in several families allowing primers developed for a certain species to amplify loci in other closely related species. Their efficiency depends on both the stability of the loci over evolutionary time and the rates of evolution of the flanking regions.
If no sequence information, for the species under investigation, or a related one, is available in the literature, or if the cross-priming strategy fails, the characterization of microsatellites has to be done as a preliminary step to an investigation.

Several methods exist in the literature for microsatellite isolation (Zane *et al.*, 2002). The most common method used to isolate microsatellites consists of the construction and screening of an enriched library of repeat motif sequences (Kandpal, *et al.*, 1994). In the past, this would include a radioactive step for the labelling of oligonucleotide probes, but nowadays this step has been replaced by chemiluminescent techniques that are safer. However, a note must be made that since the completion of the practical work for isolation of microsatellites in this thesis, more efficient methods have been developed for microsatellite isolation that do not require any form of probe-detection (see FIASCO method in Zane *et al.*, 2002).

There have been few studies on *Helicolenus dactylopteurus* and no microsatellite loci have been isolated and developed for this species. Nevertheless, some studies have been carried out on species of the same family, and cross priming was tried with primers developed for another member of the family Sebastidae: *Sebastes mentella* (Roques *et al.* 1999b), but with no success.

The objective of the present chapter was to identify, isolate and develop polymorphic microsatellite loci for *Helicolenus dactylopterus* in order to analyse the population genetic structure including identification of stocks and migration patterns and help establish a scientifically based identification of stocks for fisheries management purposes.

4.3 Materials and methods

4.3.1 Construction of enriched library

A fully non-radioactive method was chosen for isolation of polymorphic microsatellite loci. An enriched library technique based on Kandpal *et al.* (1994) with modifications by Morgan *et al.* (1999) was used (see Morgan *et al.*, 1999 for specific procedure details that may not be presented here).

Genomic DNA from liver/muscle was isolated from 100 individuals from different localities within the Azorean archipelago. Extractions were performed using the phenol-chlorophorm extraction protocol presented in Chapter 3 (Sambrook *et al.*, 1989). Extractions of 20 individuals were combined and precipitated with ethanol in order to increase the variability of the sample and obtain the required amount of DNA necessary for this protocol (≈1µg/µl).

DNA was digested for 3 hours using Mbo1 restriction enzyme (Promega) that recognises CTGA - 4 bps sequences. Fragments with CTGA sticky ends ranging from 50 to 5000bps were obtained and run in a 1% LMP agarose (Promega) gel. 200-500 bp fragments were selected by excision from the gel under UV light and purified following manufacturers

instructions for Qiagen mini columns. The next step consisted of ligating the sticky ends with complementary adapter molecules using T4 ligase (Promega) for specific PCR amplification of the fragments. Adapter oligonucleotide sequences were taken from Kandpal *et al*. (1994):

5'-GAT CGC AGA ATT CGC ACG AGT ACT AC-3'
3'-CG TCT TAA GCG TGC TCA TGA-5'

In order to increase the probabilities of finding microsatellite sequences the enrichment step of the protocol began by incubation of the purified PCR products with a synthetic biotinylated $(CA)_n$ probe (Sigma) at 50ºC for 18 hours. Fragments containing GT repeats were labelled with biotin that strongly binds with streptavidin and allows the hybridised repeat regions to be separated with streptavidin-coated magnetic beads (Promega). In a vial, hybridised fragments were concentrated against the wall with a magnet and unlabelled DNA poured off in the supernatant into another vial. Six washing cycles with magnetic selection were performed with increasing stringency to select the most specific $(CA)_n$ regions (increasing temperature and NaOH concentration). All seven washes were kept and purified through Qiagen PCR purification columns and PCR amplified using one of the adapter oligonucleotides as primer (Mbo1). PCR products were run in a 2% agarose gel that was subject to southern blotting on a nitro-cellulose membrane (Roche) to detect the richest wash in CA-repeats. The membrane was probed with a digogxygenin-labelled $(CA)_{10}$ oligonucleotide (Sigma) that binds to alkaline phosphatase in a chemiluminescent reaction using Boehringer-Mannheim Dig-Detection kit. Washes were then exposed to a light sensitive film (Kodak Bio-Max light-2) to identify the most enriched wash (strongest dig-probe signal) and purify it using a filter column.

4.3.2 Cloning and sequencing

DNA fragments rich in $(CA)_n$ repeats were ligated into plasmid vector pGEM-T using T4 ligase to transform *E.coli* (JM109 strain) (Promega). DNA fragments were present as inserts in the bacterial plasmid DNA and were multiplied as the bacteria grew into colonies. PGEM-T Easy Vector System II (Promega) includes a gene resistant to penicillin that is inserted in the β-galactosidase gene. Colonies were grown in a medium containing the substrates IPTG (isopropyl-β-D-thiogalactopyranoside) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside): the first induces production of β-galactosidase that reacts with the

second producing a blue colour. If successful cloning of an insert in the vector occurred, bacteria were unable to degrade galactose and grew into white colonies (positives).

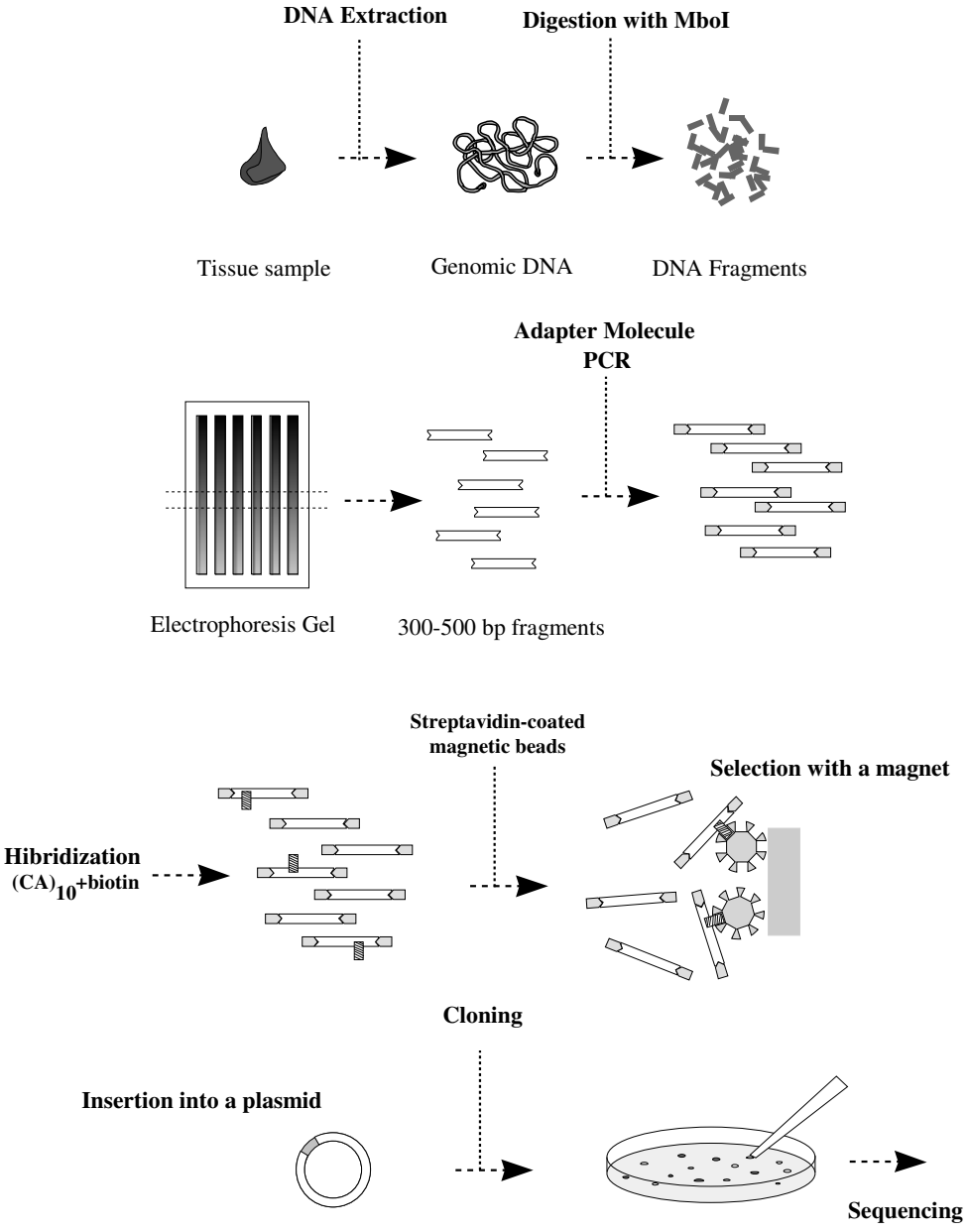


Fig.4.1- Schematic representation of the enriched library construction process and cloning.

Positives were plated out onto duplicated gridded dishes and grown overnight at 37ºC. One of the plates was used to detect microsatellite-containing clones by southern blotting with a nylon membrane and the other was used for selection and purification. Colonies with the strongest signal on the photographic film were considered to potentially contain microsatellites. These were picked and grown over night followed by purification of the plasmids with Promega Wizard Plus minipreps before sequencing on an ABI377 automated sequencer using standard M13 plasmid primers (forward – CGC CAG GGT TTT CCC AGT CAC GAC and reverse – AGC GGA TAA CAA TTT CAC ACA GGA).

4.3.3 Primer design and PCR optimisation

Primer pairs for amplification of the identified microsatellite containing sequences were designed using the program PRIMER 3 (Rozen & Skaletsky, 1998). Several parameters were taken into consideration to prevent primer mispairing and improve the performance of the primers during PCR amplification:

- Primer size should be between 18-25bp:
- Low self-complementarity and primer dimer formation (between primer pairs)
- Exclusion of highly repetitive zones.
- High GC content: G-C bonds are more stable and avoid problems with internal secondary structure. Ideally around 40-60% of the sequence should be GC.
- Similarity between Tm (melting temperatures) for primer pairs: Tm is usually 5ºC higher then annealing temperature and can be affected by $[Mg^{2+}]$ and $[K^{+}]$. Ideally Tm should be around 60ºC.

PCR amplification was performed on a Hybaid "PCR Express" thermocycler with temperature gradient block. A number of different cycling conditions were used until amplification worked reliably. Different annealing temperatures and quantities of reagents were also tested. Once reactions were individually optimised for each primer pair, multiplex reactions (with several primers at the same time) were tried in order to simplify and save some timework and cost.

Through optimisation, it was possible to amplify and genotype a quadriplex and two duplex sets of primers.

PCRs were then performed in a MWG-Biotech Primus 96 Plus machine, consisting of an initial denaturation step of 4 min at 94ºC, followed by 30 cycles of 94ºC-60s, 58ºC-45s, 72ºC-60s and a extension step of 72ºC for 20min.10 µl reactions contained approximately 20ng of DNA; 1µl 10x Qiagen buffer (Tris-HCl pH7.8, KCl, 1.5 mM Mg^{2+}); 2µl Qiagen "Q

solution"; 0.9µl DNTP's mix Perkin Elmer; 0.1µl extra dATP; 6 pmol (quadriplex) and 7 pmol (duplex) of each primer; 0.75U *Taq* polymerase (Qiagen) and chemical water.
For Genescan trials one of each pair of primers (the reverse) was ordered with a different 5' fluor-label (6-FAM; NED and HEX).

4.3.4 Analysis

The assessment of the variability of microsatellite loci was done using the GeneScan 3.1 software (ABIPrism) and analysis was performed with both GeneScan and Genotyper (Applied Biosystems) applications.
Deviations from Hardy-Weinberg equilibrium were calculated for each locus in order to assess their neutral inheritance. Calculations were performed by Arlequin version 1.1 (Schneider *et al.*, 2000) considering H_O (Observed Heterozygosity) and H_E (Expected Heterozigosity) based on genotype frequencies predicted by the HWE model.

$AA=p^2$; $Aa= 2pq$; $aa =q^2$

The test for significance of difference between H_o and H_E was performed by Guo & Thompson's (1992) analogue to Fisher's exact test (1935) (Schneider *et al*., 2000), which allows confirmation of the fit to the H-W assumptions.

4.4 Results

Around 200 colonies were successfully transformed and 33 were positive for $(CA)_n$ repeats. Twenty-five clones were sequenced and primers designed for twelve of them. From the 12 successfully amplified loci, eight produced consistent products in multiplex PCR reactions and were considered further (see characteristics of loci on Table 4.1).
Multiplex 1 (Duplex 2) contained *Hd020* and *Hd076* loci in the same PCR reaction; multiplex 2 (Quadriplex 1) contained *Hd008*, *Hd044*, *Hd063* and *Hd106* and multiplex 3 (Duplex 9) contained *Hd092* and *Hd095*. All six microsatellite loci were scored for 40 individuals randomly chosen from different areas of the Azores archipelago (Table 4.2).

Locus	GenBank Accession No.	Repeat Motif	Clone Size	Size Range
Hd 008	AY123151	$(CA)_6CCCATGTA(CA)_8CCTATGTA(CA)_{14}$	219	204-249
Hd 020	-	$(CA)_9$	161	161-167
Hd 044	AY123152	$(GT)_9$	187	183-192
Hd 063	AY123153	$(GTGTGTGTT)_4(GT)_4$	218	208-235
Hd 076	-	$(GT)_9$	212	213-217
Hd 092	AY123154	$(CA)_{21}$	182	166-206
Hd 095	AY123155	$(CA)_{12}$	120	115-191
Hd 106	AY123156	$(GT)_5TT\ (GT)_{13}$	137	135-183

Table 4.1 – *Helicolenus dactylopterus* microsatellite loci description with GenBank Accession numbers.

Locus	Primers (5'-3') – fluorescent dye	Annealing T (ºC)	Multiplex group
Hd 008	F-GCATGTGATGACCTTTGACC R-GTTACAGCGGCAAGAAACC – 6FAM	58	Q1
Hd020	F- CAGATGACACCGCACATTG R- TAAAACACCCCAACTACCCC - HEX	58	D2
Hd 044	F-AATGGGCTGAACTGTCCTTG R-CTCTGACTGCTTCCTGGGTC - HEX	58	Q1
Hd 063	F-GGCTCTGTCTATCTCTCGCC R-TTCTGAGTTCCCAAACACCC- NED	58	Q1
Hd 076	F – CTGCTGCTGCCTGTCTCAC R - ACTCCATCTCTCTCCACCCCTC - NED	58	D2
Hd 092	F-TGATGCAGTGGTGGAGAGAG R-ACCTTCTATCTGACGCGAGG –6FAM	58	D9
Hd 095	F-TTGGCTTTTTGTCGAGGG R-GCTAACATCAGCACGAATGG- NED	58	D9
Hd 106	F-AGCTTGGGCTGAAAGATGG R-TGGCAGCAGAGATGAACG – 6FAM	58	Q1

Table 4.2 - *Helicolenus dactylopterus.* PCR conditions for amplification of 8 microsatellite loci with multiplex reactions including primer sequences, fluorescent dyes, multiplex agroupments and annealing temperatures.

In general, polymorphism was high showing 3 - 21 different alleles per locus. The observed and expected heterozygosity ranged from 0.211 to 0.868, and from 0.356 to 0.925, respectively. The observed genotype frequencies deviated from Hardy-Weinberg expectations at four out of the eight loci (P<0.001) resulting from heterozygote deficiency (Table 4.3).

Locus	Sample size	No of alleles	H_O	H_E	P (H-W)
Hd 008	25	18	0.800	0.888	0.072
Hd 020	38	4	0.211	0.412	0.001***
Hd 044	37	6	0.378	0.529	0.002**
Hd 063	40	5	0.600	0.659	0.160
Hd 076	40	3	0.275	0.356	0.001***
Hd 092	38	20	0.868	0.925	0.360
Hd 095	38	21	0.684	0.880	0.000***
Hd 106	39	18	0.641	0.785	0.408

Table 4.3 – *H.dactylopterus*. Microsatellite loci diversity and fits to Hardy-Weinberg equilibrium. *** significant P-values for P<0.001.

4.5 Discussion

Some of the microsatellite loci described were more variable in size than usually expected for this kind of marker (Goldstein & Schlötterer, 1999). In some cases this variation can result from complex repeat patterns (e.g. *Hd008* and *Hd063*), but in the case of simple dinucleotide motifs like *Hd095*, rare alleles can be the cause of such a wide range of alleles sizes. The possibility of misinterpreted readings has also to be taken into consideration, as sometimes, artifactual "stutter bands" on gels can make some loci difficult to score correctly. Overall genetic diversity was high with values of overall expected heterozygosity comparable with those found in other marine fish (García León *et al.*, 1997; Ruzzante *et al.*, 1996; Rico *et al.*, 1997; Stockley *et al.*, 2000) and even for some species of the same family (Roques *et al.*, 1999b).

4.5.1 Hardy Weinberg equilibrium deviations

Significant deviations from Hardy-Weinberg equilibrium were obtained for four of the eight loci analysed as a result of heterozygote deficiency.
The departures from the Hardy-Weinberg expected genotype frequencies because of heterozygote deficiency could be explained by several hypotheses including the presence of null alleles, selection, non-random mating within the chosen sample or population structure.

Null alleles arise when mutations occur within primer-binding sequences, which prevent the primer from annealing and amplifying one or both alleles in an individual. In such a situation, heterozygotes can be under represented and misinterpreted as homozygotes biasing estimates of allele frequencies and leading to an observed heterozygote deficiency in the population (Pemberton *et al.*, 1995). It is difficult to detect with certainty the presence of null alleles because panmixia and non-violation of any H-W assumption have to be assumed. In most cases, as in this one, definitive identification of a null allele is not possible but it can be done by pedigree analysis for instance as in Callen *et al.* (1993). Nevertheless, a more indirect estimation of null allele frequencies can be carried out using statistical methods (Chakraborty *et al*. 1992; Allen *et al*., 1995; Brookfield, 1996).
Once more, the presence of "stutter bands" on gels can lead to scoring errors revealed by an apparent deficiency of heterozygotes (O'Reilly & Wright, 1995).

Small sample size can also be responsible for misinterpretation of data, as microsatellite analysis requires large numbers of samples, from 50 to 100 individuals, to obtain accurate genotype frequencies (Ruzzante, 1998).
The Wahlund effect (1928) is also characterized by deficiency of heterozygotes. This can occur if a sampled population is divided into smaller reproductive groups. Heterozygote deficiency can also arise through inbreeding. Heterozygote deficiency is eliminated when populations are panmitic with large effective sizes, no barriers to dispersal and random mating.

Subdivision of the Azorean metapopulation into demes around the three island groups and seamounts could be responsible for the observed significant deviations from H-W expected frequencies. The 40 individuals screened with microsatellite loci come from different sampling areas distributed within the Azores archipelago, around different islands and seamounts. All these hypotheses are examined in greater detail in Chapter 6.

PART III
RESULTS

SECTION 1 – BLUEMOUTH

CHAPTER 5
GENETIC POPULATION STRUCTURE OF *Helicolenus dactylopterus* (SCORPAENIDAE) IN THE NORTH ATLANTIC OCEAN USING MtDNA SEQUENCES

5.1 Abstract

Aspects of the life history of *H. dactylopterus* suggest that populations located in different geographic areas may exhibit marked genetic differentiation as a result of low dispersal. In this chapter, partial sequences of the mitochondrial control region (D-loop) and cytochrome *b* (Cyt *b*) were used to test the hypothesis that *H. dactylopterus* disperses between continental margin, island and seamount habitats on a regional or even oceanic scale in the North Atlantic Ocean. Individuals were collected from 5 different geographic areas: Azores, Madeira, continental Portugal (Peniche), Cape Verde and the Northwest Atlantic. D-loop (415bp) and cyt *b* (423bp) regions were partially sequenced for 208 and 212 individuals, respectively. Analysis of variance amongst mitochondrial DNA sequences based on F-statistics and AMOVA demonstrated significant genetic differentiation between populations in different geographic regions specifically the Mid-Atlantic Ridge (Azores)/ NE Atlantic (continental Portugal, Madeira) compared to populations around the Cape Verde Islands and in the NW Atlantic. Some evidence of intraregional genetic differentiation between populations was found. Minimum spanning network analysis revealed star-shaped patterns of haplotype frequency suggesting that populations had undergone expansion following bottlenecks and / or they have been colonised by jump dispersal events across large geographic distances along pathways of major ocean currents. Mismatch disequilibrium analysis indicated that Azores and northwestern Atlantic populations fitted a model of population expansion following a bottleneck estimated to be between 0.64 and 1.2 million years before present. Phylogenetic analysis also suggested that invasion of the NW Atlantic slope by populations originating on the eastern side of the Atlantic may have occurred.

5.2 Introduction

Many bathyal fish species (living at 200 – 2000m depth) have large geographic ranges that span one or more oceans. Populations of benthopelagic species inhabit continental slopes, the slopes of oceanic islands and seamounts that may be separated from each other by thousands of kilometres of the deep ocean. The question arises as to whether such species have life histories that are characterised by extremely high dispersal or if their present day distributions have resulted from past dispersal events when oceanic conditions and the configuration of geographic features were different. Such historic structuring of populations is exhibited by shallow–water fish such as anchovies (reviewed in Grant & Bowen 1998).

Since the mid- 20th century major fisheries have developed which target deep-water fish species living on the slopes of continents, oceanic islands and on seamounts (Koslow *et al*., 2000). These have targeted markedly different guilds of fish to those traditionally exploited by fisheries. In particular robust, deep-bodied species, with "K-type" life strategies, typical of high-energy benthic habitats located on seamounts and continental slopes have been subject to trawl and long-line fisheries. These have included orange roughy (*Hoplostethus atlanticus*), rock-fish (*Sebastes* spp) and slender armourhead (*Pseudopentaceros wheeleri*). They are generally long-lived, with low natural rates of mortality and sporadic recruitment to populations (Rogers 1994, Koslow *et al.* 2000). The life- history of many deep-sea fish species, however, includes an extensive larval phase. This suggests that they may exhibit high levels of dispersal and, as a result, should exhibit a lack of stock structure on oceanic, regional and sub-regional scales. However, seamounts and oceanic islands maybe isolated from each other and from continental margins by large geographic distances. Current-topography interactions may also generate trapped parcels of water around these features (e.g. Taylor columns on seamounts) acting as larval retention mechanisms (Rogers 1994).
Many studies of seamount and oceanic island-associated species to date suggest that populations do exhibit panmixia across large geographic distances on regional or even oceanic scales, as expected from aspects of life history, including: slender armourhead (*Pseudopentaceros wheeleri* Hardy 1983), wreckfish (*Polyprion americanus* Bloch & Schneider 1801) and alfonsino (*Beryx splendens* Lowe 1834) (Martin *et al.* 1992, Sedberry *et al.* 1996, Hoarau & Borsa 2000). For some deep-water species of fish there is, however, evidence for genetic differentiation among populations at the trans-oceanic, oceanic and regional scales including roundnose grenadier (*Coryphaenoides rupestris* Gunnerus 1765), Greenland halibut (*Reinhardtius hippoglossoides* Jordan & Snyder 1901), rockfish

(*Sebastolobus alascanus* (Bean 1890) and *S. altivelis* (Gilbert 1896)), ling (*Genypterus blacodes* (Forster 1801)), hoki (*Macruronus novaezealandiae* (Hector 1871)), oreos (*Allocyttus niger* (James, Inada & Nakamura 1988), *A. verrucosus* (Gilchrist 1906)) and others (reviewed in Creasey & Rogers 1999, Rogers 2003).

The existence of morphologically different geographic populations of bluemouth is inconsistent with features of its life history (see Chapter2). However, this species is known to have internal fertilization with brooding of eggs until the early stage of development (early embryo) consistent with zygoparity (Sequeira *et al.* 2003) and larvae occur in the zooplankton. Tagging experiments around the Azores archipelago strongly suggest that adult fish lead a very sedentary life style, as many tagged specimens have been recaptured, after more than one year, exactly in the same places as they were originally caught and tagged (Menezes unpubl. data). Thus, although adults are sedentary, larval-mediated dispersal is likely to occur in this species allowing genetically effective migration over considerable distances.

The aim of this study is to investigate the genetic population structure of bluemouth in the North Atlantic Ocean to determine whether this species can disperse over large (inter-oceanic) distances or whether larval transport is limited. Extensive larval dispersal will result in panmixia amongst populations at the regional and oceanic scale whereas limited dispersal will lead to marked genetic structure amongst populations. Historical factors, such as the effects of the Last Glacial Maximum, may have also played a role in determination of the current distribution of this species and the genetic structure of populations (e.g. Muus *et al*. 2001, Marko 2004). To test this hypothesis, *H. dactylopterus* was collected using a stratified sampling scheme from the Macaronesian archipelagos (Azores, Madeira, Cape Verde), continental Portugal (Peniche), and the NW Atlantic (off the coast of the USA - South Carolina). Single samples were collected from localities on an oceanic scale; multiple samples were collected on a regional scale in the NE Atlantic and on a sub-regional scale in the Azores Archipelago including the three island groups and the Azores Bank (38°10'N 29°00'W). Genetic population structure was analyzed amongst populations by comparisons of haplotype frequencies in partial sequences of the mitochondrial cytochrome *b* gene (cyt *b*) and mitochondrial control region (D-loop).

