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Molecular Ecology and Evolution of an Acorn Barnacle, *Balanus improvisus* (Darwin)

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ABSTRACT

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MOLECULAR ECOLOGY AND EVOLUTION OF AN ACORN BARNACLE, *Balanus improvisus* (Darwin)

by Sanit Piyapattanakorn

Balanus improvisus is a euryhaline barnacle, but is mostly found in estuaries under low salinity conditions. Populations occur world wide, but its distribution is patchy. In the British Isles, the populations are mostly found at the top reaches of estuaries and abundant populations are more stable than small populations.

The reproductive biology and population dynamics of *B. improvisus* were investigated. This barnacle species prefers to settle close to its conspecifics (0.4-0.8 cm), but there were more isolated individuals of *B. improvisus* (up to 3 cm apart) found compared with *E. modestus* (not more than 1.6 cm apart). This could be the result of competition for space between these two barnacles or a consequence of the ability of *B. improvisus* to self-fertilise, which allows the species to reproduce without the requirement of neighbouring individuals. In southern England, the breeding season of *B. improvisus* was between March and October, and the putative period of planktonic larvae release was from July to August. Settlement of this barnacle occurred between July and September.

The population genetic structure of this barnacle was investigated both at the local- (within the U.K and Baltic) and broader- (between the U.K. and the Baltic) levels using mtDNA PCR-RFLP analysis of the COI-COIII genes. The results of the local-scale study suggest that the populations around the British Isles are isolated and that the level of gene flow between the estuaries is low. In contrast, the level of gene flow between the populations within the Baltic is high. This indicates that the planktonic larvae of this barnacle are capable of dispersing throughout the Baltic. In the broad-scale study, there was no significant genetic differentiation between the British and Baltic populations. This suggests that the level of gene flow between the two regions is high. Combination of the results from both local- and board-scale studies indicated that the populations of this barnacle species in the two regions could be derived from the same gene pool. This migration between the regions could be facilitated by ships. Genetic differentiation found among the British populations could be the result of differences in the founders during colonisation, after which the local larval supply (larval retention) plays an important role in maintaining the genetic identity of the populations.

The evolution and origin of a unique intergenic spacer found in *B. improvisus* were investigated. The results suggest that this spacer is not passed on the evolutionary lineage of Thoracica, but that it is restricted to a particular evolutionary lineage of a few barnacles in the *B. amphitrite* group (*B. improvisus* and *B. eburneus* so far). The phylogenetic analysis in this study showed that the insertion of this spacer could have occurred after the separation of *B. improvisus* and *B. eburneus* from the other Balaninae. With the evidence obtained so far, the origin of this spacer remains unclear.

The mating strategies of *B. improvisus* were examined using microsatellite analysis. The results showed that *B. improvisus* has the ability to self-fertilise and that self-fertilisation occurred in the individuals with only 2.0cm separation. Cross-fertilisation was found between individuals 0.8cm apart. Therefore, it is likely that self-fertilisation occurs only when the cross-fertilisation cannot take place. There was no evidence for multiple-fertilisation and sperm transfer via the water column.

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Abbreviations

AFLP	amplified fragment length polymorphism
AMOVA	analysis of molecular variance
APS	amonium persulfate
asp	aspartate
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
bp	base pair
BSA	bovine serum albumin
COI	cytochrome oxidase subunit I
COII	cytochrome oxidase subunit II
COIII	cytochrome oxidase subunit III
CTAB	hexadecyl trimethylamonium bromide
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
ddATP	dideoxyadenosine triphosphate
ddCTP	dideoxycytidine triphosphate
ddGTP	dideoxyguanosine triphosphate
ddTTP	dideoxythymidine triphosphate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleoside triphosphates
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
Fam.	family
g/l	gram per litre
IAA	isoamyl alcohol
IPTG	isopropyl B-D-thiogalactopyranoside
kb	kilobase
leu	leucine
lys	lysine
met	methionine
mg	milligram
$MgCl_2$	magnesium chloride
ml	millilitre
mRNA	messenger RNA
MSN	minimum spanning network
mtDNA	mitochondrial DNA
mya	million year ago
NaCl	sodium chloride
NADH	nicotinamide adenine dinucleotide hydride
NaOH	sodium hydroxide