5.3 Material & Methods

5.3.1 Sampling and DNA extractions

The majority of *Helicolenus dactylopterus* individuals (see Table 3.1) were collected in the Madeira, Azores and Cape Verde archipelagos during the ARQDMAD-P97, ARQDAÇO-P00 and ARQDCAB-P00 cruises on board of the RV "Arquipélago" of the Department of Oceanography and Fisheries - University of Azores (Menezes *et al.*, 1998; map Fig.5.1). These samples were collected using a "stone-buoy" type of bottom long-line gear (Menezes *et al.*, 1998). Other individuals were collected at the fisheries market of Peniche (Portugal) and samples from the USA were sent by Dr. George Sedberry from the South Carolina Department of Natural Resources.

Liver and muscle samples were removed from fresh fish and stored in 95% ethanol immediately after collection. In the case of NW Atlantic samples, fins were collected and stored in a sarcosyl/urea preservative solution. Total genomic DNA was extracted from small (1-3mg) sections of tissue following a phenol\chloroform protocol based on Sambrook *et al.* (1989). The extracted DNA was resuspended in elution buffer and stored at –20ºC until further utilization.

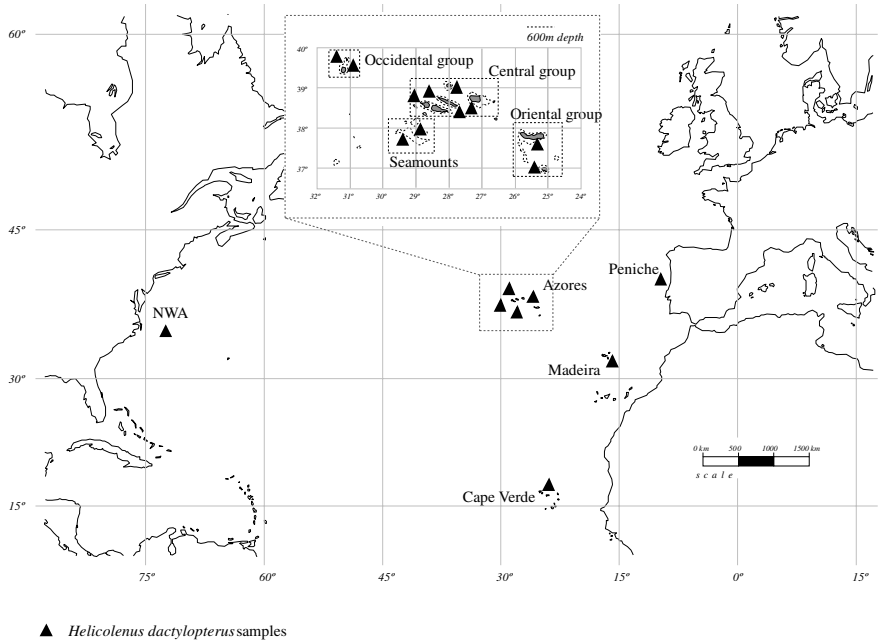


Figure 5.1. North Atlantic map with *H.dactylopterus* sampling sites.

5.3.2 PCR amplification and Sequencing

The mitochondrial control region (415 bp) was amplified for 208 individuals by PCR using two universal primers: L-Pro-1 (5'-ACT CTC ACC CCT AGC TCC CAA AG-3') and H-DL-C-1 (5'-CCT GAA GTA GGA ACC AGA TGC CAG-3') described by Ostellari *et al.* (1996). PCR reactions of 10 µl total volume containing 1µl 10x buffer (Qiagen Crawley, West Sussex, U.K.; Tris-HCl, KCl, $MgCl_2$, pH 7.8); 1.25µl $MgCl_2$ (Qiagen); 0.6 µl DNTP mix (Applied Biosystems, Warrington, Cheshire, U.K.); 0.1 µl Taq polymerase (Qiagen); 1.5µl of template (10-20 ng DNA); 5.05 µl H_2O and 0.5 µl of each primer (10pmol/µl), were conducted on a Perkin Elmer DNA Thermal Cycler 480 under conditions as follows: 4mins at 94ºC, followed by 30 cycles of denaturing at 92ºC for 60s, annealing at 50ºC for 60s and extension at 72ºC for 60s; finishing with an extension step at 72ºC for 5 min.

The cyt *b* region (423bp) was amplified for 212 individuals using the same PCR reagents and quantities as for D-loop but using the universal primers CYB-GLU-L-CP (5'-TGA CTT GAA GAA CCA CCG TTG -3') and CB2-H (5'- CCC TCA GAA TGA TAT TTG TCC TCA-3') described by Palumbi *et al.* (1991). PCR cycles were performed in an MWG-Biotech Primus 96 plus thermocycler under the following conditions: 94ºC for 4min, followed by 30 cycles of 94ºC for 50s, 56ºC for 30s and 72ºC for 50s; finishing with an extension step at 72ºC for 5 min.

All amplified products were purified using a QIAquick PCR Purification Kit (Qiagen) following the supplier's instructions. Sequences were obtained by a cycle-sequencing reaction based on a dideoxynucleotide chain termination method by Sanger *et al.* (1977). Each purified PCR product was used in a cycle sequencing reaction using Applied Biosystems Big Dye Terminator Cycle Sequencing Ready Reaction Kits (Applied Biosystems) under the following conditions: 10 µl reactions – 4µl Dye; 0.16 µl primer; 1µl sample and 4.84 µl H_2O - at 96ºC for 10s, 50ºC for 5s, 60ºC for 4min during 25 cycles and a holding step at 4ºC. The resulting cycle sequencing fragments were cleaned up using a DyeEx Spin Kit (Qiagen) following the supplier's instructions. Finally, products were visualized using an Applied Biosystems Prism 377 automated sequencer.

5.3.3 Sequence alignment

Both mitochondrial partial sequences were sequenced in both directions to check the validity of the sequence data. One example of cyt *b* and d-loop sequences were used to search

Genbank for similar sequences using the Basic Local Alignment Search Tool (BLAST) available on the NCBI website (http://www.ncbi.nih.gov/BLAST/). The most similar sequences obtained from BLAST searches were added as outgroups for phylogenetic analysis to the data sets obtained for *Helicolenus dactylopterus* in the present study. These included cyt *b* partial sequence data for *Sebastes emphaeus* (AF030725), *Sebastes hopkinsi* (AF030751), *Hozukius emblemarius* (AB096132) and *Helicolenus hilgendorfii* (NC003195, AB096133, AB096134). D-loop sequences were obtained from *Sebastes inermis* (AB071270.1) and *Helicolenus hilgendorfii* (AP002948). All sequences were aligned using CLUSTAL X (Thompson *et al.*, 1997). Alignments were checked by eye and repeated using different values for parameters. For cyt *b* all parameters were as default with Pairwise parameters set at Gap Opening Penalty 10, Gap Extension penalty 0.1 and Multiple Alignment parameters set at Gap Opening Penalty of 10, Gap Extension 0.2. D-loop alignment parameters were default except for pairwise and multiple alignment parameters that were both set at Gap Opening Penalty 10 and Gap Extension Penalty 5.

5.3.4 Population genetic analysis

Eight different geographic populations were defined *a priori*: NW Atlantic, Cape Verde, Peniche (continental Portugal), Madeira, Azores Central group, Azores Oriental group, Azores Occidental group and Azores seamounts (Azores Bank).

Intrapopulation diversity was analysed by estimating gene diversity (h)- the probability that 2 randomly chosen haplotypes are different (Nei, 1987), and nucleotide diversity (π) - the probability that 2 randomly chosen homologous nucleotides are different (Tajima, 1983; Nei, 1987).

Hierarchical genetic differentiation and the significance of group and population structure were tested using analysis of molecular variance (AMOVA) (Excoffier *et al.*, 1992) and F-statistics (Wright, 1951) respectively. All population analyses were performed using Arlequin version 2.0 (Schneider *et al.*, 2000).

5.3.5 Phylogeographic analysis

Phylogenetic analysis was carried out on both Cyt *b* and D-loop sequences along with outgroup taxa in order to ascertain whether results obtained using population genetic analysis were reflected in the geographic distribution of haplotypes. Because mitochondrial DNA sequences often have unequal nucleotide base frequencies and transition/transversion ratios

markedly different from 2, these parameters were estimated using the programme Tree-Puzzle Version 5.0 (Strimmer & von Haesler, 1996). This programme also identified sequences that were identical and these were reduced to a single sequence for subsequent analyses to save on computing time and to minimise the size of output trees.

Sequence data was subsequently analysed using distance (Neighbour-Joining) and Maximum Parsimony methods. All analyses used default parameters except for Expected Transition/Transversion ratios and nucleotide frequencies that were estimated from the data. The HKY85 model of sequence evolution (Hasegawa *et al.*, 1985) was used in all analyses where appropriate as this most closely simulates the evolution of mitochondrial DNA sequences. It was assumed that mutation rates were similar across the analysed partial sequences and Gamma Distribution was set at default. The sampling error of Neighbour-Joining and Maximum Parsimony Trees was analysed using bootstraps of 10,000 replicates where possible followed by the construction of Majority Rule Trees. For Maximum Parsimony analysis, for D-loop sequences, only 100 bootstraps were possible because of computational limitations. In order to attempt to reduce homoplasy cyt *b* sequences were also analysed using Transversions only, again using 100 bootstraps because of computational limitations. All phylogenetic analyses were carried out using Phylip vers. 3.6a3 (Felsenstein, 2002).

Because phylogenetic analysis makes assumptions that are invalid at the population level (ancestral haplotypes are extinct) data were also analysed using phylogeographic techniques based on haplotype networks as implemented by the software package TCS Version 1.13 (Clement *et al.*, 2000).

5.3.6 Neutrality and Demographic History

Demographic history was investigated by analysing mismatch distributions of pairwise differences between all individuals of each population using the Arlequin ver.2.00 software package (Schneider *et al*., 2000). This kind of analysis can discriminate whether a population has undergone a rapid population expansion (possibly after a bottleneck) or has remained stable over time. The mismatch distribution will appear unimodal (like a Poisson curve) if accumulation of new mutations is greater than the loss of variation through genetic drift, and multimodal if the generation of new mutations is offset by random genetic drift (Rogers & Harpending, 1992).

Arlequin vers.2.00 was also used to test for departures from mutation drift equilibrium with Tajima's D-test (Tajima 1989). The time of possible population expansions (t) was

calculated through the relationship $\tau = 2ut$ (Rogers & Harpending 1992), where τ is the mode of the mismatch distribution, u is the mutation rate of the sequence considering that $u=2\mu k$ (μ is the mutation rate per nucleotide and k is the number of nucleotides). A mutation rate of 2% per nucleotide per million years was used for cyt *b* as the mean rate for vertebrate mitochondrial DNA (Brown *et al*. 1979). The d-loop region evolves faster than this rate in fish and a mean value of 3.6% per million years was selected as the mean mutation rate estimated from species pairs located either side of the Isthmus of Panama (Donaldson & Wilson 1999). The generation time for *H. dactylopterus* was taken as approximately 14 years as ageing studies on otoliths have indicated that most fish live to 13-14 years of age. Note that this study indicated that ages of more than 30 years were not uncommon for bluemouth and individuals reaching a maximum age of 43 years for males and 37 years for females in the NE Atlantic (Kelly *et al*. 1999). 14 years is probably therefore a conservative figure for generation time in this species.

5.4 Results

5.4.1 Control region

Control region sequence variation

A total of 208 individuals were sequenced for the mt-DNA control region (415bp) and the overall diversity revealed was high with 191 different haplotypes. This agrees with previous works on teleostean fish (Lee *et al.,* 1995). Only 16 haplotypes (≅8%) were shared among different individuals, the other 160 were singletons. Nine of the shared haplotypes were represented in more than one site; while the other seven were only shared between individuals restricted to the same geographical site (Table 5.1). The most common haplotype sequence was registered in GenBank (Accession Number **AY563096**). Sequence comparisons revealed 138 divergent sites resulting mainly from transitions, followed by transversions and some insertions/deletions of single base pairs. The expected Transition/Transversion ratio was 5.38 and nucleotide frequencies were: T = 0.314, C = 0.166, A = 0.396 and G = 0.124.

Haplotypes / Site	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	n_h	n_t
Cape Verde	0	0	0	0	0	0	0	0	0	3	3	2	0	0	0	0	22	28
NW Atlantic	0	0	0	0	0	0	0	0	0	0	0	0	8	2	0	0	18	26
Peniche	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	28	28
Madeira	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	22	22
Central G.	1	1	1	1	1	0	0	1	0	0	0	0	0	0	0	0	27	27
Oriental G.	0	1	0	0	1	0	1	1	1	0	0	0	0	0	2	0	22	23
Occidental G.	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	2	25	26
Seamounts	0	0	0	0	2	1	1	0	0	0	0	0	0	0	0	0	27	28
Total	2	3	2	2	4	2	2	2	2	3	3	2	8	2	2	2	191	**208**

Table 5.1. *H. dactylopterus*. Control region. Absolute frequencies of shared haplotypes; n_t- number of individuals analyzed per site; N_h- number of haplotypes per site.

Population variability

The haplotype diversity (*h*) of the analysed populations was very high, with observed values between 0.911 in the NW Atlantic and 1.000 in several other populations. In contrast, nucleotide diversity (π) within each population was moderate to low, ranging from 0.006 in the NWA population and 0.033 in Cape Verde (Table 5.2).

Populations	**Nucleotide diversity** (π)	**Haplotype diversity** (*h*)
CAPE VERDE	**0.033**	**0.976**
NWA	**0.006**	**0.911**
MADEIRA	**0.031**	**1.000**
PENICHE	**0.029**	**1.000**
AZORES	**0.029**	**0.996**

Table 5. 2. *H. dactylopterus*. Control region. Intrapopulation nucleotide (π) and haplotype (*h*) diversities.

Phylogeographic relationships of populations

The analysis of molecular variance (Table 5.3) on the 5 specified groups (Madeira, Peniche, Azores, Cape Verde and NW Atlantic) indicated that a high proportion of the total variance was attributed to differences between the defined groups of populations with a significant value (p<0.0127), indicating geographic structure in haplotype frequencies for d-loop

between regions. Only 0.01 % of the variation was attributed to differences between populations within groups but this was also significant suggesting that, whilst the populations within the NE Atlantic did not contain a large component of the variance at the sub-regional scale, there were significant differences within the region.

Source of variation	% Total variance	Fixation indices	*P*-value
Among groups	39.86	F_{CT}= 0.3986	<0.05
Among populations within groups	0.01	F_{SC}= 0.0001	<0.001
Within populations	60.13	F_{ST}= 0.3987	<0.001

Table 5.3. *H. dactylopterus*. Control region. Analysis of molecular variance (AMOVA) results.

Estimates of genetic differentiation between all eight pre-defined populations, using F-statistics, are given in Table 5.4. The populations from the NW Atlantic and Cape Verde Islands showed high levels of genetic differentiation from all the other populations. F_{st} values were high for all comparisons with the Cape Verde Islands, ranging from 0.235 to 0.659. For comparisons with the USA continental slope populations values ranged from 0.690 to 0.766. The *p*-values associated with these comparisons were significant (p<0.0005). Significant genetic differentiation was not detected between populations within the NE Atlantic region (Azores, Peniche, Madeira).

Populations / F_{ST}values	Azores (Oriental)	Azores (Ocidental)	Azores (Central)	Azores Seamounts	Peniche	Madeira	NW Atlantic	Cape Verde
Oriental	-	-0.0115	-0.0117	-0.0116	0.0048	-0.0075	0.7583***	0.3036***
Ocidental		-	0.0167	-0.0085	0.0034	-0.0101	0.7326***	0.2711***
Central			-	0.0083	0.0069	0.0191	0.7665***	0.3317***
Seamounts				-	-0.0077	-0.0189	0.6904***	0.2346***
Peniche					-	-0.0162	0.7173***	0.2434***
Madeira						-	0.7272***	0.2349***
NWA							-	0.6587***
CapeVerde								-

Table 5.4 - *H. dactylopterus.* D-loop. Pairwise F_{ST} values between populations and significance. *p*-values: ***= significant at p<0.001 (after sequencial Bonferroni corrections).

Phylogenetic analysis

Haplotype networks were inconclusive for d-loop and were excluded from this section as a result of high variability and high level of homoplasy amongst sequences. Neighbour-joining trees showed a poor resolution amongst populations from the NE Atlantic (Azores, Madeira, European continental shelf) but Cape Verde individuals were mostly contained within 3-4 clades depending on the type of analysis and NWA individuals were always contained within a single clade. For Neighbour-Joining analysis Cape Verde individuals were most closely related or sister haplotypes to NWA haplotypes but bootstrap sampling of the data showed that this relationship was not supported and trees were characterised by a high number of polytomies (Figure 5.2). A few Cape Verde individuals were detected amongst NE Atlantic haplotypes and a few NE Atlantic individuals were detected amongst Cape Verde clades or unresolved haplotypes. Bootstrap support was extremely poor for Neighbour Joining trees apart from near the tips of the tree. *Sebastes inermis* appeared in different parts of the tree with different analyses suggesting that this sequence was so distantly related to *H. dactylopterus* that it was effectively acting as random data (e.g. Wheeler, 1990).

Demographic History and Neutrality

Since no evidence of genetic differentiation was observed in the Azores, all populations within this archipelago could be pooled as a single group to conduct tests of selective neutrality and demographic history as for intrapopulation diversity. Pairwise mismatch distributions and results of Tajima's D-test performed on each population are given in Fig 5.3. The parameters of the model of sudden expansion (Rogers & Harpending, 1992) and the goodness of fit test to the model are also given in Table 5.5. All histograms presented multi-modal curves characteristic of populations with constant size over time. Most of the populations presented moderate to highly negative Tajima's D-test values although only one, the NW Atlantic was significant. This population presents a unimodal curve with a significantly negative Tajima D-test value, indicating a sudden expansion in population size. A time of this expansion was estimated at approximately 642,000 years before present.

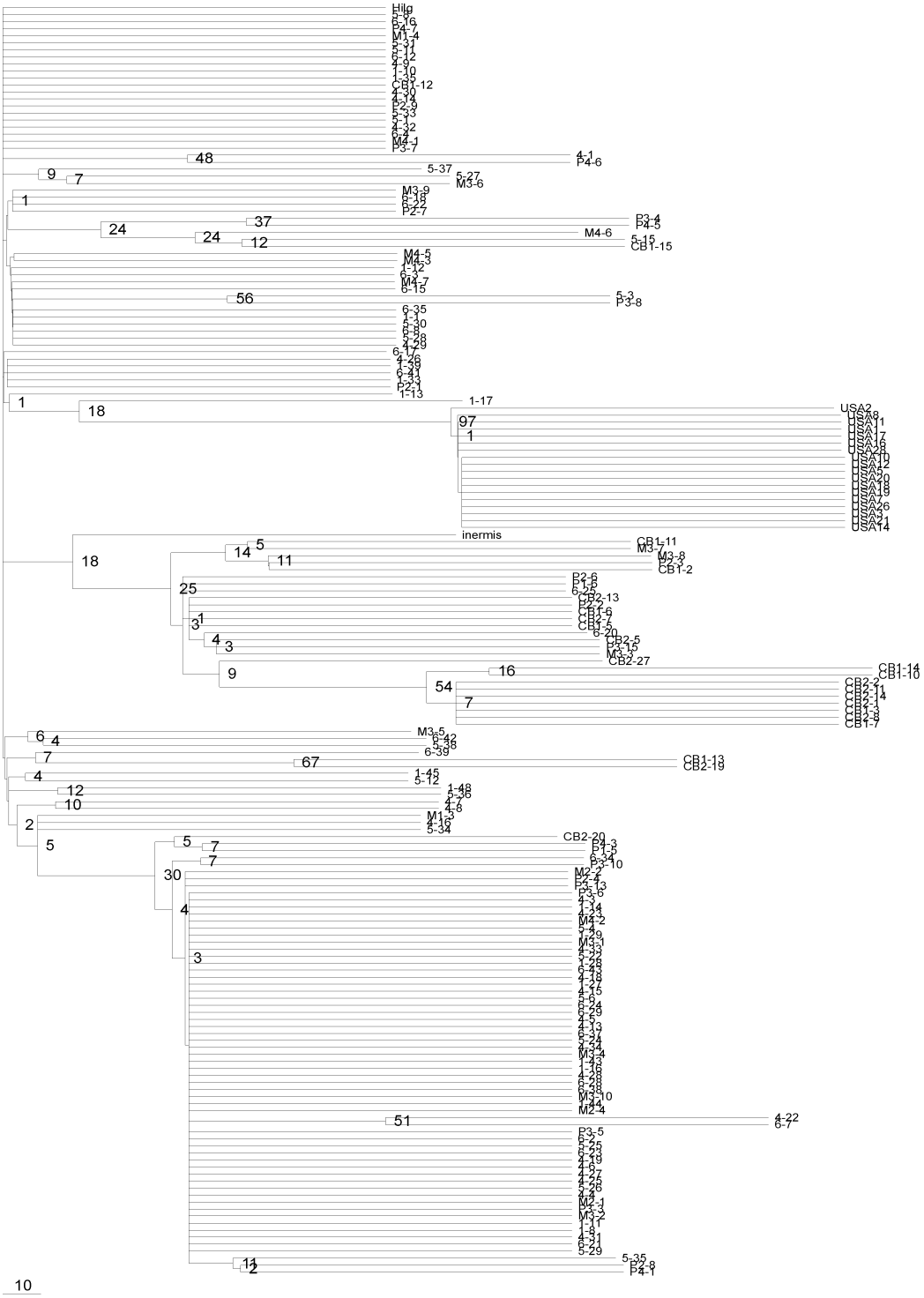


Figure 5.2. *H. dactylopterus*. Control region. Majority rule concensus parsimony tree with 100 bootstraps. Inermis = Sebastes inermis; Hilg = Helicolenus hilgendorfii (northwest Pacific); P = European continental shelf; M = Madeira; CB = Cape Verde Islands; USA = US continental slope. Samples without alphabetical prefix are from the Azores.

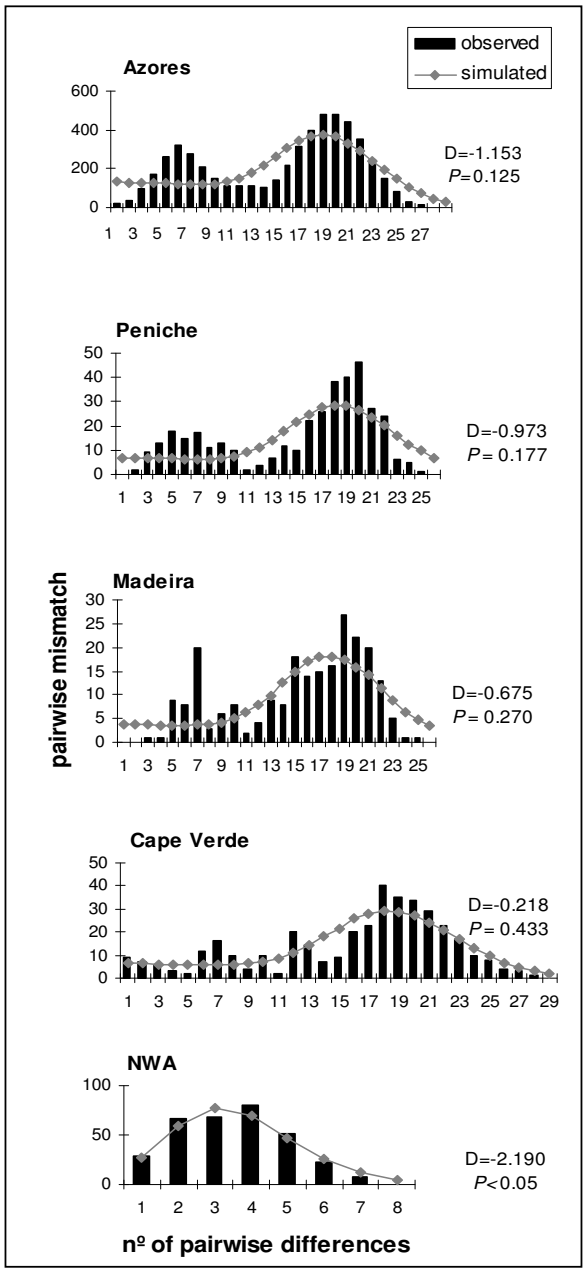


Fig.5.3 – *H.dactylopterus*. Control region. Pairwise mismatch distributions (Rogers & Harpending 1992), simulated model of sudden expansion (Rogers 1995) and results of Tajima's D-test with associated probability (Tajima 1989) for each population.

Populations	Azores	Madeira	Peniche	Cape verde	NWA
Parameters					
S	105	57	67	60	24
θ_0	0.003	0.000	0.002	0.005	0.002
θ_1	38.037	60.469	54.258	59.102	35.039
τ	18.172	17.875	18.478	18.630	2.728
Goodness of fit test					
SSD	0.008	0.013	0.012	0.006	0.003
P	0.238	0.223	0.173	0.513	0.595
R	0.004	0.018	0.009	0.009	0.034
P	0.859	0.360	0.705	0.579	0.692

Table 5.5 – *H.dactylopterus*. Control region. Parameters of the sudden expansion model and goodness of fit test to the model for each population. S-number of polymorphic sites, θ_0 – pre-expansion population size, θ_1- post-expansion population size, τ - time in number of generations, SSD – sum of squared deviations, R- raggedness index, *P*= p-values.

5.4.2 Cyt *b* region

Cyt b sequence variation

The 5'end of the cyt *b* mtDNA region (423 bp) was amplified from a total of 212 individuals altogether. The alignment of the sequences revealed 69 different genotypes defined by 70 divergent nucleotide sites. Most nucleotide variation resulted from transitions followed by transversions with an expected Transition/Transversion ratio of 5.63. The nucleotide frequencies were T = 0.302, C = 0.287, A = 0.249 and G = 0.162. The most common haplotype (GenBank Acc. No. AY563095) was found in 50 individuals from the NE Atlantic populations (Azores, Madeira, European continental slope) but this was not detected in Cape Verde and NWA individuals. 14 other haplotypes were also found shared amongst individuals from different areas.

Population Variability

Haplotype diversity (*h*), within the geographic populations was high ranging from 0.426 in the NWA population to 0.936 in Madeira. Nucleotide diversity (π) was generally low ranging from 0.002 in the NWA population to 0.007 in continental Portugal (Peniche) (Table 5.6).

Populations	**Nucleotide diversity (π)**	**Haplotype diversity (h)**
CAPE VERDE	**0.006**	**0.788**
NWA	**0.002**	**0.426**
MADEIRA	**0.007**	**0.936**
PENICHE	**0.007**	**0.860**
AZORES	**0.005**	**0.865**

Table 5.6. *H. dactylopterus*. Cyt *b*. Intrapopulation nucleotide (π) and haplotype (h) diversities.

Phylogeographic relationships of populations

The hierarchical partition of variance amongst populations tested using AMOVA (Excoffier *et al.*, 1992) was performed as previously presented for the control region (Table 5.7). The proportion of "among regions" variation is large (45.52%) and significant. Again, only a small variance component was attributable to populations within regions but this was also significant.

Source of variation	% Total variance	Fixation indices	P-value
Among groups	42.52	F_{CT}= 0.4252	<0.001
Among populations within groups	-0.07	F_{SC}= -0.0013	<0.001
Within populations	57.56	F_{ST}= -0.4244	<0.001

Table 5. 7. *H. dactylopterus*. Cyt *b*. Analysis of molecular variance (AMOVA) results.

The estimates of pairwise F_{ST} values between the different geographic areas are given in Table 5.8 together with respective *p*-values. High F_{ST} values were found in pairwise comparisons between all NE Atlantic populations and the Cape Verde and NW Atlantic. *P*-values were all significant ($p<0.0005$), as for the results obtained with the control region.

F_{ST}values	Azores (Oriental)	Azores (Ocidental)	Azores (Central)	Azores Seamounts	Peniche	Madeira	NWA Atlantic	Cape Verde
Oriental	-							
Ocidental	0.0002	-						
Central	-0.0123	0.0052	-					
Seamounts	0.0082	-0.0043	-0.0004	-				
Peniche	0.0270	0.0279	0.0188	0.0096	-			
Madeira	-0.0027	-0.0068	0.0053	0.0013	0.0069	-		
NWA	0.7910***	0.7408***	0.7714***	0.7315***	0.7159***	0.7531***	-	
Cape Verde	0.4154***	0.3668***	0.3778***	0.3119***	0.2509***	0.3440***	0.6845***	-

Table 5.8. *H. dactylopterus.* Cyt *b*. Pairwise F_{ST} values for between populations and significance. *p*-values: ***= significant at $p<0.0005$ (after sequencial Bonferroni corrections)

Phylogenetic analysis

The haplotype network derived from cyt *b* partial sequences is presented in Figure 5.4. The most common haplotype, represented by a square-shaped box (size of squares and circles is proportional to the number of haplotypes) represents individuals from the NE Atlantic (Azores, Madeira, Peniche). The fourth and fifth most common haplotypes were restricted to the NW Atlantic and Cape Verde respectively. The star-shaped phylogenies for 3 primarily NE Atlantic clades, and the Cape Verde and NWA clades are consistent with recent population expansion. The fact that different regions are almost exclusive to specific clades is consistent with results from analysis of molecular variance and F-statistics that suggest strong genetic differentiation between the NE Atlantic, Cape Verde and NW Atlantic (but see below).

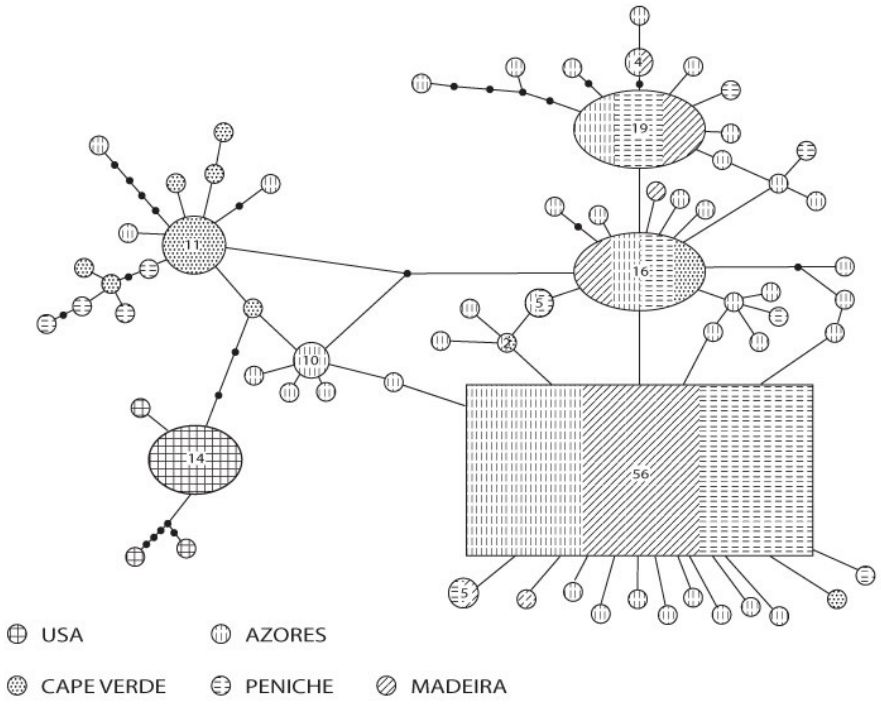


Figure 5.4. *H. dactylopterus*. Cyt *b*. Minimum spanning network analysis of haplotypes for all populations. Black dots represent putative mutational steps between haplotypes.

Neighbour-joining and maximum parsimony analysis of the cyt *b* haplotype data (Figure 5.5) also showed poor resolution for NE Atlantic populations and poor bootstrap support for the trees as a whole. However there were similarities in the topology of all trees for cyt *b* analyses, including Tranversion-only analysis, in that NW Atlantic haplotypes formed a single clade and this was most closely related to Cape Verde haplotypes that were found mostly in 3 clades. A few Cape Verde individuals exhibited NE Atlantic haplotypes and a few Cape verde haplotypes were found in the NE Atlantic islands. A single small clade of haplotypes from the Azores only was also consistently resolved but with poor support. This may contribute to the small but significant variance component detected by analysis of molecular variance at the sub-regional scale. *Sebastes* spp did not consistently occur in one place on the tree though for Neighbour-Joining analysis, if designated as the outgroup. Again this is consistent with a distant relationship between *Sebastes* spp and *Helicolenus* resulting in a large degree of homoplasy in the data.

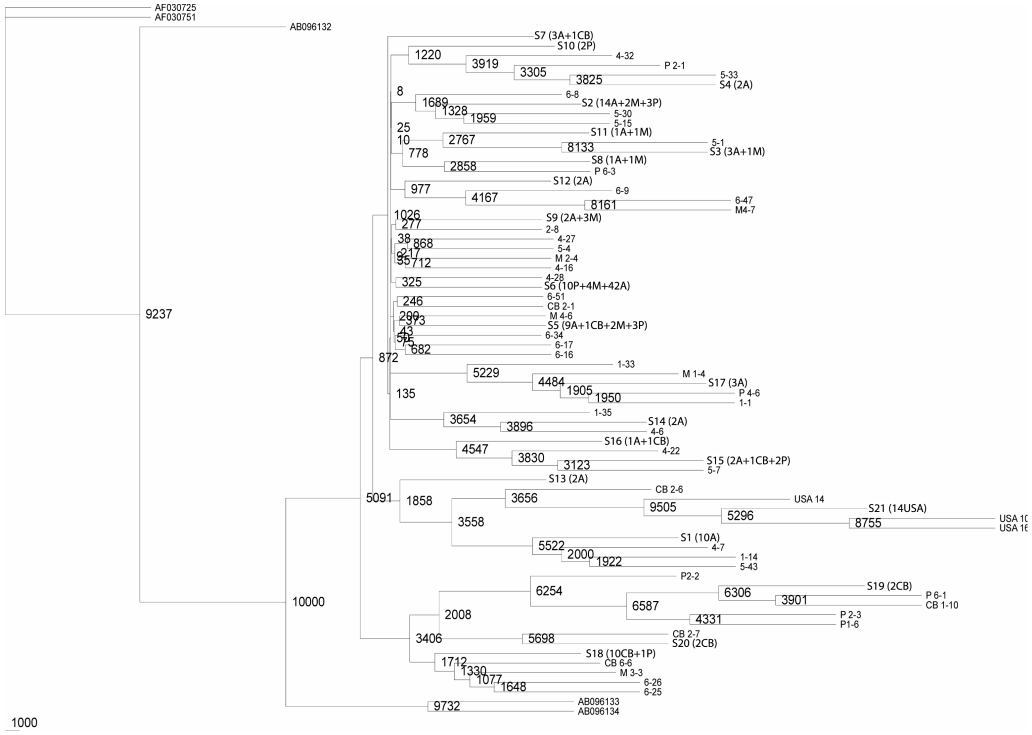


Fig.5.5.- *H. dactylopterus*. Cytb. Majority rule concensus neighbour-joining tree with 10,000 bootstraps. AF030725 = *Sebastes emphaeus*; AF030751 = *Sebastes hopkinsi*; AB069132 = *Hozukius emblemarius*; AB096133 & AB069134 = *Helicolenus hilgendorfii* (northwest Pacific); P = European continental shelf; M = Madeira; CB = Cape Verde Islands; USA = NWA continental slope. Samples without alphabetical prefix are from the Azores.

Demographic History and Neutrality

Pairwise mismatch distributions and results of Tajima's D-test performed on each population - Azores, Madeira, Peniche, Cape Verde and NWA – are given in Fig 5.6. The parameters of the model of sudden expansion (Rogers & Harpending, 1992) and the goodness of fit test to the model are also given in Table 5.9. For Azores and NWA mismatch distributions were close to an estimated Poisson model curve and presented significantly negative Tajima's D-values, indicating more rare nucleotide sites than it would expected under a neutral model of evolution. The Madeira population could not be fitted to an expansion model. For Cape Verde and Peniche populations Tajima's D-values were not significantly negative, and the mismatch distributions were closer to multi-modal curves, normally characteristic of stable populations over time. The estimated times of expansion for populations from the Azores and the NWA were 1.02 and 1.24 million years respectively.

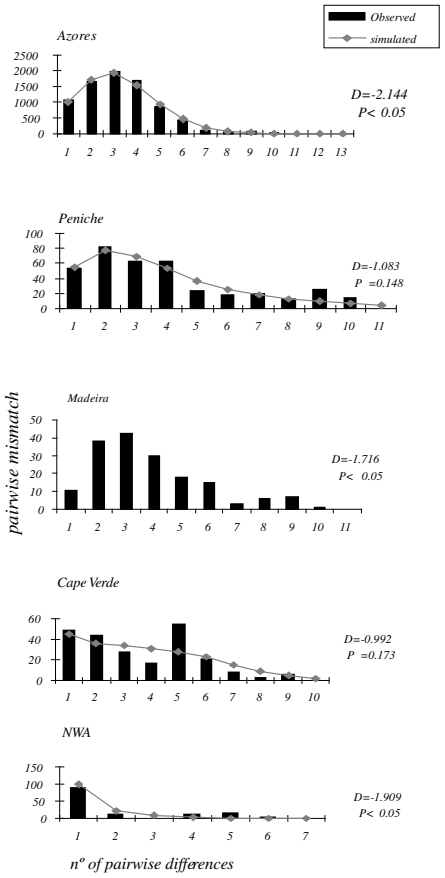


Fig 5.6 – *H.dactylopterus*. Cyt *b*. Pairwise mismatch distributions (Rogers & Harpending, 1992), simulated model of sudden expansion (Rogers, 1995) and results of Tajima's D-test with associated probability (Tajima, 1989) for each population

Populations	**Azores**	**Madeira**	**Peniche**	**Cape verde**	**USA**
Parameters					
S	42	19	17	13	7
θ_0	0.003		2.472	0.005	0.404
θ_1	15.079		620.625	4.073	0.405
τ	2.473		0.701	4.544	3.000
Goodness of fit test					
SSD	0.001	No fit	0.005	0.022	0.034
P	0.852		0.727	0.561	0.135
R	0.024		0.024	0.061	0.351
P	0.789		0.867	0.582	0.634

Table 5.9 – *H.*dactylopterus. Cyt *b*.Parameters of the sudden expansion model and goodness of fit test to the model. S-number of polymorphic sites, θ_0 – pre-expansion population size, θ_1- post-expansion population size, τ - time in number of generations, SSD – sum of squared deviations, R- raggedness index, *P*= p-values.

5.5 Discussion

5.5.1 Genetic variation

Nucleotide ratios are consistent with previous findings that the mtDNA control region is an A-T rich region for fish and other vertebrates. Nucleotide and haplotype diversity of *H. dactylopterus* populations can provide some information on the history of bluemouth populations in the North Atlantic. High genetic variation (*h*) and low to moderate nucleotide diversity (π) were found in all populations analysed for both mtDNA markers. This pattern of genetic diversity can be attributed to a population expansion after a low effective population size caused by bottlenecks or founder events (Grant & Bowen, 1998). Such an explanation is also consistent with the star-shaped haplotype networks detected for cyt *b* in populations of *H. dactylopterus* (Fig.5.4). Mismatch distribution analysis further supports a population expansion, for populations in the Azores and the NW Atlantic (Fig.5.3 and 5.6). In such cases, the rapid growth of a population leads to the retention of new mutations especially in mtDNA sequences, known to evolve several times faster than nuclear DNA (Brown *et al.*1979). Such patterns of diversity, haplotype networks and mismatch equilibrium strongly suggest a historical influence on the genetic structure of *H. dactylopterus* populations as estimated by analysis of haplotype frequencies.

5.5.2 Population structure

F-statistics, analysis of molecular variance, haplotype network and phylogenetic analysis all indicate marked genetic structure in *H. dactylopterus* populations at the inter-regional scale. There appears to be no effective gene-flow between the northeastern and northwestern Atlantic populations of this species and little or no gene-flow between the Cape Verde Islands and populations on the Mid-Atlantic Ridge (Azores), Madeira and the European Continental slope (Peniche). However, a few "Cape Verde" haplotypes were sampled from NE Atlantic populations and vice versa. This may represent occasional migrants between these localities though the overall level of genetic exchange must be below that required to homogenise populations, or there is a barrier to gene flow between these populations (i.e. they are separated species). These haplotypes may also represent historical migration events or even homoplasy amongst haplotypes. In contrast to studies on some seamount-associated species (wreck fish, alfonsino, slender armourhead) the larval dispersive phase in *H. dactylopterus* is not sufficient to allow gene-flow between populations at the regional scale.

Occasional long-distance migration has been detected in other deep-water species that can even span the entire length of oceans (e.g.Møller *et al.*, 2003).

Within the geographic region of the NE Atlantic there is some evidence for genetic differentiation between the island groups and seamounts of the Azores, Madeira and the European continental slope. Analysis of molecular variance showed that only a small component of variance was attributable to populations within regions, however, this was significant. It must also be noted that phylogenetic analysis for both d-loop and cyt *b* data both revealed a small clade of haplotypes that only occurred in the Azores. This is suggestive of some degree of reproductive isolation of Azores populations but the limited sample sizes in this investigation, and the poor resolution of phylogenetic analyses, limit the interpretation of these data. Migration between these populations is probably occurring by larval transport in the Azores and Canaries currents. Adult migration is not consistent with behavioural observations for this species but these data are limited at present so this cannot be ruled out.

More detailed investigations of the populations within this region, using high-resolution genetic markers to detect fine-scale genetic structure, are presented in the next chapter.

5.5.3 Demographic Evolution of *H. dactylopterus* populations

A strong historical influence on the genetic population structure of marine organisms in the North Atlantic has been suggested for other demersal fish species (e.g. Carr *et al*. 1995, Pogson *et al*. 1995, Muus *et al.* 2001, Bargelloni *et al.* 2003, Stockley *et al.* 2005). In particular, the last glacial maximum is thought to have been responsible for the extermination or reduction of populations of marine organisms in both the North Atlantic and North Pacific. The survival of some species in refugia is thought to be responsible for genetic signatures of bottlenecks followed by recent population expansion. Mismatch equilibrium tests and Tajima's D are both indicative of population bottlenecks followed by expansion in at least two of the sample locations, the Azores and the NW Atlantic. The haplotype network and phylogenetic analyses of the NE Atlantic, Cape Verde and NWA populations suggest historical dispersal events between these populations followed by population expansions. The phylogenetic trees and minimum-spanning networks are similar to extinction-recolonization models of other fish taxa (e.g. sardine evolution; Grant & Bowen, 1998).

The polarity of the relationships between the NW Atlantic and Cape Verde and other NE Atlantic populations inferred by using *Helicolenus hilgendorfii* as the outgroup taxon suggest that the eastern Atlantic populations are likely to have been the source of migrants for the

NW Atlantic population of *H. dactylopterus*. Migration between the NE Atlantic and Cape Verde Islands is limited in the present day and if it exists at all it is sporadic. Dispersal routes may include larval dispersal via the Canaries current and adult dispersal along the continental slope. This is consistent with data that suggests that current western Atlantic populations of some marine invertebrates such as *Asterias rubens*, *Littorina obtusata* and *Nucella lapillus* were founded from the eastern Atlantic (e.g. Wares & Cunningham 2001). For these species, trans-Atlantic migration is thought to have occurred after the last glacial maximum, which was much more severe in the NW Atlantic than the NE, resulting in elimination of populations of marine organisms on the eastern coast of North America (Wares & Cunningham 2001, Hewitt 2003). The last glaciation was sufficiently severe not only to influence intertidal and near-shore species but also impacted deep-water species living in the Atlantic probably through effects on the planktonic stage of the life-cycle and/or by influencing food supply for adult populations. Refugia in the eastern Atlantic allowed survival of populations throughout this episode of climatic cooling. The low genetic diversity associated with USA populations is also consistent with long-distance or jump-dispersal events between the eastern and western Atlantic rather than stepping-stone or Gaussian modes of dispersal (reviewed Hewitt, 2003). Intuitively, it may seem unlikely that deep-sea species were strongly impacted by previous glaciation events, but the larvae of *H. dactylopterus* are planktonic and would therefore be vulnerable to changes in sea-surface temperatures and other physical factors. In addition, food chains in the deep sea, with the exception of chemosynthetic communities, are dependent on surface productivity. Any changes that impact surface productivity patterns are likely to have knock-on effects on food webs as indicated by recent changes in community structure of deep-sea animals (e.g. Billett *et al.* 2001, Wigham *et al*. 2003). An east to west migration is counter to evidence that some tropical Atlantic taxa migrated from the western Atlantic to the eastern Atlantic via the Atlantic Equatorial Undercurrent (Muus *et al.* 2001). Historical dispersal for *H. dactylopterus*, across the Atlantic would have to occur at the larval stage via the Northern Equatorial Current.

The estimated times for expansion in *H. dactylopterus*, however, are not consistent with the end of the last glacial maximum, but are much older ranging from 0.64 – 1.2 million years for the NWA population to 1.02 million years for the Azores population. These times are more in agreement with data available for populations of decapod crustaceans from the North Atlantic / Mediterranean region (200,000 – 400,000 years; Stammatis *et al*., 2004) but even exceed these estimates markedly. The period encompassed by the expansion times for *H. dactylopterus* populations in the NWA and Azores coincide with a period of major global

climatic change, known as the Mid-Pleistocene Revolution. This period began with a major advance in ice sheets, as detected through marine oxygen isotope ($\delta^{18}O$) records around 900,000 years ago, that also coincided with major shifts in ocean biogeochemistry (Becquey & Gersonde, 2002). This was followed by a shift to 100,000-year glacial cycles at approximately 650,000 years ago (Mudelsee & Schulz, 1997). A further significant event, known as the Mid-Brunhes Event occurred 300,000 – 400,000 years ago and was associated with an increase in glaciation of the northern hemisphere (Jansen *et al*., 1986; Becquey & Gersonde, 2002). Whilst the estimates of times of population expansion from this study may be subject to considerable margins of error it is clear that the time of expansion for *H. dactylopterus* populations, in the North Atlantic, is much older than the LGM. The extensive time period since expansion may explain the morphological differences between populations of this species and together with phylogeographic data may even cast doubt on the conspecificity of at least some *H. dactylopterus* sub-species (see Emerson *et al*. 1999 for a comparable terrestrial example). Significantly it suggests that glaciations prior to the Last Glacial Maximum may strongly influence the genetic structure of populations of marine species in the North Atlantic / Mediterranean. Some of these events were more severe than the Last Glacial Maximum and may have had geographically wider and more profound impacts on marine organisms.

CHAPTER 6
HIGH-RESOLUTION ANALYSIS OF *Helicolenus dactylopterus* (SEBASTIDAE) POPULATION STRUCTURE IN THE NORTHEASTERN ATLANTIC USING MICROSATELLITES

6.1 Abstract

The population structure of *Helicolenus dactylopterus* in Portuguese waters was surveyed using 8 polymorphic microsatellite loci. At a regional scale the geographic locations of Peniche (mainland Portugal), Madeira and Azores where sampled with a finer local scale analysis undertaken within the Azores archipelago between island groups: Occidental group, Oriental group, Central group and a seamount (Azores bank).

Contrary to previous work with mtDNA sequences, which showed little evidence for genetic differentiation of bluemouth populations within the NE Atlantic, microsatellite data revealed isolation of the Peniche population and some differentiation at the local scale within the Azores archipelago.

There were significant excesses of homozygotes over all samples, more than expected for randomly mating populations in Hardy-Weinberg equilibrium. Tests of F_{ST}, R_{ST}, S_{DW} and $(\delta\mu)^2$ genetic distances measures revealed significant differences between Continental Portugal (Peniche) and the Azores archipelago sup-populations (P<0.005). Some inter-regional differentiation was also detected within the Azores archipelago and resolved by multiscalling analysis of genetic distances. Isolation-by-distance was not confirmed by Mantel tests.

The microsatellite heterogeneity suggests that there is a strong population differentiation that may have be caused by lack of gene-flow between populations at the regional scale, hydrographical and climatic factors or more recent and on-going demographic events caused by anthropogenic actions in some regions.

6.2 Introduction

The previous chapter was dedicated to the study of the population structure of *Helicolenus dactylopterus* within the North Atlantic using mtDNA markers. The work revealed strong population differentiation between both the Cape Verde and NW Atlantic populations from the NE Atlantic populations (Azores, Madeira and Peniche). This was contrary to expectations based on potential for larval-mediated dispersal in this species but consistent with previous work showing morphological differences between geographically separated populations of bluemouth. However, a degree of reproductive isolation of the Azores archipelago was suggested by phylogenetic analyses, and analysis of molecular variance attributed a small but significant component of variance to groups within the Azores archipelago. This suggested that population differentiation maybe occurring in this species at the local geographic scale.

Significant differences in *H.dactylopterus* landing numbers (e.g. Azores and Peniche markets) can also be indicative of geographic differences in population size and perhaps demographic parameters at a fine geographic scale. This may occur if populations are acting independently as separate stocks.

MtDNA sequences are considered to be very useful for population genetic studies particularly because of their rate of evolution, which is faster than nuclear DNA (Brown *et al.*, 1979 and Meyer, 1994) (see Chapter 1). However, recent studies employing microsatellites markers have began to uncover regional and subpopulation structuring in pelagic and demersal fish species (e.g. cod) previously thought to be homogeneous over large geographical ranges when analysed using mtDNA sequencing (Ruzzante *et al.*, 1999; and 2000).