nDNA	nuclear DNA
ng	nanogram
OD	optical density
PCR	polymerase chain reaction
PIMA	PCR-based isolation of microsatellite arrays
pmol	picomole
RAHM	random amplified hybridisation microsatellites
RAPD	random amplified polymorphic DNA
REAP	the restriction enzyme analysis package
RFLP	restriction fragment length polymorphisms
RNA	ribonucleic acid
rRNA	ribosomal RNA
SDS	sodium dodecyl sulfate
SSC buffer	sodium chloride, and trisodium citrate buffer
SSCP	single-stranded conformation polymorphism
SSPE buffer	sodium chloride, sodium phosphate, and EDTA buffer
SSRs	simple sequence repeats
Subfam	subfamily
Subor.	suborder
Superfam.	superfamily
TBE buffer	tris-HCl, boric acid, and EDTA buffer
TE buffer	tris-HCl and EDTA buffer
TEMED	N, N, N', N' – Tetramethylethylenediamine
TEN buffer	tris-HCl, EDTA, and sodium chloride buffer
Tris-HCl	tris[hydroxymethyl]-aminomethanehydrochloride
tRNA	transfer RNA
v/v	volume per volume
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-B-galactoside
μg	microgram
μl	microlitre
μM	micromolar

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Chapter One

General Introduction

Barnacles are crustaceans, in spite of the presence of a calcareous shell as in molluscs (Thompson, 1830; Darwin, 1854). They belong to the subclass cirripedia, which consists of four orders, namely Ascothoracica, Rhizocephala, Acrothoracica, and Thoracica (Newman and Ross 1976). The Thoracica is the largest and most diverse group in this subclass consisting of 150 genera and 700 species (Spears *et al.*, 1994). The Thoracica can be divided into two main groups, pedunculate or stalked barnacles (Lepadomorpha) and sessile barnacles (Sessilia). The latter consist of the Brachylepadomorpha (mostly extinct), Verrucomorpha (asymmetrical sessile barnacles), and Balanomorpha (symmetrical sessile barnacles). Sessile barnacles are common organisms found in the coastal zone including the shore and also in deep water where suitable substrata are present. In contrast, stalked barnacles mostly attach to floating objects, such as drift-wood, and are mostly found immersed in the sea; this could be the result of sensitivity to dehydration in these barnacle species. A few stalked barnacle species do occur in the intertidal zone, but only in areas of extreme wave action (e.g. *Pollicipes pollicipes*, Rainbow, 1984).

1.1 Life cycle of barnacles

As in most marine sessile organisms, the life cycle of barnacles consists of three main stages: namely sessile adults, planktonic larvae (nauplii), and a settlement stage (cyprids). Adult barnacles carry fertilised eggs in their mantle cavity. The developing eggs use yolk as a source of energy for their development, and then hatch as nauplius

larval stage I, which are then released into the water column. The larvae then spend several weeks in the water column. Nauplius stage I is a non-feeding larva that survives by using the yolk reserve as a source of energy for a few days before developing to nauplius stage II (Rainbow and Walker, 1977). The nauplii from stage II to VI feed on plankton in the water column and accumulate lipid (for energy) which is later used during the non-feeding cyprid larval stage and also during metamorphosis, from which the juvenile barnacles emerge (Rainbow and Walker, 1976). Cyprid larvae play an important role in finding suitable substrata to settle upon. Generally, they prefer to settle on a roughened surface rather than a smooth one, since rough surface substrata enable more adhesion than smooth surfaces (Crisp and Barnes, 1954). They can also recognise the area where their con-specific adults are present using a chemotactile response (Crisp and Meadow, 1962; Gabbott and Larman, 1971). The chemotactile response varies from species to species (Crisp, 1974, Larman and Gabbott, 1975). Therefore, the con-specific gregarious behaviour of barnacles could be enhanced by that chemotactile response. Furthermore, settling near to con-specific species can also increase the chances of successful cross-fertilisation.

1.2 The distribution of common sessile barnacles in the north-western European region

There are several publications which focus on the distribution of barnacles and other marine organisms (e.g. see Southward and Crisp, 1956; Crisp and Southward, 1958; Crisp *et al.*, 1981; Southward *et al.* 1995). In this section, the distribution and habitat of common sessile barnacles found in north-western Europe, namely *Semibalanus balanoides* (L.), *Elminius modestus* (Darwin) *Balanus crenatus* (Bruguière), *Chthamalus stellatus* (Poli), *C. montagui* (Southward), and *Balanus improvisus* (Darwin), are described.

The common acorn barnacle, *S. balanoides* can be found on nearly all the coasts of the British Isles (Bassindale, 1964; Southward and Crisp, 1956) together with *C. stellatus* and *C. montagui*; the latter especially along the west coast of Britain (Crisp *et al.*, 1981). However, *S. balanoides* is dominant on the eastern and northern coast of the British Isles (Southward and Crisp, 1954). Being a "North Atlantic Boreal Species" (cool-water species), it spreads down from the Arctic to the southern European limit on the northern coast of France (Bassindale, 1964) and Spain (Southward *et al.*, 1995). *S. balanoides* is an intertidal barnacle of western Europe and Atlantic North America (Stubbing, 1975; Flowerdew, 1983). The habitat of this species is on exposed places found around the mid-tide level (Bassindale, 1964). On the southern coast of Britain, *S. balanoides* occurs together with *C. stellatus* and *C. montagui* (Crisp and Southward, 1958; Southward and Crisp, 1956; Bassindale, 1964; Crisp *et al.*, 1981). Usually, *S. balanoides* occupies the area below the habitats of *C. stellatus* and *C. montagui*, from high water neap tide (H.W.N.T.) to above mean tide level (M.T.L.) (Bassindale, 1964).

Elminius modestus (Darwin) has been classified as an immigrant species opportunistically travelling from Australia or New Zealand to England (Europe) on ships (Bishop, 1947; Bassindale, 1964). It was first discovered in Chichester Harbour (Bishop, 1947) and has since spread throughout the English Channel and the North coast of France (Crisp, 1958; Bishop and Crisp, 1958). On the eastern side of the English Channel, this species is abundant, while on the western side, it was initially only found in estuaries and harbours (Crisp and Southward, 1958). Since then it has spread to all rocky shores, although it is rarely found in exposed conditions (Rainbow, 1984). In the 1950s, it was generally accepted that *E. modestus* (Darwin) had its northern limit in south-western Scotland and its southern limit in France (Crisp and Southward, 1958; Southward and Crisp, 1954; Crisp and Southward, 1959). However, Hiscock and Baker (1978) have found that the northern limit of this species has extended further to Shetland, approximately 450 km North from the previous record.

Elminius modestus can be found from the top of the tidal zone to the sub-littoral zone but it is most abundant in the low intertidal and shallow subtidal zones. This species prefers sheltered places and can tolerate muddy and brackish conditions (Bassindale, 1964).

The two species of *Chthamalus* (*C. stellatus and C. montagui*) have been recognised on European western coasts from morphological (Southward, 1976) and enzyme electrophoresis studies (Dando *et al.,* 1979). Before these investigations, they were recorded collectively as *C. stellatus* (Poli) (e.g. Darwin). The distribution of these species after separation confirmed in 1976, is described as follows. The distribution of these two species is generally overlapping along the western coasts of the British Isles, France, Spain and North Africa. They also occur in the Mediterranean and Black Sea (Crisp *et al.*, 1981; Pannacciulli *et al.*, 1997). Along the eastern coast of the British Isles, they occur only on the north-eastern side of Scotland. In the Mediterranean Sea, *C. stellatus* is abundant in wave-beaten areas where there is a small number of *C. montagui* (Crisp *et al.,* 1981). In terms of vertical distribution, *C. montagui* generally occupies the area above the upper limit of *C. stellatus*, mean high water of spring tide (M.H.W.S.) to mean high water of neap tide (M.H.W.N.). Crisp *et al.* (1981) suggested that the vertical distribution of *C. stellatus* in shaded areas has an upper limit higher than in sun-exposed areas such on Bardsey, North Wales.

Balanus crenatus is the common sublittoral species and is usually found on small stones and the shells of molluscs and crustaceans (Bassindale, 1964; Crisp and Southward, 1958). The northern and southern European limits of this species are the Arctic and the west coast of France, respectively, and it can be found around the British Isles (Crisp and Southward 1958, Hayward *et al.,* 1996). In the English Channel, it can be found along the English Channel and grows in estuaries and harbours better than

on the exposed coasts in the western part of the English Channel (Crisp and Southward, 1958).

Balanus improvisus is found from North Europe (Baltic, Sweden) to France and it is the only kind of barnacle found on the coast of Finland (Bassindale, 1964). It is a euryhaline species but usually prefers low salinity condition and can be found mostly in estuaries and brackish water. Crisp and Southward (1958) observed that in the English Channel, *B. improvisus* is abundant in estuaries, from Helford and L`Aber Beniot in the west to Rother and Boulogne in the east. Commonly, the distribution of this species is patchy or non-continuous, but in the Danish Belt and on the western coast of Sweden and in the rest of the Baltic, the distribution is continuous (Barnes and Barnes, 1962; Furman 1989a). The habitat of this species is generally from low on the shore down to the sublittoral zone (Hayward *et al.,* 1996) (for more details of its distribution see also section 2.1.2)

1.3 Why study *B. improvisus*?

B. improvisus was chosen for this study because the populations are patchily distributed and occur only in estuaries around the British Isles (Furman *et al.*, 1989 (see also section 2.1.2)), unlike most other barnacle distributions, which are continuous. In Britain, its populations are also not found along the coastline between the two estuaries where the populations occur. The main interest of this study is how they can maintain their patchy populations, which mainly involves studying the sources of larval supply.