When effective population size is moderate, mtDNA sequences are more likely to provide better population markers because of greater genetic drift in this genome, but this susceptibility can also lead to misleading conclusions with regards to population substructuring in large panmitic populations (Ferguson *et al.*, 1995 and Ferguson & Danzmann, 1998).

The high levels of allelic variation together with other features (see Chapter 1) make microsatellite loci better markers to understand genetic variability at a finer-scale and to resolve microevolutionary events between closely related populations or at the intra-population level (Estoup & Angers, 1998). They permit the detection of slight departures from panmixia in geographically proximate populations with limited genetic variation or that have experienced severe bottlenecks (Wright & Bentzen, 1994).

In terms of conservation and management of exploited fish species it is important to understand the distribution of genetic diversity to delineate distinct stocks or management units. Estimating the dispersal capacity of a species is fundamental to assess its ability to re-colonize areas after a natural or anthropogenic impact. Small local populations, with low genetic diversity, which are more susceptible to extinction, are formed when gene flow is geographically restricted.

Primer pairs for eight highly polymorphic microsatellite loci were specifically developed for *Helicolenus dactylopterus* (see Chapter 4 and Aboim *et al.*, 2003) and used here. The objective of the present study was to conduct a survey on the genetic variability revealed by these markers to test the hypothesis of genetic differentiation of bluemouth populations at a finer geographic scale, within a single region of the NE Atlantic. This region includes the Portuguese waters that comprise the Madeira and Azores archipelagos and the continental slope of Europe adjacent to Portugal. 390 individuals were sampled in Portuguese waters from 6 different localities: Peniche (continental Portugal), Madeira Islands and 4 areas within the Azores archipelago (Oriental, Central, Occidental and Seamounts groups).

6.3 Material and Methods

6.3.1 Sampling

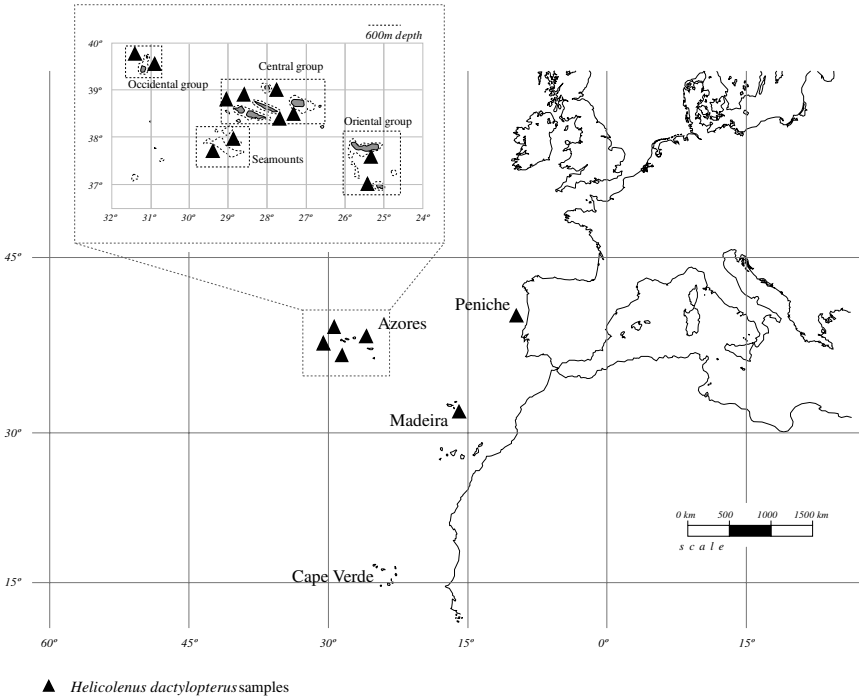


Fig.6.1. *H.dactylopterus*. Map with sampling sites for microsatellite analysis.

Samples were collected using methods described in Chapter 3. A list of the utilised samples and corresponding sample locations is presented in Table 6.1 and illustrated in Figure 6.1.
An effort was made to obtain a minimum number of 80 individuals per location as indicated by Ruzzante (1998) which suggests an optimum sample size of between 50 and 100 when working with microsatellite data.

Location	Area	Cruise	Date	Sample size
Azores	Oriental group	ARQDAÇOP00	May 2000	88
	Ocidental group	ARQDAÇOP00	June 2000	80
	Central group	ARQDAÇOP99 ARQDAÇOP00	 May 2000	45 34
	Seamounts	ARQDAÇOP00	May 2000	80
Madeira	Madeira	ARQMADP97	June 1997	26
Portugal	Peniche	-	August 2001	80

Table 6.1 – List of utilized samples for microsatellite analysis.

6.3.2 Microsatellite amplification and Genotyping

A detailed description of the primer development, DNA extraction protocols, PCR and running conditions are given in Chapter 4 (see also, Aboim *et al.*, 2003). Genotyping of microsatellite loci was performed by labelling reverse primers from each pair with a different 5'-fluorescent dye, taking into account requirements for multiplex PCR reactions and electrophoresis gels: Hd 008 (6-FAM); Hd020 (HEX); Hd044 (HEX); Hd 063 (NED); Hd 076 (NED); Hd 092 (6-FAM); Hd 095 (NED) and Hd106 (6-FAM) (Table 4.2).
PCR products were diluted 4 times before screened on an ABI 377 automated sequencer. Gel lanes were analysed using GeneScan® ABIPrism software and detected peaks were transferred to Genotyper®ver.2.00 ABIPrism for further analysis.

6.3.3 Data Analysis

Intrapopulation genetic diversity was estimated from the allelic composition of 6 putative populations. Allele frequencies, estimates of number of alleles and probability tests for gene and genotypic differentiation were estimated using GENEPOP vers. 3.1 software (Raymond & Rousset, 1995). Arlequin ver 2.0 (Schneider *et al.*, 2000) was used to estimate observed and expected heterozygosities and to test for conformation with Hardy-Weinberg equilibrium expectations using a test analogous to the Fisher's exact tests (Guo &

Thompson, 1992). The Arlequin program was also used to carry out an analysis of molecular variance (AMOVA).

To test whether a particular locus contributed disproportionately to any heterozygote deficiencies, single and multilocus F_{IS} estimators were calculated as in Weir & Cockerman (1984) using F-STAT ver.2.9.3 (Goudet, 1995). The null hypothesis of no linkage disequilibrium was tested for all possible pairs of loci in each population and for all populations using the same software, as well as, the estimation of extent of genetic differentiation among pairs of samples with pairwise F_{ST} analysis (estimated by θ; Weir & Cockerman, 1984).

Because of a current debate concerning the most suitable statistic for quantifying population differentiation with microsatellite loci, the genetic variation apportioned among samples was also estimated with unbiased R_{ST} (estimated by *Rho*; Goodman, 1997) which assumes a stepwise mutation model and adjusts for differences in sample and allele sizes variances, using the RST-CALC computer programme (Goodman, 1997).

For this study, genetic distances between populations were estimated through D_{SW} (Shriver *at al.*, 1995) and $(\delta\mu)^2$ (Goldstein *et al.*1995) using the Populations vers. 1.2.28 software (Langella, 1999). To better understand the genetic population variation a multidimensional scaling of genetic distances was also performed on both D_{SW} and $(\delta\mu)^2$ using Primer ver.5.2.0 software package (Clarke & Gorley,2001). This approach has the advantage of detecting non-hierarchical aspects of genetic variation without excluding hierarchies and linearity of other hierarchical analyses (Lessa, 1990).

The hypothesis that subtle genetic differences have arisen as a result of geographic isolation, in which dispersal between sites may be correlated to geographic distance – the stepping stone model – was tested through the correlation between pairwise $F_{ST}/(1 - F_{ST})$ values and the logarithm of geographic distances between populations. Statistical significance was inferred with a Mantel Test (10000 permutations) (Mantel, 1967) as in Genepop vers. 3.3 (Raymond & Rousset, 1995). Distances among samples were measured as the shortest distance between localities in kilometres (Km).

The existence of null alleles was tested with MicroChecker (Van Oosterhout, 2004) as well as other possible genotyping errors (i.e. stuttering and large allele dropout) within the microsatellite data set.

The program Migrate (Beerli & Felsenstein, 2001) was used to estimate relative effective population sizes. Migrate uses a Markov chain Monte-Carlo based Maximum-likelihood (MCML) approach based on an expansion of the coalescent model to estimate θ. Theta (θ) is equal to $4N_e\mu$, where N_e is the long-term (inbreeding) effective population size and μ is the

mutation rate for the microsatellite data set. As microsatellite mutation rates (μ) are reported to vary in several orders of magnitude (10^{-5} to 10^{-2} per locus; Weber & Wong, 1993), θ-estimates were not used to assess actual effective population sizes but, assuming that the mutation rate is the same for all microsatellite loci, were validated as estimators of relative effective population sizes for comparison between samples.

6.4 Results

6.4.1 Bluemouth microsatellite loci

Eight microsatellite loci were isolated and amplified for 390 bluemouth individuals across 6 sample locations. All loci were polymorphic across all populations and were used for the subsequent analyses. The overall variability and characteristics of microsatellite loci are described in Table 6.2 (See also Chapter 3 and Aboim *et al.,* 2003).

Across all six samples combined, the average number of alleles per locus was relatively high (k = 20) ranging from 38 alleles for *Hd008* to a minimum of 7 alleles for *Hd020* and *Hd076*. These were also the loci with the highest and the lowest observed and expected heterozygosities.

Locus	GenBank Accession No.	Repeat Motif	Size Range	n	N_A	HW	F_{IS}	F_{ST}	R_{ST}
Hd 008	AY123151	$(CA)_6CCCATGTA(CA)_8$ $CCTATGTA(CA)_{14}$	203-249	357	38	***	0.137	-0.002	-0.0008
Hd020		$(CA)_9$	117-169	374	7	***	0.371	0.050	0.0099
Hd 044	AY123152	$(GT)_9$	178-192	382	8	***	0.284	0.005	-0.0037
Hd 063	AY123153	$(GTGTGTGTT)_4\,(GT)_4$	209-244	385	9	**	0.075	0.003	-0.0010
Hd 076		$(GT)_9$	209-221	383	7	***	0.165	0.010	0.0098
Hd 092	AY123154	$(CA)_{21}$	165-215	380	27	***	-0.007	0.021	0.0000
Hd 095	AY123155	$(CA)_{12}$	110-194	371	34	Ns	0.278	0.002	0.0001
Hd 106	AY123156	$(GT)_5TT\,(GT)_{13}$	125-191	388	32	***	0.157	0.013	0.0286

Table 6.2 - *H.dactylopterus.*Microsatellites genetic variability. N= sample size; N_A = number of alleles; HW= significance of deviation from Hardy-Weinberg Equilibrium *** = P<0.001, ** = P<0.05; F_{IS} = variance of allele frequencies within populations; F_{ST} and R_{ST}= estimates of overall genetic differentiation between populations.

6.4.2 Genetic Variation

Table 6.3 lists the allele sizes in base pairs and their frequency in each population. All loci were highly polymorphic for *Helicolenus dactylopterus.* A total of 162 alleles were found in the 390 sampled individuals. The number of alleles per locus varied considerably, ranging from 7 to 38. There were private alleles in all populations and Peniche presented the highest number with 8 private alleles. There did not appear to be large differences among geographic sites in the number of alleles resolved, except for Madeira, which had a significantly smaller number of sampled individuals.

Allele frequencies distributions were multimodal and exhibited many rare alleles.

Locus 008			Na= 21.5			
Allele	**Oriental Group n=70**	**Central Group n=55**	**Ocidental Group n=76**	**Seamounts n=64**	**Madeira n=21**	**Peniche n=71**
195	0.007	0	0	0	0	0
197	0	0	0	0.008	0	0
198	0	0.009	0	0	0	0
201	0	0	0.013	0	0	0.007
203	0.029	0.009	0.026	0.023	0	0.021
205	0.007	0.009	0.020	0.023	0	0.021
207	0.021	0.045	0.007	0.016	0.024	0.007
209	0.014	0.009	0.013	0.031	0.024	0.021
211	0	0.009	0.013	0.008	0	0.014
213	0.021	0.009	0	0	0.024	0
215	0.007	0	0.020	0.016	0.024	0.007
217	0	0.009	0	0	0	0
218	0	0	0	0	0	0.007
219	0.014	0.009	0.039	0.016	0.048	0.014
221	0.021	0.045	0.007	0.031	0.024	0.021
223	0.150	0.100	0.105	0.125	0.071	0.077
225	0.236	0.227	0.276	0.242	0.286	0.190
227	0.114	0.127	0.112	0.133	0.071	0.148
229	0.057	0.045	0.039	0.047	0.143	0.049
230	0	0.027	0	0	0	0
231	0.100	0.100	0.132	0.078	0.048	0.120
233	0.100	0.145	0.079	0.094	0.071	0.148
235	0.036	0.036	0.013	0.016	0.024	0.028
236	0	0.009	0	0	0	0
237	0	0	0.013	0	0.071	0.014
239	0	0.009	0.026	0.008	0	0.021
240	0	0	0	0	0.024	0
241	0.007	0	0	0.008	0.024	0.007
243	0.014	0	0	0	0	0.007
245	0.014	0.009	0	0.023	0	0.021
247	0.007	0	0.020	0.008	0	0
249	0.007	0	0	0.023	0	0
251	0	0	0	0	0	0.007
253	0.007	0	0.020	0.016	0	0
255	0.007	0	0.007	0	0	0
261	0	0	0	0	0	0.014
263	0	0	0	0	0	0.014
269	0	0	0	0.008	0	0

Observed alleles	23	21	21	23	16	25
Private alleles	1	3	0	2	1	4
Allelic richness	13.96	13.56	13.87	15.01	16.00	15.03

Locus 020				Na= 4.67		
Allele	**Oriental Group n=72**	**Central Group n=54**	**Ocidental Group n=74**	**Seamounts n=70**	**Madeira n=25**	**Peniche n=79**
117	0	0	0	0.007	0	0
159	0.007	0	0	0	0	0
161	0.042	0.306	0.014	0.043	0.080	0
163	0.750	0.565	0.838	0.714	0.840	0.810
165	0.181	0.093	0.122	0.214	0.040	0.146
167	0.021	0.037	0.020	0.021	0.020	0.038
169	0	0	0.007	0	0.020	0.006
Observed alleles	5	4	5	5	5	4
Private alleles	1	0	0	1	0	0
Allelic richness	3.81	3.86	3.41	3.85	4.66	3.11

Locus 044				Na= 5.33		
Allele	**Oriental Group n=70**	**Central Group n=64**	**Ocidental Group n=75**	**Seamounts n=72**	**Madeira n=23**	**Peniche n=78**
178	0	0	0.027	0	0	0
180	0	0	0.007	0	0	0
182	0	0.008	0.027	0	0	0.013
184	0.693	0.633	0.567	0.611	0.674	0.622
186	0.014	0.023	0.033	0.014	0	0.115
188	0.214	0.242	0.280	0.326	0.326	0.186
190	0.057	0.039	0.013	0.014	0	0.045
192	0.021	0.055	0.047	0.035	0	0.019
Observed alleles	5	6	8	5	2	6
Private alleles	0	0	2	0	0	0
Allelic richness	4.12	4.84	5.95	3.83	2.00	4.97

Locus 063				Na= 6.17		
Allele	**Oriental Group N=72**	**Central Group n=64**	**Ocidental Group n=77**	**Seamounts n=73**	**Madeira n=22**	**Peniche n=77**
209	0.069	0.062	0.091	0.103	0.068	0.071
216	0.021	0.008	0.006	0.007	0	0
218	0.368	0.406	0.429	0.274	0.364	0.448
225	0.007	0.031	0	0.007	0	0
227	0.458	0.391	0.396	0.473	0.455	0.409
234	0.014	0.016	0.013	0.007	0	0
236	0.049	0.070	0.065	0.130	0.114	0.065
243	0	0.016	0	0	0	0.006
244	0.014	0	0	0	0	0
Observed alleles	8	7	6	7	4	5
Private alleles	1	0	0	0	0	0
Allelic richness	5.83	6.17	4.70	4.86	4.00	4.21

Locus 076				Na=4.67		
Allele	**Oriental Group n=73**	**Central Group n=59**	**Ocidental Group n=75**	**Seamounts n=72**	**Madeira n=25**	**Peniche n=79**
209	0.007	0.008	0.013	0.035	0.020	0.006
211	0.027	0	0.013	0	0	0
213	0.048	0.008	0	0.049	0.200	0.051
215	0.767	0.737	0.787	0.688	0.660	0.797
217	0.137	0.246	0.180	0.222	0.120	0.146
219	0.014	0	0	0	0	0
221	0	0	0.007	0.007	0	0
Observed alleles	6	4	5	5	4	4
Private alleles	1	0	0	0	0	0
Allelic richness	4.44	2.71	3.25	4.03	3.84	3.19

Locus 092				Na= 19.83		
Allele	**Oriental Group n=70**	**Central Group n=59**	**Ocidental Group n=76**	**Seamounts n=72**	**Madeira n=25**	**Peniche n=78**
165	0	0.017	0	0.007	0	0
167	0.036	0.051	0.026	0.028	0.020	0.006
169	0.093	0.102	0.086	0.132	0.080	0.026
171	0.014	0.017	0.033	0.014	0	0
173	0.029	0.025	0.020	0	0	0.019
175	0.079	0.110	0.099	0.090	0.040	0.026
177	0.057	0.059	0.046	0.049	0.120	0.237
179	0.036	0.034	0.033	0.021	0.100	0.205
181	0.043	0.034	0.013	0.021	0	0.006
183	0.043	0.017	0.013	0.028	0.060	0.013
185	0.007	0	0.020	0.028	0	0
187	0.029	0.051	0.039	0.042	0.020	0.019
189	0.043	0.051	0.013	0.049	0.040	0.013
190	0.007	0	0	0	0	0
191	0.036	0.059	0.099	0.132	0.120	0.032
193	0.043	0.102	0.151	0.069	0.060	0.103
195	0.121	0.144	0.086	0.090	0.140	0.045
197	0.086	0.068	0.112	0.062	0.080	0.205
199	0.057	0.017	0.046	0.042	0.060	0.013
201	0.079	0.008	0.026	0.049	0.040	0.019
203	0.029	0.008	0	0	0.020	0.013
205	0.007	0.017	0	0.021	0	0
207	0.014	0.008	0.020	0.014	0	0
209	0	0	0.007	0	0	0
211	0.007	0	0	0	0	0
213	0.007	0	0.013	0	0	0
215	0	0	0	0.014	0	0
Observed alleles	24	21	21	21	15	17
Private alleles	2	0	1	1	0	0
Allelic richness	17.72	16.09	15.76	16.41	14.44	11.37

Locus 095				Na= 21.83		
Allele	**Oriental Group n=68**	**Central Group n=55**	**Ocidental Group n=77**	**Seamounts n=71**	**Madeira n=24**	**Peniche n=76**
110	0.007	0.027	0.013	0.007	0.021	0.026
112	0.007	0.027	0.006	0.035	0.021	0.007
114	0.051	0.073	0.071	0.042	0.042	0.079
116	0.037	0.036	0.058	0.021	0.021	0

118	0.044	0	0.019	0.021	0	0.013
120	0.029	0.164	0.032	0.007	0.146	0.046
122	0.279	0.227	0.318	0.275	0.250	0.263
124	0.132	0.064	0.058	0.099	0.042	0.079
126	0.110	0.064	0.110	0.099	0.083	0.145
128	0.088	0.100	0.097	0.197	0.125	0.151
130	0.059	0.045	0.065	0.028	0.062	0.033
132	0.044	0.045	0.019	0.056	0.042	0.020
134	0.007	0.018	0.013	0.007	0.042	0.013
136	0.007	0	0	0.007	0	0
138	0.015	0	0.026	0.014	0	0
140	0.007	0.009	0.006	0	0.042	0.007
142	0.007	0.009	0.006	0	0.021	0
144	0.007	0.009	0.006	0	0	0.007
148	0	0	0.006	0	0	0
150	0	0	0	0.007	0	0
152	0	0.009	0	0.007	0.021	0.007
154	0.007	0	0.006	0	0	0.007
156	0.015	0.009	0.019	0.007	0	0
158	0.007	0.009	0.013	0	0	0.013
160	0.015	0.009	0.013	0.007	0	0.020
162	0	0.018	0	0.021	0	0
164	0	0	0	0.007	0	0
166	0	0.009	0	0.014	0.021	0
168	0	0	0.013	0.014	0	0
174	0.007	0.009	0	0	0	0
176	0	0	0	0	0	0.007
178	0.007	0	0	0	0	0.026
180	0	0.009	0	0	0	0.026
194	0	0	0	0	0	0.007
Observed alleles	24	23	23	23	16	22
Private alleles	0	1	1	2	0	2
Allelic richness	14.26	15.20	14.20	13.55	15.18	13.48

Locus 106			Na= 23.5			
Allele	**Oriental Group n=71**	**Central Group n=66**	**Ocidental Group n=77**	**Seamounts n=70**	**Madeira n=25**	**Peniche n=79**
125	0.014	0	0.013	0.021	0	0.006
129	0	0	0.006	0	0	0
131	0	0	0	0.007	0	0
133	0.014	0.030	0.026	0.007	0.060	0.038
135	0.451	0.439	0.461	0.436	0.320	0.209
137	0.063	0.076	0.084	0.079	0.120	0.120
139	0.028	0.038	0.019	0	0.040	0
141	0.014	0.015	0.006	0.014	0	0
145	0	0	0.006	0.007	0.020	0.006
147	0.007	0.015	0.000	0.007	0.020	0.019
149	0.007	0	0.013	0.007	0.040	0.006
151	0.007	0.015	0.013	0.007	0.020	0.006
153	0	0	0.006	0.021	0	0
155	0.014	0.008	0	0.007	0	0.025
157	0.014	0.015	0.026	0	0	0.013
159	0.028	0.030	0.026	0.014	0	0.006
161	0.028	0.015	0.006	0.014	0	0.019
163	0.021	0.015	0.013	0.014	0.020	0.006
165	0.021	0.023	0.026	0.021	0.020	0.019
167	0.042	0.015	0.013	0.036	0.060	0.108
169	0.042	0.038	0.013	0.029	0.100	0.114
171	0.042	0.030	0.013	0.021	0.080	0.057
173	0.014	0.030	0.052	0.036	0.020	0.044
175	0.049	0.038	0.065	0.100	0.040	0.044

177	0.028	0.030	0.026	0.043	0.020	0.063
179	0.028	0.023	0.026	0.021	0	0.006
181	0.014	0.030	0.019	0.007	0	0
183	0.007	0.023	0.006	0.007	0	0.025
185	0	0.008	0.013	0	0	0.013
187	0	0	0	0.014	0	0.006
189	0	0	0	0	0	0.013
191	0	0	0	0	0	0.007
Observed alleles	24	23	26	26	16	26
Private alleles	0	0	1	1	0	2
Allelic richness	15.36	15.74	14.88	14.86	14.80	15.55

Table 6.3 – *H.dactylopterus*. Microsatellites allele frequencies across 6 populations estimated trough Genepop 3.3 (Raymond & Rousset, 1995). Alleles are represented in base pairs. n = number of individuals; N_A= mean number of alleles per population.

There was no evidence of linkage disequilibrium among pairs of loci within each population, as permutation tests showed no significant p values (P>0.05) for 161 out of 169 comparisons (data not shown). Eight (4.9%) comparisons showed a significant value for linkage disequilibria, but this dropped to no significant values after Bonferroni correction (Rice, 1998).

Some random individuals failed to amplify, even after attempting re-amplification: 37(9.3%) individuals for locus *Hd008*, 18 (4.5%) for locus *Hd020*, 12 (3.0%) for locus *Hd044*, 9 (2.3%) for locus *Hd063*, 10 (2.5%) for locus *Hd 076*, 14 (3.6%) for locus *Hd092*, 22 (5.6%) for locus *Hd095* and 6 (1.5%) for locus *Hd106*.

This phenomenon, seem to be explained by the presence of mutations within the primer binding section causing the presence of null alleles. The probability of this is quite high for 5 loci as checked by Microchecker and presented in Table 6.4, even knowing that the percentage of non amplifying loci is quite small comparing to the total amplified loci in most cases (<5%).

	Hd008	**Hd020**	**Hd044**	**Hd063**	**Hd076**	**Hd092**	**Hd095**	**Hd106**
Occid.G.	yes	no	yes	no	no	no	yes	no
Orien.G.	yes	yes	yes	no	yes	no	yes	no
Central G.	yes	yes	yes	no	no	no	yes	yes
Seamounts	yes	no	no	no	no	no	yes	yes
Madeira	no	yes	no	yes	yes	no	yes	yes
Peniche	yes	yes	yes	no	no	no	yes	yes

Table 6.4 – *H.dactylopterus*. Microsatellites. Probability of null alleles existence per locus per population calculated with MicroChecker (Oosterhout *et al.*, 2004)

6.4.3 Heterozygosity and Hardy-Weinberg Equilibrium

Estimates of variability at eight microsatellite loci within all populations sampled are shown in Table 6.5. Within samples, mean observed heterozygosity (H_O) ranged from 0.670 in the Occidental group to 0.720 in the Central group of Azores; and mean expected heterozygosity (H_E) ranged from 0.506 in Madeira to 0.626 for the Azores seamounts population. Most populations showed a deficit in heterozygotes for the majority of loci.