It is an arduous task to determine the sources of the planktonic larvae of marine benthic organisms because the local populations of adults do not necessarily produce the larvae caught in local water or which sustain the local sedentary population. If the

larvae disperse further from their parents to become the new recruits of other populations, each population may be supplemented by the larvae from many populations. Although the investigation of larval sources is a difficult task, one potential way of carrying out these studies is the use of molecular methods to identify which population produced which larva (finding the natal site of larvae). Alternatively indirect methods may be used to estimate the level of gene exchange (gene flow) between populations.

1.4 Common molecular techniques used in population studies

Molecular methods have been widely used to answer several ecological questions at both the enzyme and DNA level (Ayala and Kiger, 1984; Avise, 1994; Ferraris and Palumbi, 1996). Therefore, these techniques have become powerful tools for many ecologists since they have enabled them to gain information which would otherwise have been difficult to obtain using field experiments alone.

Most molecular methods in ecology are now dependent on the PCR procedure (Polymerase Chain Reaction) (Saiki *et al.*, 1985; Palumbi, 1996a). The basics of the PCR technique involves the use of primers (short oligonucleotides), which may be commercially available, to anneal onto both upstream and downstream sides of the DNA sequence of interest at sites which are complementary to primers. The target DNA or gene is then replicated by thermostable DNA polymerase (*Taq* polymerase). It can then be amplified by a number of cycles. Generally, the PCR condition involves three steps per cycle of reactions, namely denaturation, primer annealing, and primer extension (see Innis *et.al.*, 1990). This technique thus allows researchers to multiply the copy number of the gene of interest. Only a small amount of DNA is required to serve as a DNA template.

Molecular techniques have been rapidly developed for use in ecological studies. Current techniques are usually easy to use and access, saving in time, inexpensive, and provide more information in terms of genetic variation. Therefore, in the following section, the general information of genetic markers mostly used in ecological studies will be described.

1.4.1 Protein techniques

1.4.1.1 Allozymes and Isozymes (Enzyme Polymorphism)

Allozymes are different forms of an enzyme encoded by different alleles at the same gene locus. They differ from isozymes, which are encoded by different loci. Due to differences in size and electric charge, different forms of proteins can be separated by electrophoresis on supporting media in a specific buffer (Ayala and Kiger, 1984). The supporting media can be made from starch, polyacrylamide, or cellulose acetate. This protein based-method has several applications in ecology such as taxonomic, phylogeographic (Avise, 1975; Ayala, 1983), and population studies (Burton, 1983; Ayala and Kiger, 1984). The advantages of this method are that it is inexpensive, fast and reliable. However, the data from this technique cannot always be resolved. For instance, the substitution or deletion of an amino acid may sometimes have no affect on the overall charge, or bring about a significant difference in the size of a polypeptide chain (Avise, 1975) because some amino acids have the same charge (e.g. lysine, arginine and histidine having positive charges and aspartic and glutamic acids having negative charges). Furthermore, substitutions and deletions in a DNA strand giving rise to a particular protein may not be revealed by enzyme electrophoresis. Another limitation is the requirement for living organisms or freshly frozen materials and a large amount of tissue. For this reason, it cannot be used with fossil or museum specimens (Avise, 1975). There are also a small number of polymorphic loci for studying genetic variation by allozyme analysis (Karl and Avise, 1992; Garcia *et al.,* 1994). Therefore,

screening of a large number of different enzymes is required. Finally, there are many reports about selection affecting the outcome with this method. For example selection at the mannose-6-phosphate isomerase (*Mpi*) locus associated with environmental stress was found in *S. balanoides* (Holm and Bourget, 1994; Schmidt *et al.*, 2000) and selection was also seen at the aminopeptidase-1 locus which was associated with salinity as found in *Mytilus edulis* (Theisen, 1978; Koehn *et al.*, 1980). Thus, there can be a problem assuming they are neutral markers.