Significant departures from Hardy-Weinberg equilibrium were detected for 30 out of 48 comparisons, of which 17 remained significant after a sequential Bonferroni procedure. All populations presented a significant global deficit of heterozygotes for all loci. Only in a few cases, did loci present an excess of heterozygotes: *Hd092* for three populations (Central G., Oriental G. and Seamounts), Hd063 for Central G. and *Hd008* for Madeira. However, these heterozygotes excesses were never statistically significant.

The non - conformity with the Hardy-Weinberg predicted genotype frequencies and the global heterozygozity deficiency is also shown in the significantly positive F_{IS} values (Table 6.5).

	LOCATION					
LOCUS	**Oriental G.**	**Central G.**	**Ocidental G.**	**Seamounts**	**Madeira**	**Peniche**
Hd008						
N_A	**23**	**21**	**21**	**23**	**16**	**25**
H_0	0.700	0.704	0.776	0.766	1.000	0.817
H_E	0.890**	0.893***	0.879	0.896*	0.890	0.904*
F_{IS}	0.212***	0.205**	0.114***	0.143**	-0.128	0.108**
Hd020						
N_A	5	4	5	5	5	4
H_0	0.194	0.204	0.230	0.386	0.160	0.241
H_E	0.419***	0.601***	0.297*	0.455	0.327**	0.323*
F_{IS}	0.522***	0.653*	0.194***	0.133	0.456**	0.257**
Hd044						
N_A	5	6	8	5	2	6
H_0	0.329	0.422	0.427	0.444	0.304	0.333
H_E	0.474**	0.551*	0.600**	0.522	0.463	0.567***
F_{IS}	0.308***	0.220***	0.290*	0.150	0.328	0.413***
Hd063						
N_A	8	8	6	7	4	5
H_0	0.569	0.719	0.623	0.616	0.318	0.610
H_E	0.663	0.677*	0.656	0.685*	0.681***	0.633
F_{IS}	0.126	-0.062	0.043	0.092	0.523***	0.026
Hd076						
N_A	6	4	5	5	4	4
H_0	0.315	0.356	0.307	0.458	0.200	0.291
H_E	0.393***	0.399	0.361	0.488**	0.558***	0.353**
F_{IS}	0.198*	0.109	0.126	0.041	0.620***	0.151
Hd092						
N_A	24	21	21	21	15	17
H_0	0.957	0.864	1.000	0.972	0.880	0.821
H_E	0.944	0.931	0.925	0.932	0.935*	0.848***
F_{IS}	-0.015	0.071	-0.081*	-0.044	0.055	0.033
Hd095						
N_A	24	23	23	23	16	22
H_0	0.632	0.636	0.610	0.662	0.583	0.645
H_E	0.878***	0.902***	0.868***	0.864***	0.902***	0.878**
F_{IS}	0.281***	0.293***	0.294***	0.234***	0.355***	0.263***
Hd106						
N_A	24	23	27	26	16	26
H_0	0.761	0.606	0.701	0.700	0.600	0.696
H_E	0.791	0.803***	0.772	0.792	0.891**	0.910***
F_{IS}	0.030	0.238*	0.092***	0.115*	0.314***	0.234***
Multilocus						
Mean H_E	**0.516**	**0.563**	**0.584**	**0.626**	**0.506**	**0.557**
Mean H_O	**0.682**	**0.720**	**0.670**	**0.704**	**0.706**	**0.677**
H-W	***	***	***	***	***	***
F_{IS}	**0.178*** **	**0.211*** **	**0.122*** **	**0.107*** **	**0.269*** **	**0.177*** **

Table 6.5 – *H.dactylopterus.*Microsatellites.Summary of genetic variation whithin the studied locations. N_A, number of alleles; H_O, Observed heterozygosity; H_E, Expected heterozygosity; F_{IS}, inbreeding coefficient. Significant values: (*=*P*<0.05, **=*P*<0.01; ***=*P*<0.001).Underline values= significant H-W desviations after sequential Bonferroni correction.

6.4.4 Population substructuring and genetic distances

Hierarchical AMOVA was used to test for genetic structuring defined by the 3 main geographical regions (Peniche, Madeira and Azores). To avoid losing statistical power, the Madeira population was excluded from the initial AMOVA (I) because of its smaller sample

size (N=25) compared to the other samples (58<N<80). A second AMOVA (II) including the Madeira sample was also performed. Both AMOVAs revealed that the greatest percentage of the variance was explained by within sup-populations (97.97% and 98.8% respectively), and only a non-significant 1.54% or 1.12% was explained by variance among groups (Table 6.6). Locus by locus AMOVA showed that *Hd020*, *Hd106* and *Hd092* have a greater contribution for the percentage of variance among groups (data not shown).

	Source of Variation	**% Total Variance**	**Fixation indices**	***P*-value**
AMOVA I	Among groups	1.12	F_{CT}= 0.0112	0.083
	Among Populations within groups	0.58	F_{SC}= 0.0059	<0.001
	Within Populations	98.30	F_{ST}= 0.0170	<0.001
AMOVA II	Among groups	1.54	F_{CT}= 0.0154	0.199
	Among Populations within groups	0.58	F_{SC}= 0.0059	<0.001
	Within Populations	97.98	F_{ST}= 0.0211	<0.001

Table 6.6 - *Helicolenus dactylopterus*. Microsatellite Analysis of Molecular variance (AMOVA) results. AMOVA I does not include the Madeira sample.

Multi-locus estimates of F_{ST} values for each population were high, indicating some level of differentiation between populations sampled.

The analogous R_{ST} coefficient, calculated by averaging the variance components over loci, also revealed a significant structuring of genetic variation among populations. The different loci did not contribute equally to the population differentiation as can be seen by looking at the single locus F_{ST} and R_{ST} values that ranged from 0.000 to 0.050 (Table 6.2).

In agreement with previous work with other markers (see previous Chapter and Aboim *et al.*2005), microsatellites demonstrated genetic uniformity across Portuguese Macaronesian island populations (Azores and Madeira archipelagos). However, in contrast to mtDNA sequences, microsatellites detected more subtle, and significant levels of population differentiation between Peniche and Azores samples.

Pairwise comparison of F_{ST} values revealed the Peniche sample to be relatively distinct from the Azorean samples - Seamounts, Oriental, Ocidental and Central groups (Table 6.7). The R_{ST} coefficient also confirmed the high degree of genetic isolation between Peniche and the Azorean archipelago populations, except for the Seamounts group.

(with no Bonferroni corrections)

F_{ST}\ R_{ST}	Oriental G.	Central G.	Ocidental G.	Seamounts	Madeira	Peniche
Oriental G.	-	0.0089	-0.0042	-0.0037	-0.0058	0.0138*º
Central G.	0.0086	-	0.0062	-0.0011	0.0177	0.0261*
Ocidental G.	0.0016	0.0135**	-	-0.0008	0.0051	0.0171**
Seamounts	0.0013	0.0110**º	0.0045	-	-0.0023	0.0076
Madeira	0.0019	0.0096	0.0056*º	0.0038	-	0.0165
Peniche	0.0144***	0.0275***	0.0155***	0.0219***	0.0083	-

(after sequencial Bonferroni corrections)

F_{ST}\ R_{ST}	Oriental G.	Central G.	Ocidental G.	Seamounts	Madeira	Peniche
Oriental G.	-	0.0089	-0.0042	-0.0037	-0.0058	0.0138
Central G.	0.0086	-	0.0062	-0.0011	0.0177	0.0261*
Ocidental G.	0.0016	0.0135*	-	-0.0008	0.0051	0.0171*
Seamounts	0.0013	0.0110	0.0045	-	-0.0023	0.0076
Madeira	0.0019	0.0096	0.0056	0.0038	-	0.0165
Peniche	0.0144*	0.0275*	0.0155*	0.0219*	0.0083	-

Table 6.7 – *H.dactylopterus*. Microsatellites. Population pairwise F_{ST} (below diagonal) and R_{ST} (above diagonal) comparisons. F_{ST} estimated by FSTAT 2.9.3 (Goudet,1995) and R_{ST} estimated by R_{ST}Calc (Goodman, 1997). Significant values (*=P<0.05, **=P<0.01; ***=P<0.001 and º = non significant after sequencial Bonferroni correction).

Genetic differences between populations were also tested using two pairwise measures of genetic distance D_{SW} (Shriver et al., 1995) and $(\delta\mu)^2$ (Goldstein, 1995),which are shown in Table 6.8.

Populations	Oriental G	Central G	Ocidental G	Seamounts	Madeira	Peniche
Oriental G	-	177.994	174.364	186.944	156.91	200.227
Central G	0.074	-	179.892	191.846	162.332	204.588
Occidental G	0.033	0.068	-	189.301	159.01	203.645
Seamounts	0.046	0.091	0.064	-	171.239	211.673
Madeira	0.055	0.104	0.081	0.787	-	184.219
Peniche	0.271	0.309	0.333	0.265	0.259	-

Table 6.8 – *H.dactylopterus*. Microsatellites.*G*enetic distances between populations. Below diagonal D_{SW} (Shriver *et al.*, 1995) distances. Above diagonal $(\delta\mu)^2$.(Goldstein, 1995).

The multidimensional scaling of genetic distances using D_{SW} (Shriver *et al.*, 1995) is presented in Fig. 6.2 and 6.3. In these graphs or dimensional plots the relative distances of the points represent the relative dissimilarities of the samples. Points that are close together represent samples with smaller genetic distances, hence, less genetic differentiation, and points further apart correspond to more differentiated populations. Oriental, Central and Occidental Groups are tightly grouped demonstrating that there is high genetic similarity between these populations. Madeira and Peniche samples are shown quite far apart from the

rest of the populations consistent with a high level of differentiation relative to the Azores archipelago. The Seamounts population also shows a high distance (but lower than Madeira and Peniche vs Azores- see 3D plot).

Fig. 6.2 – Two-dimensional (2D) scaling plot of pairwise stepwise weighted genetic distances (D_{SW} – Shriver *et al.*, 1995).

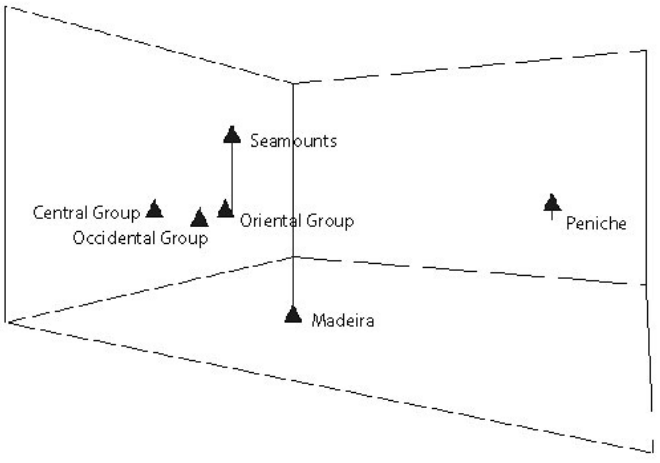


Fig.6.3 – Tri-dimensional (3D) scaling plot of pairwise stepwise weighted genetic distances (D_{SW} – Shriver *et al.*, 1995).

6.4.5 Isolation by distance

The hypothesis that the geographic distance between populations, i.e, isolation by distance, alone, was responsible for the genetic differentiation was excluded. The relationship between $F_{ST}/(1-F_{ST})$ values and the logarithm of geographic distances in kilometers showed a non-significant but positive correlation (r=0.152; P=0.151) (Fig. 6.4). The absence of significant correlation between genetic and geographic distances suggests that genetic differentiation estimated, as Fst values between samples, cannot be explained solely by geographic isolation.

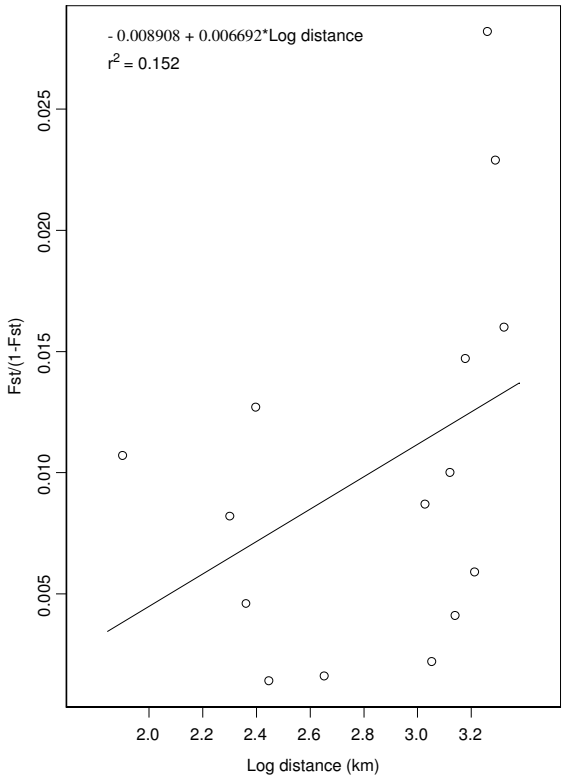


Fig.6.4 – *H.dactylopterus.* Relationship and regression line between genetic differentiation [$F_{ST}/(1-F_{ST})$] and the Log geographical distance.

6.4.6 Effective Population Size

The average θ-values estimated with Migrate software, for the entire microsatellite data set, were 9.4300 in the Azores archipelago, 0.9356 in Madeira and 4.0593 in Peniche (mainland Portugal). All θ-estimates varied significantly between the three samples. The very low value obtained for Madeira might be related to the small number of samples obtained and analyzed for this population. However, the significant difference obtained between Peniche and Azores samples with a smaller effective sample size for Peniche cannot be neglected.

6.5 Discussion

6.5.1 Microsatellite Genetic Variability

Microsatellite loci display high levels of polymorphism in *Helicolenus dactylopterus*, suitable for population structure analysis. This agrees with polymorphism found in mtDNA markers (see Chapter 5) for this species, which was also relatively high for this type of marker.

However, the mean expected heterozygosity over all populations found in *H.dactylopterus* using microsatellites is much lower (H_E = 0.559) compared with other marine fish such as sea bass (H_E = 0.779) and herring (H_E = 0.889) (García de León *et al.*, 1997; O'Connell *et al.*, 1998; Shaw *et al.*, 1999b).

All loci showed significant deviations from Hardy-Weinberg equilibrium due to heterozygote deficiencies translated into high positive F_{IS} values, except one. Disequilibria can result from several nonexclusive factors such as null alleles, population differentiation, selection or evolution.

Regarding the high probabilities of nonamplifying alleles presence in some loci, it is worth mentioning that this phenomenon may underestimate the degree of heterozygosity and cause deviations from Hardy Weinberg equilibrium and non-Mendelian inheritance of alleles.

There is also the probability of mis-scoring of alleles in heterozygous individuals because of stutter bands in genotyping gels. This is a common problem in microsatellite analyses of populations and occurs especially in long dinucleotide or composed repeats. During PCR slipped-strand mispairing can occur and may form extra shadow bands that appear as several peaks instead of a single product (Shinde *et al.*, 2003). Some of the loci showed some stutter

bands, but analysis of allelic frequencies and values show no significant evidence of this (data not shown).

6.5.2 Heterozygote deficiency

Heterozygotes deficiencies have often been detected in other marine fishes (García de León *et al.*, 1997; Rico *et al.*, 1997) and hypothetically explained as a combination of several technical factors such as null alleles and biological factors including inbreeding, the Wahlund effect, selection and assortative mating.

Heterozygotes deficiency can be explained by mating among relatives, hence inbreeding, within the sampled populations. This hypothesis is plausible in *H.dactylopterus* has adults are found to be very sedentary and fertilization is internal, which enhances chances of non-random matting between individuals. However, the pelagic larvae of this species should act to counter these affects unless mechanisms for larval retention are occurring because of larval behavior or through physical mechanisms such as island mass effects (e.g. Hernandezleon, 1991) or Taylor column formation (seamounts only; reviewed in Rogers, 1994).

Another possibility is that heterozygote deficiency may be caused by small reproductive subunits within the sampled populations that are present but not detected. This is known as the Wahlund effect (Wahlund, 1928) and is analogous to the effects of inbreeding, as it also causes high positive observed F_{IS} values (Table 6.3). The average frequency of homozygotes among subpopulations is always greater than would be expected with random mating; because the mixture of two or more populations in Hardy-Weinberg equilibrium with different allele frequencies produces a mixed population with a greater heterozygote deficiency than expected for the average HWE allele frequencies (Hartl, 2000).

The existence of the Wahlund effect in this case is possible as sample sites are quite large and can comprise more than one population. For instance, in the Madeira archipelago, samples came from different places in the archipelago that are far apart from each other, and in Peniche, samples were also collected from different areas. In the case of Azores, it seems more improbable that a Wahlund effect exists as sampling areas were more controlled and restricted, however, a temporal Wahlund effect could also occur and should be taken into account. Each population could represent several subunits of individuals recruited in different years or sampling may have occurred at a time when different reproductive stocks were mixed outside of the breeding season. The mating behavior and reproductive strategies

of *H.dactylopterus* are not completely studied and several details need to be explained in order make such remarks. Comparisons of population structure at different time periods and analysis taking into consideration the age of individuals would be useful to test these hypotheses.

Null alleles have been reported in several microsatellite loci studies as a possible cause for heterozygotes deficiency. The presence of null alleles results in false homozygotes causing deviations from Hardy-Weinberg proportions and biasing all population analyses based on allelic frequencies. Five of the microsatellites used in this study showed high probability of failure in PCR amplification due to mutations within the primer-annealing sequence. So, the hypothesis that null alleles can put in cause the population analysis has to be considered.

It is not possible to conclude, without error, which of the hypotheses presented above explains the heterozygotes deficit observed and probably more than one may be involved.

6.5.3 Population differentiation

Evidence of population structure at fine geographical scales has been shown in several marine fish species in several parts of the world using different molecular markers (e.g. Ruzzante *et al.*, 1996; 1998). Actually, within the analyzed area a study on *Pagellus bogaraveo* (Stockley, 2005) has also shown evidence for the same pattern of population differentiation between the Azores and the Portuguese continental slope as found here for *H.dactylopterus*.

Highly significant levels of population differentiation based on both F_{ST} and R_{ST} were found for *H.dactylopterus* populations on a regional scale across the NE Atlantic (Table 6.7). Isolation-by-distance does not seem to be the main cause of the differentiation found, as no significant correlation was found between geographic and genetic distances of populations.

The detection of relatively small-scale population units conforms to studies on bluemouth behavior, reproduction and tagging. Internal fertilization and sedentary adults can promote differentiation between samples.

Low gene-flow between populations caused by physical, hydrographical and historical demographic factors are surely playing a major role in shaping the genetic structure of the species at this regional scale.

Results show a clear isolation of Peniche and Azores samples. Looking at the geographic location of Peniche it seems feasible that the level of isolation between bluemouth off of mainland Portugal and those off the Azores is higher than between Madeira and Peniche.

Several seamounts exist between these two locations (Epp & Smoot, 1989) and Madeira is closer to the continental shelf, permitting demersal fish to migrate more easily between them. Looking at the circulation pattern of surface currents in the NE Atlantic region it is also possible that larval dispersal through current transport is easier between Peniche and Madeira through the Canaries current than between Peniche and Azores (see fig 2.1 - Chapter 2).

The fact that Madeira sample is also much smaller than the others (only 25 individuals) can also be the main reason for not finding such evident genetic differentiation between this population and the others.

In addition to geographic and ecological factors, historical events can also be invoked to explain the genetic discontinuity between populations. It is possible that a combination of both historical and contemporary ecological factors has effectively isolated Peniche or Azores bluemouth populations.

Strong historical influences on the genetic population structure of *H.dactylopterus* were previously hypothesized on the basis of mtDNA sequence data (Chapter 5). The Azores population showed evidence of bottlenecks followed by expansion events in the past.

The reproductive isolation of Azores populations proposed in the previous chapter is here confirmed and enhanced by these more sensitive and polymorphic markers.

It would be useful and interesting to test for bottlenecks and times of expansion using allelic data to compare with the ones obtained with mtDNA. However, specific tests for demographic past events such as recent bottlenecks are not so common for microsatellite data. Most of them, such as for example that of Cornuet & Luikart (1996), assume Hardy-Weinberg equilibrium in the tested populations, which is not the case here.

The isolation encountered is also supported by the θ-estimates that point to a significantly smaller effective population size of the Peniche sample when compared with the Azores archipelago. Landing numbers observed in Peniche and Azores markets support these results, as Peniche numbers seem to be lower than Azorean ones, specially if one takes into consideration that sometimes several other Scorpaenae species are misidentified as bluemouth in this area (Moura, 1995; Moreira *et al.,* 2000; Lotaçor, 2003).

This might be due to anthropogenic factors such as different resources' exploitation levels in both areas, or it can also be attributed to biological characteristics of the species such as specific reproductive behavior or assemblages.

However, further analysis using temporal sets of samples would be useful on trying to understand the demographic history of populations using microsatellites markers.

The data presented here also suggests the evidence of some degree of population structure between subpopulations of the Azores archipelago. F_{ST} analysis shows some significant differentiation of Central Group subpopulation from some other groups of the archipelago. This might be influenced by the fact that the Central group sample is constituted by individuals belonging to two different collection years. The observed genetic relationships could have been caused by temporal instability of allele frequencies, although some studies in teleost marine fishes have demonstrated that allelic shifts over small periods of time are not so common (Grant & Utter, 1984). This is probably especially the case in relatively long-lived species of fish such as *H.dactylopterus.* For this reason it would also be interesting to analyze temporal sets of samples which could reveal interesting results on the demographic history of these populations (Beaumont, 1999). However, such separation should not be completely neglected as some evidence of internal differentiation within the Azores archipelago has already been come from mtDNA data although this was not significant.

Multidimensional scale analysis of genetic differences provided further evidence of a possible population separation within the Azores archipelago. The genetic distance between the Seamounts population from the others of the archipelago is quite striking. No previous analyses have revealed genetic isolation between seamount populations although this has been hypothesized as resulting from physical retention of larvae by hydrodynamic processes around these topographic features. One of the explanations for the maintenance of benthic or bentho-pelagic populations in isolated and patchy environments is the combination of several patterns of circulation flow and water masses. It has been described that around islands and seamounts typical flow patterns such as Taylor Columns (Roden, 1987 and 1991), Island mass effect (Sander, 1981; Hernandezleon, 1991; Caldeira *et al.*, 2002) and other hydrographic processes may induce retention of larvae or high production up-welling inducing adults to stay feeding on this habitats (Rogers et al., 1994; Mullineaux & Mills, 1997; Rissik & Suthers, 2000). But contrary to what expected it has been difficult to find evidence for genetic isolation of seamount communities.

As pointed out before, the presence of null alleles can highly biased the population analyses based on allelic frequencies. So, all those analyses were repeated excluding loci with strong probability of null alleles. When loci *Hd008, Hd020, Hd044, Hd095* and *Hd106* were omitted from the data, some slight differences were found (see Appendice 1). For example, on F_{ST} and R_{ST} pairwise comparisons some differences in population structure within the

Azores archipelago were found. Most pairwise significant values were maintained, with no doubts for the Peniche population isolation. However, some differentiation within the Azores archipelago was lost, nominally, no significance was found between the Central and Occidental groups, but a significant differentiation was found between Seamounts and Occidental groups. This seems to emphasize some kind of isolation of the Occidental Group, which might be caused by its geographic location. The occidental group is located in a different tectonic plate and separated from the other islands and seamounts by the Mid-Atlantic Ridge, which might influence migration of demersal species. Some differentiation was also lost on the genetic distances analysis, with no significant separation of the Madeira and Seamounts group populations from the rest, has obtained for the entire data set.

Looking at the previous chapter, it was hypothesized that Cape Verde individuals might be considered to belong to a different species as no sufficient gene flow seems to exist between NE Atlantic (Azores, Madeira and Peniche) and this archipelago. It should be interesting to analyse the data set excluding those individuals with Cape Verde exclusive haplotypes. The number of samples in this situation is probably of a number too small compared to the high numbers used for microsatellite analysis, but maybe it would influence the obtained results.

6.5.4 Contrast between microsatellite and mtDNA evidence

The results presented in this chapter contrast sharply with those derived from the analysis of sequence variation in the cytochrome *b* and control regions of mtDNA, which did not show strong evidence of subpopulation structure within the NE Atlantic region (see Chapter 5 and Aboim *et al.*, 2005). The contrast between the results is even more remarkable when one considers that the assayed individuals for nuclear DNA variation were the same or were drawn from the same aggregations as the ones assayed for mtDNA variation. These results support the assertion that nuclear DNA has a much higher potential for detecting variation and genetic differences than mitochondrial DNA (Ward & Grewe, 1994; Carvalho & Hauser, 1995; Park & Moran, 1995). However, some authors have found exactly the opposite, with mtDNA revealing more significant differentiation between populations than microsatellite data as has been seen in, for example, toothfish (Appleyard *et al.*, 2002; Shaw *et al.*, 2004).
This reinforces the statement that more than one marker should be used in molecular studies whenever necessary as their resolution and sensitivity to different factors diverge.
The encountered differences in this study can be explained by several factors that rely mainly on the fact that mtDNA is haploid and maternally inherited which makes the

effective population size of this marker ¼ of a nuclear marker like microsatellites. Because of this, mtDNA is more susceptible to genetic drift effects and demographic effects reflecting historical divergences between populations. Microsatellites have a higher mutation rate and effective population size that make them more sensitive to recent divergences than the effects of genetic drift. Differences in the level of homoplasy can also influence the results obtained by each marker.
But the more accurate explanation for the detected differences is probably the number of samples analyzed rather than the mutation rate of each marker.

6.5.5 Relevance for Management and Conservation

The obtained data have important implications for the conservation of genetic diversity in *H. dactylopterus* and other deep-sea fish populations with similar characteristics. If populations are depleted in one region through fishing activities then it is unlikely that they will be recolonised from adjacent geographic regions by immigration over the short to medium timescale. If overexploitation occurs within a very limited area on a local scale, then targeted populations are likely to be replenished from other proximate sub-populations. This suggests that a useful management strategy for this species is one that protects at least some sub-populations within each region in which *H. dactylopterus* is found. Only such a strategy will be likely to assist in the survival of the species and preservation of intraspecific genetic diversity. Management measures that attempt to limit fishing pressure across a wide geographic area will reduce the genetic diversity of the entire regional population. Such a strategy can have disastrous consequences if control of exploitation is ineffective or quotas are set at the wrong level as has occurred for other deep-sea species such as orange roughy (Rogers, 1994).

SECTION II – ALFONSINOS

CHAPTER 7
GENETIC POPULATION STRUCTURE OF TWO CLOSELY RELATED SPECIES, B*eryx splendens* and *Beryx decadactylus* (Berycidae) IN THE NORTHEASTERN ATLANTIC USING MtDNA SEQUENCES

7.1 Abstract

The geographic variation of two closely related species *Beryx splendens* (alfonsino) and *Beryx decadactylus* (imperador) in the Northeast Atlantic was investigated using partial sequence analysis of mitochondrial cytochrome oxidase-b (cyt *b*) and the mitochondrial control region (CR).

Alfonsinos and imperadors are sympatric species with largely overlapping distributions, and present very similar morphological, biological and life history characteristics. However, the species were found to differ widely in terms of the variability of mtDNA and spatial distribution of haplotypes.

The mitochondrial control region (421 bp) and cyt *b* (273bp) were sequenced from individuals captured in Azores, Madeira and Cape Verde archipelagos. *B.splendens* showed no significant population structure indicating that it forms a panmitic population in the Northeast Atlantic. In contrast, strong evidence for isolation of the Cape Verde population from Madeira and the Azores was detected for *B. decadactylus*. Following these results it was decided to perform a finer scale analysis on imperador by analysing samples from Peniche (mainland Portugal) and dividing the Azores archipelago into several different sub-sampling groups. This confirmed the differentiation between Cape Verde population and all the other samples and also detected a significant differentiation between Peniche and one of the Azores sub-samples (Central Group).

Mismatch distribution analysis points to a demographic history of a sudden expansion/bottleneck around 1 million years before present for *B.splendens* for the entire Northeast Atlantic population. For *B.decadactylus* no evidence of expansions or bottlenecks was found.

The contrast in the genetic population structure of two related deep-sea fish regarded as having similar life-history characteristics is striking. Implementation of correct management strategies for these two species is necessary and must take into account genetic evidence of panmixia in *B.splendens* and particularly the presence of discrete genetic stocks of *B.decadactylus*.

7.2 Introduction

Given the lack of physical barriers in the oceans it is difficult to conceive that marine species can exhibit structured populations at the regional scale (Palumbi, 1994; Ward *et al.*, 1994). Nevertheless, this has already been shown in many studies, and even the present work has demonstrated, that life-history characteristics of species and physical parameters of the oceans can have strong effects on the genetic variability of populations and cause differentiation at large and small scales. Several marine fish species, pelagic and demersal, are known to be differentiated in smaller demes across the oceans or even smaller regions contradicting the long held idea of general panmixia and extensive gene flow across marine environments (Baker *et al,* 1995; Aboim *et al.*, 2005).

Following this line of thought, it is also difficult to believe that sympatric and closely related fish species with morphological, biological and ecological similarities may exhibit different phylogeographic patterns. However, some studies have proven the contrary, showing that subtle differences in behaviour, life-history strategies, reproduction and recruitment may induce contrasting population structures and demographic histories among very similar species (Bargelloni *et al.*, 2003; Zardoya *et al.*, 2004)

In this chapter, two closely related congeneric species will be compared in terms of genetic variability and population structuring.

Beryx splendens (alfonsino) and *Beryx decadactylus* (imperador) are two deep-sea demersal fish species, with important commercial value and are highly exploited wherever they are abundant (Lehodey *et al.*, 1994; Rico *et al.*, 2001; Akimoto *et al.*, 2002). In the Northeast Atlantic Ocean they are caught in substantial quantities by several fisheries around seamounts and continental slopes, normally as by-catch in mixed-species fisheries. However, in some regions such as the Canary Islands, *Beryx splendens* is the main target species of small-scale fisheries.

B.splendens and *B.decadactylus* are morphologically very similar and they are often sold as the same in markets. Catches are usually recorded as *Beryx spp.* as a whole (Figure 2.8) and no separate statistics exist for these species except sometimes at a regional scale; e.g. Azores (Lotaçor, 2003 – see Chapter 2 Figures 2. 9 and 2.11).

Relatively little is known about the life history and biology of these two species, especially about *B.decadactylus*. Some work exists on the reproduction (Lehodey *et al.*, 1994 & 1996; Gonzalez *et al.*, 2003), development (Massey & Horn, 1996; Anibal *et al.*, 1998; Rico *et al,* 2001*)*, distribution (Busakhin, 1982; Ivanin, 1987; Relini *et al*, 1995) and behavior

(Galaktionov, 1984) of *B.splendens* and its life-history characteristics are normally assumed to be the same for *B.decadactylus*. No specific studies on *B. decadactylus* are available, except for some comparative studies with *B.splendens* on feeding habits (Dürr & González, 2002) and larval stages (Mundy, 1990).

Both species are benthopelagic (Maul, 1981) with a depth distribution mainly between 300 – 1,300m around seamounts and continental slopes at tropical and temperate latitudes of the world's oceans (Shotton, 2005). They are typical of the actively swimming deep-bodied type of deep-sea demersal fish. Fecundity is high and the pelagic larval stage is thought to last for several months allowing long-distance dispersal.

The only relevant difference in these two species seems to lie in population size, as alfonsino seems to be more abundant than imperador, a fact confirmed by information on catches and availability of the species in certain geographic localities (Lotaçor, 2003). Imperador also seem to achieve bigger lengths then alfonsino and in some areas, such as the Azores, they are found at greater depths (Menezes *et al.*, 2001). These subtle differences in fish-catch statistics may indicate a more conservative life-history for imperador than that of alfonsino.

This is not only one of the first population genetic studies on *B.decadactylus* but also an innovative approach in terms of the comparison between the two species of *Beryx* at the molecular level. It is also an important contribution to fisheries management, as it seems that fishery biology has showed little interest in analyzing the two species separately.

The present work provides information on the genetic variability of mtDNA in these two related species as well as on their geographic genetic structure and demographic history in the Northeast Atlantic area. The genetic analysis of geographic structure is important in exploited species because it may reveal sub-populations or stocks used for scientifically-based fisheries management.

7.3 Material and Methods

7.3.1 Sampling and DNA extractions

Liver or muscle samples of both *Beryx splendens* and *Beryx decadactylus* were collected from Madeira, Azores and Cape Verde archipelagos following the procedures presented in Chapter 3. Some extra samples of *Beryx decadactylus* were collected from Peniche (mainland Portugal) and different areas within the Azores archipelago for a finer scale geographic analysis (Fig.7.1). A list of samples is presented in Table 3.1 (see Chapter 3).
Storage of tissues and DNA extractions were also performed in the same way as explained in Chapter 3.

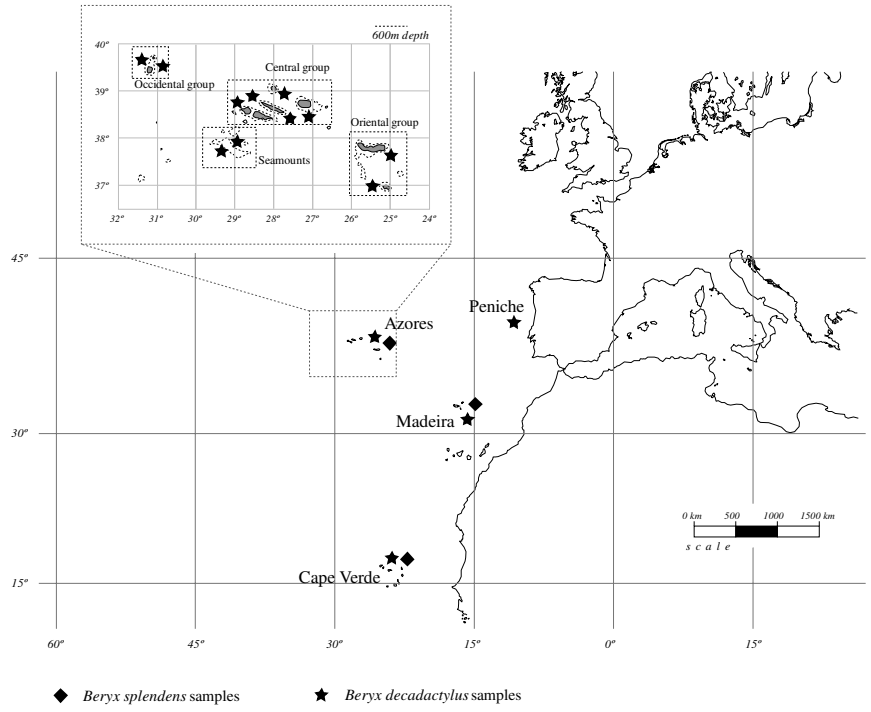


Fig.7.1 – *Beryx spp.* sampling map.

7.3.2 PCR amplification and Sequencing

The mitochondrial control region (CR) was amplified for both species by PCR using two universal primers L-Pro-1 (5'-ACT CTC ACC CCT AGC TCC CAA AG-3') and H-DL-C-1 (5'-CCT GAA GTA GGA ACC AGA TGC CAG-3') described by Ostellari *et al.* (1996).

PCR reactions of 10 µl total volume containing 1µl 10x buffer (Qiagen Crawley, West Sussex, U.K.; Tris-HCl, KCl, $MgCl_2$, pH 7.8); 1.25µl $MgCl_2$ (Qiagen) ; 0.6 µl DNTP mix (Perkin Elmer); 0.1 µl Taq polymerase (Qiagen); 1.5µl of template (10-20 ng); 5.05 µl H_2O and 0.5 µl of each primer (10pmol/µl), were conducted on a Perkin Elmer DNA Thermal Cycler 480 under conditions as follows: 4mins at 94ºC, followed by 30 cycles of denaturation at 92ºC for 60s, annealing at 50ºC for 60s and extension at 72ºC for 60s; finishing with an extension step at 72ºC for 5 min.

The cyt *b* region was also amplified for both species using the same PCR reagents and quantities as for D-loop but using the universal primers CB1-L (5'- ATC CAA CAT CTC AGC ATG ATG AAA-3') and CB2-H (5'- CCC TCA GAA TGA TAT TTG TCC TCA-3') described by Horau & Borsa (2000). PCR cycles were performed in an MWG-Biotech Primus 96 plus thermocycler under the following conditions: 94ºC for 4min, followed by 30 cycles of 94ºC for 50s, 53ºC for 30s and 72ºC for 50s; finishing with an extension step at 72ºC for 5 min.

All amplified products were purified using a QIAquick PCR Purification Kit (Qiagen) following the supplier's instructions. Sequences were obtained by a cycle-sequencing reaction based on the dideoxynucleotide chain termination method by Sanger *et al.* (1977). Each purified PCR product was used in a cycle sequencing reaction using Applied Biosystems Big Dye Terminator Cycle Sequencing Ready Reaction Kits (Applied Biosystems, Warrington, Cheshire, U.K.) under the following conditions: 10 µl reactions – 4µl Dye; 0.16 µl primer; 1µl sample and 4.84 µl H_2O - at 96ºC for 10s, 50ºC for 5s, 60ºC for 4min during 25 cycles and a holding step at 4ºC. The resulting cycle sequencing fragments were cleaned up using a DyeEx Spin Kit (Qiagen) following the supplier's instructions. Finally, products were visualized using an Applied Biosystems Prism 377 automated sequencer.

7.3.3 Statistical analysis

Thirty-one control region sequences and twenty-five cyt *b* sequences of *Beryx splendens* from the Azores archipelago were included in the analysis from Stockley (2001) and analysed together with the rest of the samples. Sequences were aligned and edited using Clustal X (Thompson *et al*, 1997).

General genetic variability, population genetic statistics, mismatch distribution and neutrality tests were all estimated using Arlequin (Schneider *et al.*, 2000).

Levels of inter- and intrapopulation genetic diversity were quantified by indices of nucleotide (π; Nei 1987) and haplotype (h; Nei 1987) diversity, number of polymorphic sites (S), occurrence of indels and substitutions, and base composition percentage.

Genetic homogeneity between populations was tested by the F_{ST} -statistic (Wright, 1951). Pairwise comparisons and their statistical significance were assessed under the null distribution hypothesis (panmixia), by performing 10,000 permutations. *P*-values were adjusted with the sequential Bonferroni correction (Rice, 1998). An analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992) was used to evaluate the extent to which sequence variation was partitioned among areas and populations.

Phylogenetic relationships among haplotypes were analysed constructing a network using the program Network ver. 4.0.8.1 (available on www.fluxus-engineering.com). A Reduced Median Network (Bandelt *et al.*, 1995) was used after a Median Joining logarithm (Bandelt *et al.*, 1999) because of the high number of haplotypes and homoplasy.

Mismatch distribution analysis was used to explore the demographic history of both species. Observed distributions of the number of pairwise differences between sequences were tested against the sudden population expansion model (Rogers & Harpending, 1992) in order to discriminate if populations have remained stable over time or undergone a rapid population expansion. The validity of the expansion model was tested by Tajima's *D* neutrality test (Tajima, 1989). This test is normally used to test selective neutrality, but is also sensitive to population history, and can be used in mismatch distributions because population growth predicts significant negative d-values. The time and magnitude of an inferred population expansion was determined by calculating the expected mean pairwise differences (θ), and units of mutational time (τ) where $\tau=2ut$ (u=the mutation rate over the fragment essayed; t= time in generations), and $\theta=2nu$ (n= effective population size) (Rogers & Harpending, 1992). The average mutation rate for fish mtDNA control region used was 3.6% per nucleotide per million years (Donaldson & Wilson, 1999); and for cyt *b*, which evolves more slowly, was 2% (Brown *et al.*, 1979).

Generation times were taken from (http://www.fishbase.org) which indicates a mean of 9.7 years for *B.splendens* and 11.5 years for *B.decadactylus*.

7.4 Results

Populations	N (CR/cytb)	Haplotypes (CR/cytb)	Private haplotypes	h (CR/cytb)	π (CR/cytb)	reference
B.splendens						
Cabo verde	**25/21**	**22/10**	**6/6**	**0.990/0.833**	**0.014/0.008**	**This study**
Madeira	**28/34**	**26/15**	**19/7**	**0.995/0.736**	**0.016/0.006**	**This study**
Azores	**25/32**	**21/8**	**12/2**	**0.987/0.900**	**0.016/0.008**	**Stockley(2001)**
Total	78/87	57/22	-	0.991/0.823	0.015/0.007	
B.decadactylus						
Cabo Verde	**15/9**	**9/3**	**6**	**0.905**	**0.008**	**This study**
Madeira	**17/6**	**11/2**	**4**	**0.735**	**0.004**	**This study**
Azores						**This study**
Oriental G.	**25**	**13**	**6**	**0.903**	**0.004**	**This study**
	23	**12**	**4**	**0.862**	**0.004**	**This study**
OccidentalG.						
Central G.	**28**	**8**	**4**	**0.743**	**0.003**	**This study**
Seamounts	**23**	**8**	**2**	**0.771**	**0.003**	**This study**
Peniche	**28/8**	**9/1**	**1**	**0.828**	**0.003**	**This study**
Total	159/23	70/5	-	0.821	0.004	

Table 7.1 – *B.splendens* and *B.decadactylus*: descriptive statistics for sequence variation in populations for both d-loop and cyt b.

7.4.1 *Beryx splendens*

Control region variation

A total of seventy-eight nucleotide sequences of the mitochondrial control region 5'-end (448 bp), were used in this analysis. Twenty-five sequences from Azores were taken from Stockley (2001) and the other 53 were sequenced for the present study.

The overall diversity of haplotypes was extensive, totalling 57 different haplotypes detected among the 78 individuals sequenced, based on variation of 46 polymorphic sites, caused mainly by substitutions (19 transversions and 25 transitions) and 5 indels.

Base composition was strongly biased: with adenine and thymine occurring at mean higher percentages of 32.46% and 27.79% respectively, while cytosine occurred at 23.65% and guanine only at 16.10%. These biases are similar to those found on the mt-DNA control region of other teleost marine fishes (Jean *et al.*, 1995; Quinteiro *et al.*2000).

5'- AGGATTCTAAACTAAACTATTCTCTGCCGCCCCAGCCCGCCCCA-CATATATG
TCCTA-GAAAGCTAGTATAGACATATATGTATTATCACCATGAATCGAATTTA
ACCATTTTCAATGGTGCCTCGGTACATAAATGTAGTTCCACTATTTGTCGAA
CTTAAACACTCACACATCAATACAAATACAAAGGTGTACATAAAGCAATACT
GGAATAACCAATAAATTAATTAATTCACGTGACAGCCGACATTTAAGACCGA
ACACAACTCGCATCGGTTGAGTTATACCATGCACTCAACACCTCGTCAACTC
TCAGATTCTCAAGTGTAGTAAGAAACCACCATCAGTTGATTCCTTAATGCAT
ATCATGCTTGATGGTCAGGGACAAAACTCGTGGGGGTTTCACTTAGTGAAC
TATTTCTGGCATCTGGTTCCTACTTCAGGA – 3'

Fig.7.2 – *Beryx splendens* most common haplotype of mtDNA control region.

A total of 46 haplotypes were unique and the dominant CR haplotype for *Beryx splendens* was only found in 5 individuals (see Fig.7.2). As a result, overall haplotype diversity (h) was high averaging 0.991 and ranging from 0.987 in Azores and 0.995 in Madeira. Nucleotide diversity (π) was low with a mean of 0.0153, ranging from 0.014 in Cape Verde and 0.0160 in Azores.

The number of haplotypes per sample did not show much variation but the number of private haplotypes showed more divergence: Cape Verde had 17 private haplotypes in a total of 22 haplotypes, the Azores 11 out of 21 and Madeira 19 out of 26 (Table 7.2).

Cytochrome b variation

A 273 bp sequence of the mtDNA cytb region was sequenced for fifty-five individuals from Cape Verde and Madeira and were combined with another 32 sequences of Azorean individuals taken from the work of Stockley (2001).

When all sequences were compared 22 different haplotypes were detected, diverging on 32 polymorphic sites resulting from 24 transitions, 12 transversions and no indels.

The most abundant haplotype was shared by 29 individuals (8 from Cape Verde, 14 from Azores and 7 from Madeira) and is represented in Fig. 7.3. Two other shared haplotypes were found in all samples, all the others were shared between two samples and only 13 were unique haplotypes.

5'- CCAAATCCTCACAGGACTTTTCCTAGCCATACACTACACCTCCGACATCGCTAC
GCCTTCTCATCAGTAGCCCACATCTGCCGAGATGTAAACTACGGATGACTAATC
GAAACCTACATGCCAACGGAGCATCTGTCTTCTTCATCTGCATCTACATACACT
CGGCCGAGGACTATACTACGGCTCCTACCTATATAAAGAAACCTGAAACACCT
GTAGTCCTACTCCTGCTAGTAATAATAACCGCTTTCGTAGGCTACGTACTCC -3'

Fig.7.3 – *Beryx splendens* most common haplotype of mtDNA cythocrome *b* region.

As expected for a non-coding region and from other works on the genetic variability of the cyt b, overall haplotype diversity for *B.splendens* mtDNA cyt b region was high (h= 0.823) but lower than CR, ranging from 0.736 in Azores to 0.9002 in Madeira. Nucleotide diversity was low (π = 0.007), ranging from 0.006 in Azores to 0.008 in both Cape Verde and Madeira (Table 7.2).

Geographic variation

Genetic homogeneity between geographic locations was tested by pairwise F_{ST} analysis (Wright, 1951). Pairwise comparisons (Table 7.2) revealed no significant heterogeneity between any pair of samples when analysing control region or cyt b sequences.

F_{ST}	Azores	Cape Verde	Madeira
Azores	-	0.031	-0.012
Cape Verde	-0.006	-	0.019
Madeira	-0.020	-0.018	-

Table 7.2 – *Beryx splendens* control region (below diagonal) and cyt *b* (above diagonal) Pairwise F_{ST} values between populations.

The lack of geographical subdivision within the overall data set was also apparent in the analysis of molecular variance (AMOVA) with non-significant F_{CT} values for both mtDNA regions (Table 7.3).

Source of Variation	% Total variance	Fixation indices	P-values
Control region			
Among populations	-1.48	F_{ST}= -0.0148	0.864
Within populations	101.48		
Cyt b			
Among populations	0.94	F_{ST}= 0.0094	0.083
Within populations	99.06		

Table 7.3 – *Beryx splendens*. Control region and Cyt *b*. Analysis of molecular variance (AMOVA) results.

The haplotype network derived from cyt b partial sequences is presented in Figure 7.4. The most common haplotype, represented by the biggest circle box (size of circles is proportional to the number of haplotypes) represents individuals from the 3 different analysed populations

(Azores, Madeira and Cape Verde). None of most common haplotypes were restricted to any of the populations and no specific clades seem to exist confirming the F_{ST} results of no apparent genetic differentiation between geographic areas. The general star-shaped phylogenies of the network are consistent with recent population expansion.

Haplotype networks were inconclusive for D-loop and were excluded from this section as a result of high variability and high level of homoplasy amongst sequences.

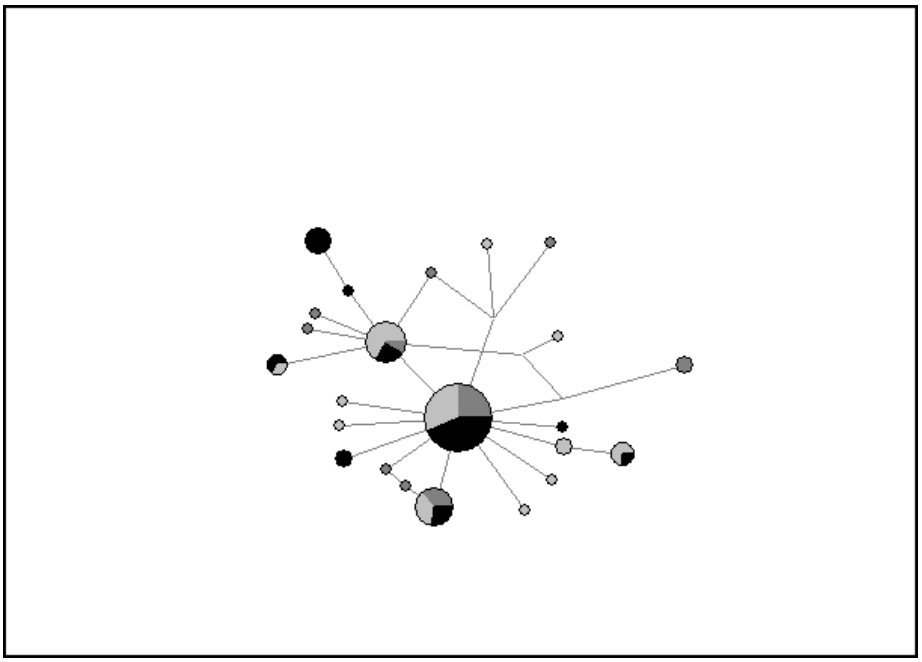


Fig.7.4 – *B.splendens* cyt b minimum spanning network analysis of haplotypes for all populations. Circles sizes are relative to haplotype number. Black= Azores, Dark Grey= Cape Verde and Light Grey= Madeira.