1.4.2 DNA techniques

In eukaryotic cells, there are two types of DNA: nuclear DNA (nDNA) located in the nucleus and mitochondrial DNA (mtDNA) located in mitochondria (found in the cytoplasm). In the following sections, the use of nDNA and mtDNA as genetic tools in ecological studies will be discussed.

1.4.2.1. Nuclear DNA

A. The use of variable number of tandem repeat sequences (VNTR)

There are two types of these repeat sequences, minisatellites having repeat lengths of 7-65 bp (Jefferey *et al.*, 1985), and microsatellites or simple sequence repeats (SSRs) having 1-6 bp repeat lengths. Minisatellite can be accessed in two ways: multi-locus DNA fingerprinting and single-locus DNA fingerprinting. Multi-locus DNA fingerprinting is the utilisation of a minisatellite as a probe to hybridise with digested DNA transferred onto a nylon or nitrocellulose membrane (for more technical details see Jeffreys *et al.*, 1985, Bruford *et al.*, 1998). Single-locus DNA fingerprinting is very similar to multi-locus DNA fingerprinting in its protocol details. The difference is the use of a single minisatellite locus (see Bruford *et al.*, 1998 and Verheyen *et al.*, 1994). The genetic information from single-locus DNA fingerprinting arises from the size variants at a single Mendelian locus. Therefore, it is simpler to score than the information from a

multi-locus one. However, it requires a lot of effort and is time consuming to obtain useful single-locus probes (Armour *et al.* 1990; Bruford *et al.*, 1998).

The microsatellite approach uses the PCR technique to allow the amplification of a specific-locus containing a simple sequence repeat, which is mostly less than 100 bp in length. Dinucleotide repeats, such as (CA)n, (GA)n and (AT)n, have been used widely because they occur frequently along the genome. However, tri- and tetranucleotide repeats have also been utilised (e.g. (GGT)n, (AAT)n, and (GATA)n). Based on the basic PCR technique, the protocol for the microsatellite marker method is similar to a normal PCR reaction, but radio-labelled nucleotides or fluorescent 5'end-labelled PCR primers are used instead of normal nucleotides and PCR primers. The PCR product is then denatured and subsequently run on denaturing polyacrylamide gels. Genetic information results from the variation in the number of repeats in the repetitive sequences (Polymorphisms), which is visualised by autoradiography or by using an automatic sequencer employing the fluorescent labelled primers (Strassmann *et al.,* 1996; David and Menotti-Raymond, 1998). The advantages of this genetic method are that a small amount of DNA is required, and the genetic data is easy to manage and score. The disadvantages are that it is difficult and time consuming to isolate and characterise the microsatellite markers, which involves genomic library construction following the screening of bacterial colonies containing repeated sequences. Developing microsatellite markers can now be achieved using an enrichment protocol (Amour *et al.,* 1994; Fleischer and Loew, 1996). This can enhance the chances of obtaining positive bacterial colonies. This marker can also be developed using RAPD, (Random Amplified Polymorphism DNA, (see section 1.4.2.2)) together with Southern-blotting and DNA hybridisation techniques called 'random amplified hybridisation microsatellites' (RAHM) (Cifarelli *et al.,* 1995).

B. Random amplified Polymorphic DNA (RAPD)

RAPD methods have been developed from DNA fingerprinting which requires high molecular weight genomic DNA, more experience in molecular techniques, and is expensive (Weatherhead and Montgomerie, 1991). The reasons why RAPD has been developed are to simplify the technique and reduce costs. RAPD can eliminate the problems encountered with other techniques such as the requirement for large quantities of DNA in the restriction fragment length polymorphism (RFLP) methods and for the DNA sequence information necessary for other PCR (polymerase chain reaction) based methods (Hadrys *et al.,* 1992). The principle of the RAPD technique is that it is PCR-based, uses arbitrary primers (usually 10 bases long), and requires non-degraded and pure DNA. The RAPD profile can be visualised by gel (agarose) electrophoresis with ethidium bromide staining (William *et al.,* 1990).