7.4.2 *Beryx decadactylus*

Control region variation

Considerably less variation was found among *B. decadactylus* mtDNA control region than among *B. splendens*. From a total of 60 individuals, 24 haplotypes were defined by 21 divergent sites in 421bp of the control region 5'-end. No indels were detected and only 4 transversions were found in a total of 22 substitutions.

Haplotype diversity (h= 0.892) was still high but relatively lower compared to alfonsino and to other teleost marine fish control region sequences. Results were more consistent with

haplotype diversity values found for other more conservative regions of the mtDNA, e.g. cyt b.

Only three haplotypes were shared among individuals from different localities, the most common haplotype was shared by 19 specimens (7 from Azores, 3 from Cape Verde and 9 from Madeira) and is presented in Fig.7.5. 17 haplotypes (around 71%) were unique.

5'-GGATTCTAAACTAAACTATTCTCTGCCGCCCCAGCCCGCGCCCACATATA
TGTCCTAGAAAGCTAATATAGACATATATGTATTAACACCATGAATCGA
ATTTAACCATTTCTAATAGTGCCCTGGGACATACCTGAACTATCAACAC
ATGTCGAGATTAAACATTCATACATCAACACAAATACAAAGGTGGACAT
AAAGCAATACTGAAATATCTAACAGATTGATGACTTCATGTGATATCCG
ACATTTAAGACCGAACACAACTCGCATTGGTTAAGTTATACCACGAATC
CAACATCTCGTCAACCCTCAGATTCTCAAGTGTAGTAAGAAACCACCAT
CAGTTGATTCCTTAATGCATATCATGCTTGATGGTCAGGGACAAGTGGT
CGTGGGGGTTTCACTTAGTGAACTATTT - 3'

Fig.7.5 – *Beryx decdactylus*. Control region. Most common haplotype.

Cyt B variation

The mitochondrial cyt b region (313 bp) of *Beryx decadactylus* was sequenced for 30 individuals from the different sampled sites. No more individuals were sequenced because haplotype diversity was very low, showing that genetic diversity of cyt b for this species is insufficient for it to be used as a variable molecular marker for studies of genetic differentiation. This result was expected given the low variation of the control region, which usually has the highest mutation rate of the mitochondrial genome. Consequently, for imperador mtDNA cyt b region was considered to be uninformative and dropped from this analysis.

Geographic variation and population structuring of Beryx decadactylus

Analysis of molecular variance (Table 7.4) showed that 9.38% of the total variation was partitioned among populations (P<0.001).

Source of variation	% Total Variance	Fixation indices	P- value
1.One gene pool			
Among populations	9.38	0.0938	<0.001
Within populations	90.62		
2. Four gene pools (Madeira, Peniche, Cape Verde, Azores)			
Among groups	11.59	F_{CT}= 0.1159	<0.05
Among populations within groups	-0.46	F_{SC}= -0.0053	<0.001
Within populations	88.88	F_{ST}= 0.1112	<0.001

Table 7.4 – *Beryx decadactylus.* Control region. Analysis of Molecular variance (AMOVA) results.

Statistically significant differences in haplotype frequencies were found in two out of the three pairwise comparisons (Table 7.5). The Cape Verde sample was highly differentiated from the other two (P<0.001; even after sequential Bonferroni correction).

F_{ST}	Azores	Cape Verde	Madeira
Azores	-	-	-
Cape Verde	0.135**	-	-
Madeira	-0.024	0.164**	-

Table 7.5 – *Beryx decadactylus.* Control region. Pairwise F_{ST} values between 3 populations.*** = significant at P<0.001 (after sequential Bonferroni correction)

The results presented above showed a clear differentiation between Cape Verde imperadors and the other two Macaronesian archipelagos. Consequently, it was decided to perform a more detailed analysis of the population structure of this species including additional samples that would permit a finer-scale geographical analysis.

Pairwise comparisons of F_{ST} between populations confirmed the results obtained earlier by showing significant statistical differences between Cape Verde sample and all the other populations (Table 7.6). However, significant values for population differentiation were also found between Peniche (mainland Portugal) and one of the Azorean sub-samples (Central group). There was no significant heterogeneity among samples within the Azores archipelago permitting samples to be pooled according to major geographic areas in a subsequent analysis.

F_{ST}	Ocidental G	Oriental G	Seamounts	Central G	Peniche	Madeira	Cape Verde
Ocidental G	-	0.4144	0.6306	0.8468	0.0991	0.8288	0.0000
Oriental G	-0.0006	-	0.3784	0.2072	0.0360	0.6126	0.0000
Seamounts	-0.0119	0.0049	-	0.3606	0.1531	0.5135	0.0000
Central G	-0.0219	0.0108	0.0063	-	0.0000	0.8919	0.0000
Peniche	0.0355	0.0621	0.0194	0.0909***	-	0.01802	0.0000
Madeira	-0.0227	-0.0119	-0.0099	-0.0279	0.0811	-	0.0000
Cape Verde	0.1680***	0.1901***	0.1851***	0.2205***	0.2602***	0.1643***	-

Table 7.6 – *Beryx decadactylus.*Control region. Pairwise F_{ST} (below diagonal) and associated P values (above diagonal) among populations. *** = significant at $P<0.001$ (after sequential Bonferroni correction)

An analysis of molecular variance based on the partitioning of variation across the four major geographical areas sampled (Cape Verde, Madeira, Peniche and Azores (Table 7.4), showed that most of the variation (88.88%) was within samples, but there remained a significant portion (11.59%; $P<0.05$) as a result of differences between regions.

Phylogenetic relationships of haplotypes represented in a network form also show a quite high rate of haplotypic diversity and homoplasy. The most common haplotypes are not exclusive for any population. The higher number of representatives of the Azores population is mainly the result of a higher number of individuals sampled for this site, as all subpopulations were pooled together for this analysis. However, a small clade with predominantly of Cape Verde exclusive haplotypes is present in the network, supporting the isolation of the Cape Verde population from the rest of the samples as shown by the F_{ST} analysis (Fig. 7.6).

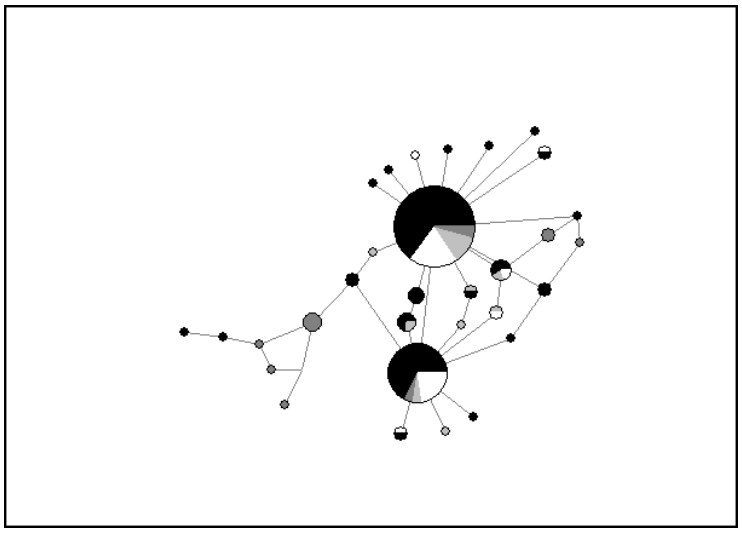


Fig.7.6 – *B.decadactylus* control region minimum spanning network analysis of haplotypes for all populations. Circles sizes are relative to haplotype number. Black= Azores, Dark Grey= Cape Verde and Light Grey= Madeira and White= Peniche.

7.4.3 Demographic History patterns

Beryx splendens

Since no evidence of genetic differentiation was observed for *B.splendens*, all samples were grouped as one to conduct tests of selective neutrality and demographic story. Pairwise mismatch distributions and results of Tajima's *D* test performed for each mtDNA region on this species are given in Figure 7.7.

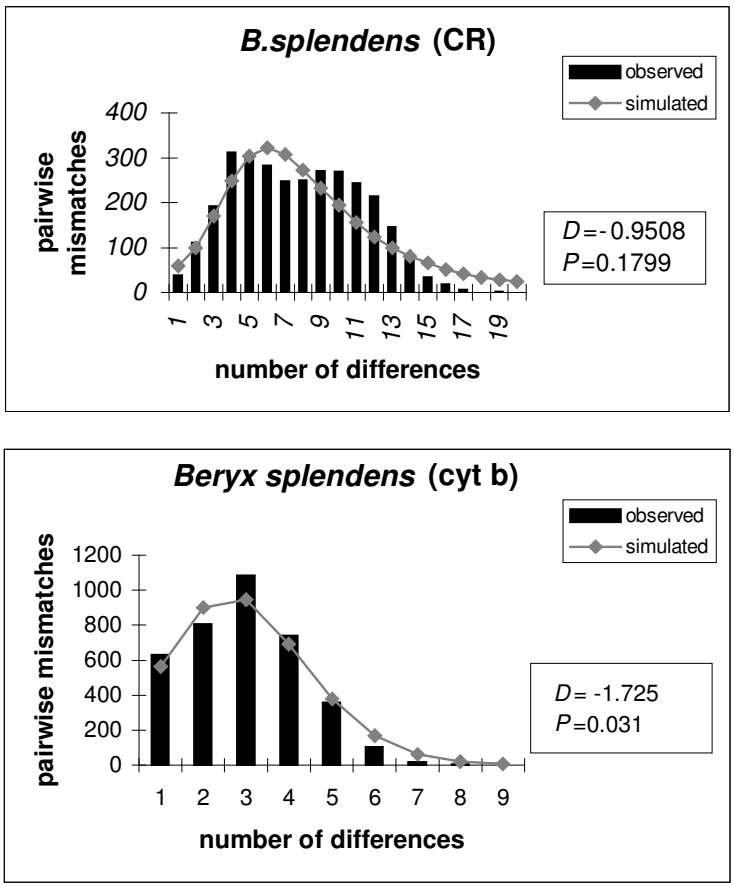


Fig. 7.7 – *B.splendens.*Control region and Cyt *b*. Mismatch distributions and Tajima's D-test results for the NE Atlantic population.

The cyt b haplotypic network shows no evidence of population structure for alfonsino population in the North Atlantic has haplotypes from different regions do not group in specific clades, instead, they are spread randomly throughout the entire network. However, the star-shape of the network clearly shows a sudden expansion probably following a bottleneck in this population's history (Fig.7.6).

Mismatch distribution analysis was performed and a poisson distribution of pairwise differences was found for both mitochondrial regions, indicating the possibility of expansion of the population in the past. This results from the rate of the accumulation of new mutations which is greater than the loss of variation through drift. However, only for cyt b was Tajima's *D*-value significantly negative, indicating more rare polymorphic nucleotide sites than would be expected under a neutral model of evolution, and indicating that a sudden expansion in population size may have occurred ($P<0.05$).

An approximate time of expansion was calculated for alfonsinos of the NE Atlantic using cyt *b* data suggesting that the sudden expansion in population size occurred around 1 million years ago.

Mismatch distribution analysis and tests for selective neutrality were also performed on each population separately in case the pooling of data masked bottlenecks in individual populations. No indication of this was observed (data not shown).

B.decadactylus

For *B.decadactylus* mismatch distribution analysis was performed separately for each population because evidence of genetic population structure was found.

Pairwise mismatch distributions and results of Tajima's *D* test for *B.decadactylus* mtDNA control region are given in Figure 7.7.

All mismatch distributions were close to a Poisson curve but none presented significantly negative values for Tajima's D-test. The Oriental group population could not be fitted to an expansion model, while for the Cape Verde population Tajima's *D*-test was not negative and distribution was closer to a bimodal curve.

The assumption of genetic "equilibrium" was also tested by pooling all samples irrespectively of their geographic origin and by pooling all the Azores sub-samples into one for Tajima's D test. Unambiguously, all tests indicated the same pattern, no significant departures from the equilibrium hypothesis were found (data not shown).

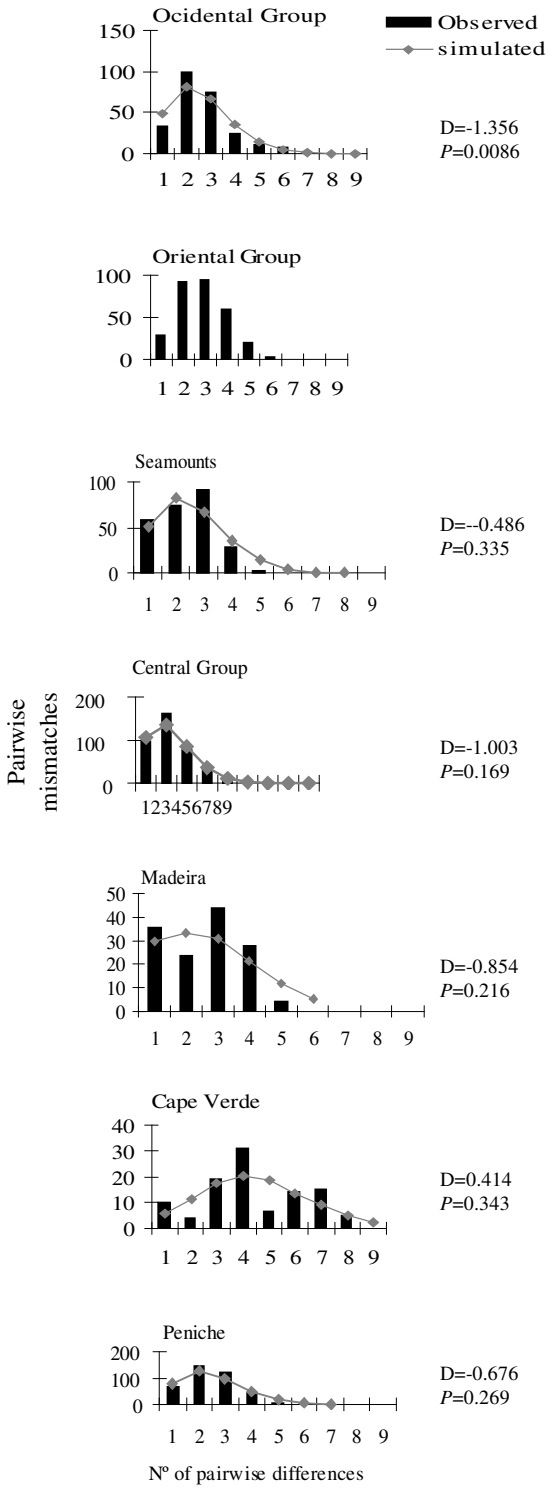


Fig 7.7 - Mismatch distributions and Tajima's D-test results for *B.decadactylus* populations.

7.5 Discussion

This study provides a comprehensive genetic analysis and comparison of the Northeast Atlantic populations of alfonsino and imperador.
It is the first work determining population genetic structure of *B.decadactylus* and expands previous work by Stockley (2001) and Horau & Borsa (2001) on *B.splendens*.

7.5.1 *B.splendens* vs *B.decadactylus*; genetic variation and distribution

Overall, conclusions point to differences in the genetic variability and phylogeography between these two closely- related species.
Summary statistics showed clear differences in the molecular variation of alfonsinos and imperadors. Nucleotide and haplotype diversity indices in *B.splendens* were consistent with those found in other marine fish species, including orange roughy (Smith, 1986; Elliott *et al.*, 1994), grenadier (Wilson & Waples, 1980) and mackerel (Nesbo *et al.*, 2000; Zardoya *et al.*, 2004). However, *B.decadactylus* showed lower genetic diversity at the intra- and inter-specific level. The variation of the mtDNA regions was much lower when compared to *B.splendens* and with other demersal fish species, such as hoki (Baker *et al.*, 1995) and bluemouth (Aboim *et al.*, 2005) for example.
However, these low levels of variability were more comparable to those found for the Northeast Atlantic species *Pagellus bogaraveo* (Stockley et al., 2005).
In terms of genetic population structure analysis, results indicate that alfonsino is panmitic in the Northeast Atlantic waters, agreeing with previous works on the genetic variability of this species that indicated extensive gene flow and no population differentiation at inter- or intra-oceanic scale (Horau & Borsa, 1999). Marine fishes with high dispersal capabilities and worldwide distributions are believed to show low levels of differentiation across oceans and smaller geographic scales, specially the ones associated with seamounts and other isolated geographic features. Alfonsino, according to the results presented here, seems to conform to this pattern.
In contrast, imperador populations show statistically significant levels of genetic structuring. The Cape Verde population is differentiated from the other populations analysed. The present results agree with previous findings on genetic structuring of *Helicolenus dactylopterus* in the North Atlantic based on mtDNA sequencing and microsatellite DNA analysis (see Chapters 5 and 6; Aboim *et al.*, 2005). It is interesting to note, that despite the cosmopolitan nature of imperador and the supposed similarities in life history with alfonsino

(high larval dispersal capability, external fertilization) a similar panmictic distribution is not found. Instead, imperador seems to be another example of a marine fish species that exhibits marked population structure with no apparent physical barriers to migration or dispersal and therefore gene flow.

Several hypotheses might be put forward to explain the occurrence of different phylogeographical patterns in *B.splendens* and *B.decadactylus* but are based on limited information from studies on the Azores area (Menezes *et al*., 2001).

Population differentiation depends directly on gene flow. Species with higher capabilities of dispersal and migration may across oceanographic barriers and do not show population differentiation whereas other species respond differently to barriers or have lower dispersal ability. No direct measures of dispersal ability exist for these species; however, known differences in terms of depth distribution may cause slightly different responses to hydrographical, dynamic and anthropogenic processes. *B.decadactylus* often occurs at greater depths than *B.splendens* occupying slightly different strata, which may lead to different responses to hydrographic features.

Regarding population size, information suggests that *B.splendens* census size is usually higher than *B.decadactylus* as more specimens are caught during survey cruises and exist in higher numbers on the market. This can have an important direct influence on genetic variation, as in populations with lower effective sizes are more affected by random genetic drift and gene flow tends to decrease. This can be an explanation for the isolation of the Cape Verde population. Lower abundances directly influence the number of individuals that may be able to migrate over larger distances and permit gene-flow between regions. Populations with smaller effective population sizes also tend towards rapid fixation of haplotypes, which may be why there is a higher number of private haplotypes found in the Cape Verde population.

It can also be the case that the apparent lower population sizes observed in *B.decadactylus* may be a reflection of a present day bottleneck provoked by anthropogenic factors that have not been detected through other methods such as demographic analysis as these studies have not been carried out.

It is also interesting to note that *B.decadactylus* presents a higher longevity as well as a mean individual weight, which is significantly higher than *B.splendens*. This can be translated in longer generation times, which can influence the way historical events influence the species differently.

Finally, the fact that high genetic variation found in *B.splendens* can strongly influence the spatial genetic analysis cannot be neglected. Molecular markers with high variability may

require bigger sampling numbers in order to "dilute" the genetic variability found. In the case of *B.splendens*, maybe some structuring could be found if higher levels of sampling could be acquired. Previous genetic studies on this species (Hourau & Borsa, 2000 and Stockley, 2001) have also neglected that possibility.

Although none of the available information on Beryx spp. biology seems to explain *per se* the divergent pattern observed, the incompleteness of current knowledge does not allow to safely exclude or hold any of the above mentioned hypotheses.

These findings lead to the conclusion that it is important to study further the biology and ecology of these species, as it is likely that the life histories do exhibit some major differences important for the interpretation of these data.

7.5.2 Demographic history

Observed distribution of pairwise differences between cyt b haplotypes of *Beryx splendens* fitted the distribution predicted for a population that has experienced a major demographic expansion (or a reduction followed by an expansion). Haplotype and nucleotide diversity values also pointed to a bottleneck followed by a sudden increase in population size (Grant & Bowen, 1998), as well as the star-shaped haplotype network (Fig.7.4)

If the estimated time is correct, this expansion occurred around 1 million years ago, much earlier than the Last Glacial Maximum (16,000 years ago). This agrees with the results obtained for *Helicolenus dactylopterus* (see Capter 4) and corroborates the suggestion that glaciations prior to the LMG may have had strong impacts on North Atlantic populations of marine species (Aboim *et al.*, 2005; Chapter 4). Several events may have been sufficiently severe to strongly influence the genetic structure of marine species that are still detected nowadays.

For *Beryx decadactylus* no significant departures from neutrality were found still mismatch distributions and star-shaped networks seem to point out for possible occurrence of bottlenecks. This might be due to the fact that current statistics seem unable to capture recent contractions or expansions of population sizes induced by anthropogenic factors for example. In the case of *Beryx decadactylus* the genetic variability of populations might have had been influenced by exploitation or still not have recovered from severe bottlenecks.

But once more, the question remains, how such related species have been so influenced in such different ways by climatic changes and historic events. The answer continues to hide probably behind unknown differences in the biology, ecology and behaviour of these two species.

7.5.3 Conservation and Fisheries management

The results have important implications for fisheries management of both species in the Northeast Atlantic.

Alfonsinos and imperadors are normally considered together in terms of fishery statistics. This is because both species are taken in the same mixed-species fisheries. Management or data reports often refer to both species as *Beryx spp.* not differentiating one from the other. As a result, stocks of both species are managed in the same way and often together.

Alfonsino is normally captured in higher quantities and sometimes is the target species of some small fisheries, while imperador is usually a by-catch species.

However, while the lack of structure found in alfonsino in the Northeast Atlantic is consistent with a "one stock" management, the population structure found for imperador is quite different showing marked genetic differentiation between regions and possibly at the within region scale. This points to a serious flaw in current strategies for reporting and management of deep-sea fisheries for these species. Correct management of this resource must take in account the genetic evidence presented by this study that there are discrete genetic stocks of imperador. Hence, conservation actions should concentrate on both species separately, and imperador cannot be neglected towards alfonsino as it as been the case until now. This is especially the case, as the former species appears to have a smaller population size and more conservative life history than the latter.

These results together with observation of oscillations in fish concentrations around seamounts after years of intensive fishing (Vinnichenko, 2002) reinforce the need for more studies and proposals for conservation of these habitats against overexploitation through mixed-species fisheries.

More studies on the biology, behaviour and population genetics of the two species, and especially on *B.decadactylus* (from which very little is known) are needed to better understand the differences revealed by this study.

PART IV
FINAL REMARKS

CHAPTER 8
GENERAL DISCUSSION AND CONCLUSIONS

8.1. Limitations to work

In order to be able to correctly assess the distribution of genetic variation within and among populations, the temporal, spatial and quantitative nature of samples has to be carefully thought and defined *a priori* (Baverstock & Moritz, 1997). Studies should be performed on a species distribution scale and should include as much samples from the same place as possible. Associated biological and environmental data should also be collected to help interpreting the genetic variation whenever possible. It has already been proven that the combination of different nature molecular markers is more informative and permits a more precise analysis and complete conclusions. On another hand it should be considered that everyday, new models and statistical approaches are developed and published. This should be taken into consideration as much as possible, as sometimes classical models become outdated. All this assumptions were taking into account when planning this work, however, some limitations were recognized but could not be overturned.

8.1.1 Sampling

Like most genetic studies on marine species, the biggest limitation of the present work is to focus only on a small number of species and/or samples and to be restricted to a certain area. Samples were obtained from multiple populations representing a hierarchy from closely spaced sites (e.g. within the Azores archipelago) to more distant geographical sites within the species overall distribution range (e.g. Cape Verde and NW Atlantic). However, the available resources did not permit that sampling was spread through the entire range of the species distribution. Places like the Mediterranean, the North Sea and NW Atlantic regions up north and south were excluded from this study because it was not possible to collect samples from those areas.

Special attention was also paid to the quantity of samples collected per site, taking into consideration the molecular markers chosen. But again, it was not always possible to achieve the optimal sample size. Only one sampling cruise was carried out in the Madeira archipelago, and unfortunately, the obtained samples were of a number too small for an accurate microsatellite analysis (Ruzzante, 1998). Collection of related biological and environmental data was mainly restricted to the Azores archipelago, as specially programmed investigation cruises permitted such compilation. Samples from other places

were obtained on a more sole sampling cruises context, by other teams or in markets which was not propitious for other related data collection, hence inclusion of further analyses for the study.

8.1.2 Molecular Markers

The molecular markers used throughout this study have been chosen with basis on previous works of population genetics in marine species. MtDNA sequencing has been widely used on phylogenetic, phylogeography, population structure and evolutionary studies on diverse species including teleost fish. Because of that this technique was chosen to be the basis of this work. However, recent studies using microsatellite loci for population analysis have begun to reveal genetic structuring in species previously thought to be homogeneous over geographic ranges using MtDNA. The combination of more than one kind of molecular marker, specially mitochondrial and nuclear genes, have proven to be essential to deeply understand the genetic structure of populations as they differ in nature, variability and sensitivity, as we could see for the *H.dactylopterus* species in this work.
However, time and monetary resources did not permit the development of microsatellite loci or any other nuclear DNA based technique for *Beryx spp.*, which could proven to be very interesting and useful to understand these two related species differences and particularities, or reveal regional and local population differentiation in *Beryx splendens* and *B.decadactylus* respectively.

8.1.3 Statistical Analysis

By the time this thesis was produced, several new approaches have been developed and introduced for population genetics analysis.
F_{ST} (Wright, 1951) has been the basis of population analysis in most molecular works, but its use has been recently criticized, especially in the case of microsatellites. The extremely high mutation rate of microsatellite loci does not allow for their mutation pattern to be neglected. Geneticists are still struggling to find the more realistic model but none seems to entirely fit and explain the way microsatellite work (Weber & Wong, 1993; Gaggiotti *et al.*, 1999; Balloux &Lugon-Moulin, 2002; Estoup *et al.*, 2002).
During this work, it was decided to use two of the most discussed statistics the F_{ST} (Wright, 1951) and R_{ST} (Slatkin, 1985) in order to have a comparable approach (Balloux & Lugon-Moulin, 2002). Comparison of both estimates revealed some differences, nominally the fact that less pairwise comparisions between populations were significant for R_{ST}. This might be

due to the fact that some of the microsatellite loci were composed and presented a lot of homoplasy that cannot be detected by the Infinite Allele Model of mutation presupposed by F_{ST}.

Statistical analysis excluding some data suspected to bias the analyses in anyway should be performed extensively as possible. Some of those data were detected and discussed in the light of new analysis in the appropriate chapters, but some of those were left behind. For example, analysis excluding microsatellite loci propitious to null alleles was performed, however, the possibility of excluding individuals with mtDNA haplotypes exclusive from Cape Verde, hence possibly belonging to another species, was not performed because of the extremely time consuming process implied.

The recent introduction or lack of support of new approaches to microsatellite analysis considering historical demographic/evolutionary processes also lead to the non-inclusion of these methods in this work (e.g. Beaumont, 1999; Dyer & Nason, 2004).

8.2. Implications for the knowledge on population genetics of deep-sea demersal fish species

Most genetic studies on deep-sea demersal fish indicate that the majority of species do not exhibit homogeneous populations throughout the range of their distribution, contrary to what has been typically assumed (reviewed in Rogers, 2003). Even though demersal fish normally present wide geographic distributions, potentially high mobility as adults and dispersive larvae, most species present intra-specific genetic differentiation and some degree of isolation between populations at oceanic, regional and even local scales (Dahle, 1991; Baker *et al.*, 1995; Clark, 1999; Quinteiro *et al.*, 2000; Roques *et al.*, 2001).

An increasing variety of molecular markers exist in the literature for population genetic analysis. Sequencing of mtDNA regions has been typically used to assess population genetic structure of several demersal fish species and to also infer facts about their demographic history (Arnason *et al.*, 1992; Smith *et al.*, 1996; Kojima *et al.*, 2001). However, recent studies using microsatellite loci for population analysis, have demonstrated their usefulness in resolving genetic structuring at geographic scales over which species have previously thought to be genetically homogeneous using other genetic markers such as mtDNA sequence variation. Species such as cod (Ruzzante *et al.*, 1996, 1998; Bentzen *et al.*, 1996), whiting (Rico *et al.*, 1997) and herring are examples of this phenomenon.

Helicolenus dactylopterus is a good example of where different markers provide different levels of resolution of population structure within a species (see Chapter 5 and 6). Previous morphological studies have suggested that bluemouth from the America and European coasts represent different clades or even sub-species (Echemeyer, 1969; Barsukov, 1980). Sequencing of two mitochondrial regions and amplification of microsatellite loci performed throughout this study have proven that *H.dactylopterus* actually presents significant genetic differentiation of populations located in different areas of the North Atlantic, hence separate clades.

According to partial sequence data of mitochondrial regions, there is a distinct genetic division between NE and NW Atlantic bluemouth populations. This is also the case of other oceanic distributed species such as *Corephaenoides rupestris* (Logvinenko *et al.*, 1983), *Sebastes mentella* (Roques *et al.*, 2001) or intertidal species such as *Asterias rubens* or *Littorina obtusata* (Wares & Cunningham, 2001).

There was also evidence of population structure at a NE Atlantic regional scale based on mtDNA sequences, which revealed that little or no effective gene flow occurs between Cape Verde bluemouths and others from the Mid-Atlantic Ridge (Azores), Madeira and European Continental slope (Portugal). No conclusive genetic evidence was found for intra-regional population structure.

Microsatellites were isolated and developed for *H.dactylopterus* to assess the population structure of this species at a finer geographic scale. Eight loci were isolated and developed using a non-radioactive method and proved to be highly polymorphic and useful for assessing genetic variation. The levels of diversity observed were in accordance with those previously described for other teleost fish species (O'Connell *et al.*, 1998; Ruzzant*e*, 1998; Roques *et al.*, 1999; Stepien, 1999).

Microsatellite analysis revealed population differentiation not detected by mtDNA markers, demonstrating isolation between the European continental slope population (Peniche) and the Azores archipelago. Such local differentiation has already been found in another Azorean demersal fish species, *Pagellus bogaraveo* (Stockley, 2001) but has only been detected in a few other cases such as with Patagonian toothfish, *Dissostichus eleginoides* (Shaw *et al.,* 2004). No evidence was revealed for the genetic isolation of the Madeira archipelago; however, these results have to be interpreted with some caution. This is because in this case, the number of samples was probably insufficient for microsatellite analysis. Hence, conclusions on no differentiation of this population for *H.dactylopterus* using these molecular markers cannot be assumed with certainty.

Evidence of some degree of differentiation at an inter-regional scale was given by variation in microsatellite loci. The Central group and Seamounts subpopulations showed moderate population differentiation for some of the performed analyses. Ideally, individuals sampled for the estimation of population genetic structure should belong to the same generation within the same breeding season, because allele frequencies vary over time in finite size populations (Waples, 1998). This assumption was considered for the sampling plan; however, a reasonable sample size for the Central Group Azorean sub-population was impossible to get unless samples from different years were pooled for the microsatellite analysis. This may have influenced the genetic substructuring detected for this population but is probably unlikely given the longevity of this species.

On another hand, there are a few demersal species that do present panmictic populations at wide geographic scales and sometimes throughout their worldwide distribution range. They are normally capable of long-distance migration and are successful colonizers of fragmented habitats and isolated topographic features such as seamounts, ridges and plateaus. Examples include walleye pollock from the Berigian Sea and Gulf of Alaska (Grant & Utter, 1980; Mulligan *et al.*, 1992) pink snapper from the Hawaiian archipelago (Shaklee and Samollow, 1984), pelagic armourhead from the Hawaiian Ridge (Martin *et al.*, 1992), the wreckfish in the Atlantic and Southern pacific (Sedberry *et al.*, 1996) and *Beryx splendens* from New Caledonia, New Zealand and the Galicia bank (Hoarau & Borsa, 2000).
Beryx splendens (see chapter 7) seems to be representative of the few species that exhibit a panmictic genetic population structure over large oceanic scales. The life history of alfonsino is poorly understood but potentially highly dispersive. This has been proposed as an adaptation to exploiting fragmented habitats separated by large tracts of open oceans. This maybe counter-intuitive as it is easy to believe that seamount creatures would present more evident reproductive and genetic isolation due to the extreme isolation of the topographic features they inhabit. However, this seems to be exactly the opposite case in this species. MtDNA sequences of both control and cyt b regions revealed no population differentiation between NE Atlantic populations (see chapter 7). These results largely agree with previous studies on this species by Stockley (2001) and Hoarau & Borsa (2000) which, in the later case suggested inter-oceanic panmixia for this species.

The third case considered here, *Beryx decadactylus,* also seems to contradict the generalization that oceanic species exhibit panmixia over very large geographic scales. The ecology and life history characteristics of *B.splendens* and *B.decadactylus* are thought to be

similar in many aspects, yet the species were found to differ widely in the extent and spatial distribution of mtDNA variation. Contrary to the congener *B.splendens, B.decadactylus* exhibits strong population differentiation within the Northeast Atlantic. MtDNA sequences showed that the imperadors from Cape Verde are genetically isolated from the rest of the NE Atlantic populations as in *Helicolenus dactylopterus* and contrary to the panmitic distribution of *Beryx splendens*.

But one question remains, what factors could be responsible for breakdown of gene-flow between populations in some demersal fish species and not in others, when both adult and/or larval characteristics suggest a high dispersal capability and the potential for long distance migration?

It is well documented that the dynamic interaction between oceanographic features and specific behavioural characteristics may determine the number of distinct populations in marine species (Iles & Sinclair, 1982).

Against previous conceptions of a homogeneous deep-sea environment, topographic and hydrographic features like oceanic gyres, currents or water masses with different physical characteristics such as temperature have been considered as important in structuring populations by producing geographical clustering and regional divergence of genotypes. The life-histories of deep-sea creatures can not be separated from surface phenomena. The early stages of their life-histories are distributed in the water column, as most species possess pelagic larvae, and influence of surface processes on their genetic variation, and therefore, evolution and demographic processes, cannot be neglected. Surface currents, larval behaviour and successful recruitment thus may produce patterns in deep-sea regions, just as they do in shallower waters such as the continental platform.

High mortality of adults and larvae swept out from suitable habitats by hydrodynamic events has been suggested as one factor responsible for genetic differentiation in some pelagic fish such as the Atlantic mackerel (Nesbø *et al.*, 2000), benthopelagic fish species such as the walleye pollock (Bailey *et al.*, 1997), the patagonian toothfish (Shaw *et al.*, 2004), cod (Ruzzante *et al.*, 1999 and 2000) and the benthic dover sole (Stepien, 1999).

This is explained by the member-vagrant hypothesis of Sinclair (1988), which suggests that marine larvae/juveniles that survive to settle in appropriate habitats are passively and/or actively retained in major currents. Gene flow and genetic divergence in species with planktonic larvae thus may be structured by larval retention areas (oceanographical, physical), which are reflected in the genetic divergences among adult populations (Sinclair,

1988). This may be particularly important for species associated with isolated topographic features such as seamounts, ridges and oceanic islands.

Larval ecology of all three species evaluated here is poorly known, however, they apparently have relatively high fecundity indices and long larval pelagic (near surface) phases. The low dispersal observed in adults of *Helicolenus dactylopterus* from mark-recapture experiments suggests that gene flow primarily occurs during the pelagic egg and larvae stages. The internal fertilization characteristic of this species (Muñoz & Casadevall, 2002) along with retention of eggs until a later stage of development compared to other species like *Beryx splendens* and *B.decadactylus*, that possess external fertilization, suggests that perhaps the time for passive dispersal of eggs and larvae is reduced compared to the latter species. However, a direct relationship between the hydrodynamic events in the North Atlantic and the population structure proposed here does not seem to exist.

The prevailing surface currents in the North Atlantic may provide an efficient means of larval retention/dispersal. The Gulf Stream seems to dominate circulation patterns within the North Atlantic basin and induce larval dispersal on an oceanic cyclonic pattern, but this would allow larvae to disperse easily between NW and NE margins, which is proven here not to be the case.

The conjunction of the Azores counter-current with the Canaries current forms a cyclonic pattern of water circulation resulting in a possible stronger trap for larvae at the regional scale. The interaction of circulation with topography induces Taylor columns and other processes around seamounts, which can also work as small local scale retainers of larvae favouring local self-recruitment. But if larval retention occurs, then relationships among populations would be related to current and gyres pattern, which is not entirely the case.

Alternatively, the patterns of genetic difference may be regulated by temperature-related selection on larval survival. Surface water and deep-water masses temperatures differ significantly in different areas and distributions of marine fish larvae appear to be sensitive to such boundaries.

As theories, based only on larval drift and migration, are unconvincing and insufficient, other explanations have been put forward to explain population structure.

Geographic distance is the most obvious barrier to gene flow in oceanic species. It is easy to understand that populations closer to each other are less isolated than populations further apart. Interestingly, there are several examples where this seems not to be the case and lack of consistency with the isolation-by-distance model has also been revealed here. Divergence

between some of the more closely spaced sites and lack of divergence in others with higher proximity has been detected during this work.

External factors (hydrographical, climatic, ecological) do not seem to equally affect species that live in the same habitats or have the same distributions or that are even sympatric. The inherent biological features of the species studied here must be largely responsible for the different population structures encountered for these species. Reproductive and feeding habits are important biological factors that influence the genetic diversity of species over time and space.

Feeding ecology, for example, can make populations vulnerable to natural oscillations of their prey species. If species have specialized feeding preferences, species may, at an ecological time scale, pass through different population fluctuations in effective sizes.

Differences in swimming power, voracious behaviour of larvae and fecundity rates are other biological factors pointed out as possible factors influencing the sensitivity of species to physical barriers in the marine realm (e.g. Zardoya *et al.*, 2004). Once more the conclusion that the lack of knowledge on species biology, ecology and behaviour are obstacles to the correct interpretation of genetic data is an inescapable conclusion.

8.3. Implications for the knowledge on deep-sea demersal fish species' evolution and demographic history in the North Atlantic.

More recently, historical processes have been pointed out as important influences in present intraspecific genetic variation and speciation among marine organisms.

Cycles of global warming or cooling have provoked shifts in the temperature and oxygen-minimum water layers motivating expansion or reduction of population sizes, hence, opportunities for speciation.

Some past events, such as the Last Glacial Maximum (LGM), are known to have had a strong historical influence on the genetic population structure of demersal fish species in both the North Atlantic and the North Pacific. In several studies, the responsibility for the reduction or extermination of populations of marine organisms in the past has been attributed to the strong LGM 16,000 years ago (e.g. Wares & Cunningham, 2001).

This study suggests that climate events previous to the LGM, might have been more severe and had more impact in shaping the population genetic structure of some demersal fish species in the North Atlantic.

Analysis on the mitochondrial sequences genetic diversity based on mismatch distributions, diversity indices and phylogenetic networks performed to *Helicolenus dactylopterus* showed that the NW Atlantic and the Azores populations had suffered a sudden expansion in

effective population sizes supposedly after strong bottlenecks. The estimated time for expansion, ranging from 0.64 –1.02 million years ago, coincide with the Mid-Pleistocene Revolution, a period of major glacial cycles and shifts in ocean biochemistry that have extended from 900,000-650,000 years ago, much older then the LGM (Mudelsee & Schulz, 1997; Becquey & Gersonde, 2002). An inter-oceanic colonization process through major currents between Atlantic margins has been proposed.

Estimated times of expansion of around 1 million years ago for the panmitic NE Atlantic population of *Beryx splendens* based on cytochrome *b* data, also correspond to this period (Chapter 7). Further analysis with a larger number of samples from other areas would be necessary for further conclusions on the colonization and evolutionary history of this species.

For *B.decadactylus* no evidence for sudden expansion following severe bottlenecks was found for any of the populations analysed. Sometimes recent demographic events may cover past events. *B.decadactylus* populations may have suffered a recent bottleneck and not recovered from it yet, as *Beryx* spp. have been hit hard by deep-sea fishing in the North Atlantic in recent times (Koslow *et al.* 2000; FAO, 2004).

Assessment of species' past history using microsatellites is more difficult as the available models normally assume H-W equilibrium, which is often not the case in microsatellite allelic frequencies. However, some new coalescent methods are being introduced.

8.4.Implications for fisheries management and conservation

Deep-sea demersal fishes are an important fisheries resource on continental slopes, oceanic islands and seamounts. They have been increasingly affected by expanding of deep-sea demersal fisheries worldwide and in many areas of the North Atlantic.

Deep-sea fish are long-lived and highly vulnerable to modern fishing methods and the depletion of their stocks has been noticed at a global, regional and especially at local scales.

Understanding the population structure, dispersal capacities and demographic history of species throughout their distribution range is fundamental for the generation of a sustainable management strategy for conservation of these species.

Effective management of fisheries resources requires critical information on the population or stock structure of the exploited species, especially because marine species have wide distributions and in part because jurisdictional boundaries for resource allocation frequently overlap.

Historically, information on stock structure was derived from morphological and/or life-history data (Ihssen *et al.*, 1981), but the use of genetic data has proven to be essential in revealing accurate population structuring in marine species. Genetic data have revealed sub-structured populations despite morphological, geographical and/or behaviour data indicating the occurrence of single stocks (Berst & Simon, 1981; Allendorf *et al.*, 1987) and vice-versa (Kornfield & Bogdanowich, 1987; Avise *et al.*, 1987; Martin *et al.*, 1992).

This study has made a significant contribution to the understanding of population genetic structure of three deep-sea demersal fish species exploited in the North Atlantic as well as the historical and hydrographical factors affecting them. All three species: *Helicolenus dactylopterus*, *Beryx splendens* and *Beryx decadactylus*, support important commercial fisheries in the Azores and by-catch in several other areas of the North Atlantic where multispecies fisheries take place.

Like many others, the lack of knowledge on these three species implies that no delimitation of stocks exists; hence, that they are not regulated in any way in response to over-fishing or perceived population declines.

Despite the fact that the demersal fishery in the Azores is mainly artesanal, a decline in catches of these three species has been noticed in several islands, seamounts and ridges. Consequently, they have recently become subject of several ecological, biological and fisheries studies in the area.

For *Helicolenus dactylopterus* no fisheries management strategies exist, however, the implementation of a Total Allowed Capture (TAC) by areas has been proposed by Azorean researchers in the last few years in the ICES working group for demersal fisheries. Proof of a separation between Continental Portugal (ICES IXa) and Azores (ICES X) found in this study support the application of such measures.

For *Beryx splendens*, observed genetic homogeneity was interpreted as support for the hypothesis that the species is composed of a single, genetic panmictic unit and that gene flow is sufficient among the Macaronesian archipelagos and European Continental slope to preclude significant genetic differentiation.

This is in agreement with the measures proposed last year of an overall TAC for *Beryx spp.* including all ICES areas of the European community. However, data obtained here on *Beryx decadactylus* completely contradicts this measure, by showing that one of the species included in the *Beryx spp.* category does present population differentiation within its distribution range. Despite the fact that no differentiation was found within the ICES

territory, some data point to a possible structure within this area that might be revealed by future studies with other more sensitive markers such as microsatellites.
So, once more there's the need of emphasize the fact that *Beryx splendens* and *Beryx decadactylus* must be separated and treated as different resources on research and management reports and measures.
These findings also support the recent NEAFC resolution of upgrading some seamounts to protected areas, as there is more evidence that these unique habitats embrace several species with distinctive biological, ecological and genetic characteristics and need to be further studied before proper management strategies must be applied.

8.5.Future Work

The present work brought new insights on the understanding of population genetics and demographic history of deep-sea demersal fishes at a regional scale and also on a North Atlantic context. However, like most molecular studies, this work focused only upon a small number of species and was restricted to a certain area.

It would be important to focus on other species and to enlarge the studied area to an oceanic or worldwide scale, in order to understand the global mechanisms, historic or life-history related, that influence demersal deep-sea fishes population structure.

In order to better understand the genetic structure of the demersal community of the Azores archipelago, more species need to be studied. Genetic approaches have been previously used to study *Pagellus bogaraveo*, *Beryx splendens* (Stockley, 2001), *Polyprion americanus* (Sedberry *et al.*, 1999) and were complemented here by analyses on *Helicolenus dactylopterus*, *Beryx splendens* and *Beryx decadactylus*. Studies need to be performed on other important representatives of the Azorean demersal community in order to achieve a global knowledge and understanding of this habitat and to characterize the response of resources to anthropological or climate changes. Not only should genetic studies be carried out on unstudied species in this area, such as *Phycis phycis, Mora moro*, *Pagrus pagrus* but more ecological and biological studies should also be iniciated for the species already genetically analysed to help interpret the genetic data.

MtDNA and microsatellites markers were used here for *Helicolenus dactylopterus*. The conjunction of these two types of markers provided a very satisfactory approach to the

problems raised. However, for *Beryx spp.*, no microsatellite loci were developed in this study. The screening and amplification of microsatellite loci in these species has never been implemented and it would be important, especially in the case of *B.decadactylus*, as regional genetic structuring of populations is likely and may be revealed by these more sensitive markers as in *Helicolenus dactylopterus*.

The sampling areas utilized in this work are not completely representative of the overall geographic distribution of these species. It would be interesting to sample different areas, such as the Mediterranean sea, as several studies have already pointed to a separation between Atlantic and Mediterranean populations. Some samples for *Helicolenus dactylopterus* have already been collected from off the coast of Italy (courtesy of the IAMC-CNR Italy) and mtDNA sequencing and microsatellite analysis will be preformed on these very soon.

The highly exploited areas of the North and Norway Seas are also interesting places to analyse as studies on other deep-sea demersal species, closely related to those present in these areas, have revealed population structure (e.g. *Sebastes spp.* ; Roques *et al.*, 2001).

Conclusions

In general, this work has brought new insights and a significant contribution to knowledge on three demersal fish species: *Helicolenus dactylopterus*, *Beryx splendens* and *Beryx decadactylus*, in respect of their natural populations and interactions, demographic history and evolution. These questions were approached trough the utilization of DNA-based molecular markers, and the main conclusions are:

1) Mithocondrial cytochrome b and control regions partial sequences provided clear evidence for the existence of strong population differentiation between populations of *Helicolenus dactylopterus* in the North Atlantic Ocean.
2) Isolation of the Cape Verde populations from the rest of the NE Atlantic populations was found for both *Helicolenus dactylopterus* as well as for *Beryx decadactylus*. However, a significant number of common haplotypes between these two NE Atlantic regions suggests that some degree of gene flow exists between populations of these two species within these areas or a common ancestral origin may exist.
3) Microsatellite loci developed specifically for *Helicolenus dactylopterus* in this study revealed a very significant population differentiation at a finer geographic scale, separating the Continental Portugal population from Azores populations. Primers

developed in this study are now available for future genetic studies on this species for understanding of recruitment and larval ecology.

4) Previous conclusions on the panmictic nature of *Beryx splendens* in the Atlantic were strengthened by this work as no population differentiation was found between samples from Cape Verde, Madeira and Azores; contrary to the other two species. However, further analysis is necessary
5) The comparison of two closely related species (from the same genus and with very similar life-history characteristics) brought to light striking differences in their genetic population structure and demographic histories.

All these conclusions stress the fact that it is necessary to increase knowledge on each deep-sea species in particular in order to be able to conserve their genetic variability and apply adequate management proposals in general. It is impossible to perform genetic studies for many species from the demersal community because of time, complexity and monetary resources; however, each study has to be seen as a jigsaw piece that contributes to a gigantic puzzle.

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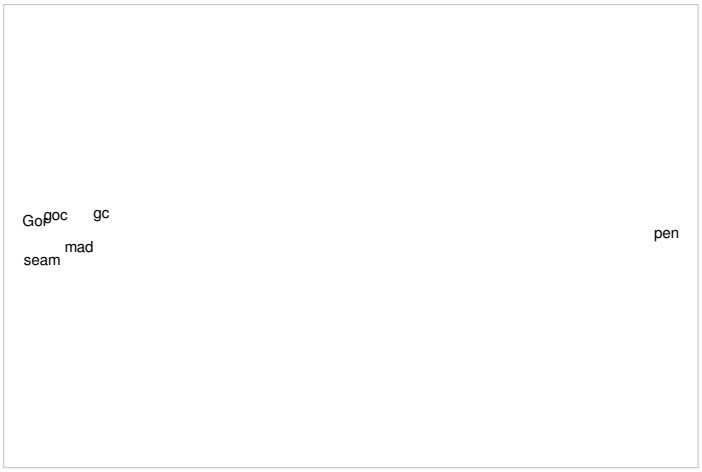
APPENDICE 1

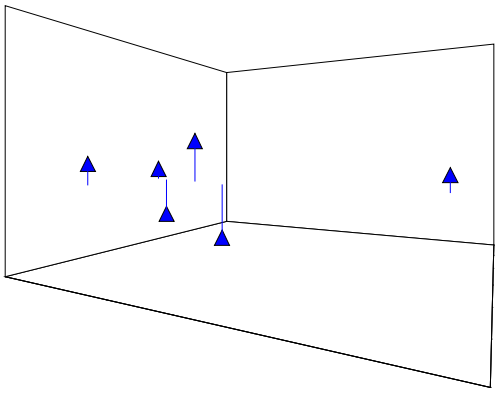
Population analysis of microsatellite excluding loci with probability of null alleles

F_{ST}	Ocidental Group	Oriental Group	Central Group	Seamounts	Madeira	Peniche
OcidentalG	-					
OrientalG	0.0028	-				
CentralG	-0.0026	0.0006	-			
Seamounts	0.0092*	0.0051	0.0004	-		
Madeira	0.00932	0.0007	0.0011	-0.0003	-	
Peniche	0.0201***	0.0235***	0.0255***	0.0390***	0.0182*	-

* = p<0.05 and *** = p<0.0001



2D Multivariate analysis of genetic distances



3D Multivariate analysis of genetic distances