There are various applications of RAPD in ecology (see Hadrys *et al.,* 1992). These are involved in taxonomic studies (Wilding *et al.*, 1998; Marillia and Scoles, 1996), kinship analysis (Welsh *et al.*, 1991), intraspecific gene flow (Bielawski and Pumo, 1997; Schierenbeck *et al.* 1997) and the design of specific probes for use in RFLP or microsatellite methods. For example, Garcia and co-workers (1994,1996) used the RAPD technique to investigate genetic variation and to detect microsatellite probes in the marine shrimp, *Penaeus vannamei.* The same method was used to design microsatellite markers in *Daphnia* (Ender *et al.,* 1996). However, RAPD also has many limitations such as the size of the primers and co-migration of similar size fragments. The main disadvantage of this technique is the sensitivity of the PCR reaction, which makes this technique unreliable and over sensitive to variations of DNA concentration in the samples. This means that polymorphism may also be caused by some uninterested factors in the PCR reaction, 'Artifactual Variation' (Ellsworth *et al.,* 1993 and Halldén *et al.,* 1996). Although the analytical information gained from RAPD is less effective and reliable than that obtained from methods using a single locus probe, it can

resolve certain questions and can potentially provide information for the next analytical step.

C. Amplified fragment length polymorphisms (AFLP) *(Vos et al., 1995)*

AFLP is a novel DNA fingerprinting technique involving three main steps in its protocol. Firstly, total genomic DNAs are digested and ligated to oligonucleotide adapters. The restriction enzymes used in the digestion consist of one rare and one frequent cutting enzyme. Secondly, the restriction fragments are selectively amplified based on the PCR technique. Selective amplification is carried out by the use of primers that extend into the restriction fragments. Therefore, only the fragments that the primers can extend, are amplified. Finally, analysis of the amplification fragments is then carried out by running the amplification products on a denaturing polyacrylamide gel and visualised by autoradiograph. The advantage of this technique is that DNA sequence information of the organism under study is not required and polymorphisms are high (Travis *et al.*, 1996). The disadvantage is that a fairly large amount of genomic DNA (~0.5 μg) is essential for the first step. It becomes clear that this technique is not suitable for small organisms.

D. Single-stranded conformation polymorphism (SSCP) *(Dean and Milligan, 1998)*

This is a PCR-based technique. The PCR product is denatured and run on non-denaturing gel. Genetic variations are detected by the differences in folding of single-stranded DNA molecules, which cause the differences in mobility of the DNA fragments. This technique is simple, and fast to use to screen large number of samples. The disadvantage is that differences in mobility of the DNA fragments arises when slightly different gel conditions are used, but this problem can be overcome by including control samples onto every gel to facilitate the comparison between gels.

1.4.2.2 Animal mitochondrial DNA (mtDNA)

Animal mitochondrial DNA has a closed-circular shape, except for a linear form in *Hydra* (Warrior and Gall, 1985) and *Paramecium* (Prichard *et al.*, 1990). Its gene contents and arrangement are highly conserved. However, gene rearrangement may occur between phyla (Staton *et al.,* 1997). Normally, an animal mtDNA consists of two rRNA genes, a large (16S rRNA) and small (12S rRNA) rRNA, twenty-two tRNA units, thirteen mRNA units coding for proteins (cytochrome b, three subunits of cytochrome oxidase, three subunits of ATP sythetase and six subunits of NADH dehydrogenase), and a control region called a D-loop in vertebrates or A-T rich in *Drosophila* (Harrison, 1989). Generally, the size of animal mtDNA is about 16,000-19,000 bp. However, in some species it can be variable. Thus, scallops show intraspecific size variation of the mitochondrial genome and the largest can be up to 39.3 kb (Gjetvaj *et al.,* 1992).

Most of the sequence in the mitochondrial genome consists of coding regions, except for the D-loop or control region, which is the only substantial non-coding region in the genome. This has been reported as the origin of replication of animal mtDNA (Wilson *et al.*, 1985). The evolutionary rate or genetic variation of mtDNA varies in different genes. Transfer and ribosomal RNA are more conserved than the others because of their functional and structural constraints (Simon *et al.,* 1994). Protein-coding genes are more variable than tRNA and rRNA genes because they are less constrained in function (silent or synonymous codon) and structure than tRNA and rRNA genes. The most variable region is the control region, because it is the only non-coding region in the whole mitochondrial genome. Point mutations and small insertions and deletions occur throughout the genome. Large insertions and deletions mostly occur in the control region, although there is some evidence that large insertions and deletions can also occur in other regions. For example, in the honeybee (*Apis mellifera*) mtDNA, a large insertion has been found between COI and COII genes (Hall and Smith, 1991 (see also section 5.1)). Mutation rates are greater in mtDNA than in nuclear DNA,

probably because of the generation of free oxygen radicals in mitochondria. Rates are specially high in the control region.

Animal mtDNA has been increasingly used as a molecular tool for population and evolutionary biology because it is a small molecule (about 1.6-1.9 kb) which is easy to access, has a rapid rate of nucleotide divergence, and arises from maternal inheritance (no recombination) (Wilson *et al.*, 1985). However, there are some disadvantages to the use of animal mtDNA such as heteroplasmy (the phenomenon of two or more mtDNA haplotypes occurring in an individual), (Monnerot, 1984, Bermingham *et al.*, 1986), paternal inheritance (Gyllensten *et al.*, 1991, Hoeh *et al.*, 1991, Zouros *et al.*, 1992; Skibinski *et al.*, 1999), and nuclear insertion (De-Xing Zhang and Whewitt 1996). Although, these phenomena have been discovered in only a few species of animals, it is necessary to take them into account in all species.

Two main methods employed with animal mitochondrial DNA are the use of restriction enzymes and sequencing of specific genes or regions. The restriction enzymes method is also called restriction fragment length polymorphism. This method involves the use of a restriction endonuclease, which recognise 4-6 bases cutting sites. Polymorphisms can be detected by the differences in length of mtDNA fragments caused by insertions or deletions and the gain or loss of cutting sites brought about base substitution in the recognition sites. The sequencing of specific genes can reveal a high resolution of mutation for population and evolutionary studies, but it is expensive, requires laborious work and is time consuming. Recently, automatic sequencers have become available to help to speed up the process, but the expense is still high and experience is also required.

1.5 Points to be aware of when using genetic markers in ecological studies

When using molecular tools in ecological studies, the background biological and ecological knowledge of the organism under study is required in order to be able to fully interpret the results obtained from genetic data, and also to select suitable techniques to be used in the study. For example, in animals where males migrate over a greater distance than females, animal mtDNA might not show any gene flow between distantly-related populations since transfer of this marker is restricted to maternal inheritance. In this situation, nuclear DNA markers could be suitable for the study because of their recombinant properties.

The level of polymorphism in the marker is also an important aspect. In paternity studies, to identify individuals at the con-specific level, highly variable genetic markers are required. Certainly animal mtDNA markers cannot be used in this type of study because they generally show only maternal genetic material. RAPD, AFLP, multi-locus DNA fingerprinting and microsatellite markers are commonly used to carry out paternal studies since they can provide high levels of polymorphisms. The disadvantage of AFLP and multi-locus DNA fingerprinting is the requirement of large amount of genomic DNA. Therefore, these techniques cannot be used in very small organisms or their small larvae. RAPD markers also have problems with reliability. The most versatile and frequently used marker for paternity studies is the use of microsatellite loci because of their high variability, reliability, and specificity. Moreover, only a small amount of DNA is required because this technique is based on PCR methodology.

In population genetic studies, if highly polymorphic markers are used, a larger sample size might be needed to detect the genetic structure of the populations under study. However, in practice, it is difficult to identify this type of problem, unless preliminary experiments have been carried out.

The availability of genetic information (DNA sequences) can facilitate the process of genetic marker development for a particular species. Marker development is very time consuming, especially for microsatellite loci, which involve complicated protocols. It is a lot easier to use established microsatellite markers than to develop novel ones. In contrast, animal mtDNA markers are much easier to access because of the availability of universal PCR primers, mitochondrial genome sequences of various organisms (invertebrates and vertebrates), and the use of degenerate PCR primers (Simon *et al.*, 1994, Palumbi, 1996a). There are also universal PCR primers available for nuclear DNA markers (see the review in Loxdale and Lushai, 1998). This shows that bioinformatic knowledge is essential and can be used to obtain invaluable information for a study.

In conclusion, there are various factors (e.g. simplicity, investment of time, cost, ease of obtaining and managing genetic data) which have to be considered in order to obtain the ideal genetic markers for a particular ecological study. Once found, it is sometimes worth conducting preliminary investigation of the markers with a small number of samples before screening all samples. In practice, it is perhaps impossible to obtain ideal markers, but those obtained by simple techniques, providing enough genetic variation, and fitting the budget, should ultimately be the most practical to use.

1.6 The Objectives and structure of this study

This study has been divided into two sections, 1) background ecological studies and 2) molecular ecological and evolutionary studies on *B. improvisus*. In the ecological section (Chapter two), the species' reproduction and population dynamics are examined. More specifically, the occurrence of the populations around the British Isles, the nearest distance within and between species, the recruitment and settlement season, and male and female reproductive activities of the major population under study in Southampton Water (Cobden Bridge, River Itchen, Southampton) were

investigated. This information could help to increase understanding of the population genetics of this barnacle.

The population genetics section involves the use of molecular methods to examine the recruitment process and mating strategies of *B. improvisus*. Firstly, the development of genetic markers *for B. improvisus* (Chapter three) was necessary since there was no genetic marker (DNA marker) available for *B. improvisus*, when this study was started. Chapter three consisted of the development of genetic markers and laboratory protocols for use in the population genetics and mating strategy studies. The markers identified include both mtDNA and nuclear DNA markers.

In Chapter four, local and broad-scale population genetics of *B. improvisus* were studied. The aim of this work is to investigate the degree of gene exchange between populations, and compare the results from populations around the British Isles, which are isolated and non-continuous, with populations in the Baltic, which are relatively continuous. Gene flow between populations should provide useful information on dispersal patterns of planktonic larvae or on sources of larval supplies. Genetic variation within and between populations was examined. A mtDNA marker and the RFLP technique developed in Chapter three were employed to detect genetic variation of *B. improvisus*.

Chapter five is dedicated to the study of the general properties and characteristics of the unique spacer found during the development of genetic markers for *B. improvisus* (Chapter three). The evolutionary significance of this spacer was also investigated using phylogenetic analysis.

In Chapter six, since the isolated individuals of *B. improvisus* have been found to possess self-fertilisation behaviour, the reproductive strategy of this barnacle species

was investigated, using genetic markers (microsatellites), to determine whether individuals use self-fertilisation behaviour or not. As far as cross-fertilisation of communal individuals is concerned, the occurrence of multiple or single fertilisation events has also been examined.

In Chapter seven, the results obtained from the previous chapters have been summarised and integrated, and the limitations of this study and future work were discussed.

Section One

The study of basic ecology and reproductive biology

of *Balanus improvisus*

Chapter Two

Background ecological studies on *Balanus improvisus*

2.1 Introduction

The objective of this section is to consider some of the ecological background of *B. improvisus*, which can be used to support genetic studies of its populations (see chapter 4). Relatively little is known about the natural history and ecology of this barnacle species, especially at upper estuarine sites. The main emphasis of this Chapter was on the distribution and temporal occurrence of the populations around the British Isles (cf Furman, 1989a.), reproductive biology and recruitment dynamics, which were also important because of their direct relevance to the population genetics study.

2.1.1 The identification of *B. improvisus*

In British estuaries, the most commonly found barnacles are *Elminius modestus*, *Semibalanus balanoides*, *Balanus crenatus* and *B. improvisus*. *Elminius modestus* is distinct from the others, because it has four wall plates (the others have six wall plates). As in *Chthamalus spp.*, *S. balanoides* has six wall plates, but its anterior wall (rostrum) is much wider than its operculum. In contrast, in *Chthamalus spp.*, the rostrum is only slightly wider than its operculum (see fig. 2.1). These characteristics can be used for separating these two types of barnacles: Balanoidea and Chthamaloidea (see section 5.1). *Semibalanus balanoides* is also easily distinguished from other Balanidae, since it is the only species having a membranous base out of the Balanidae. The most difficult distinction to make is between *B. crenatus* and *B. improvisus*. These two barnacles are

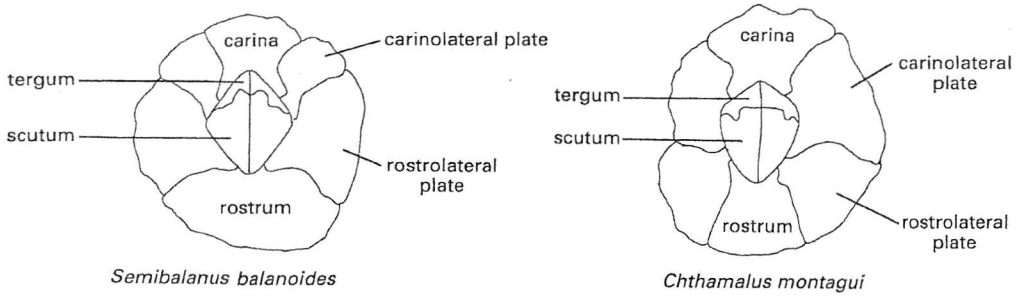
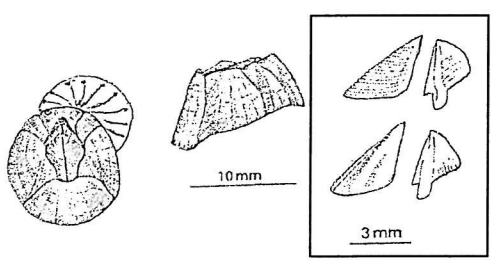


Figure 2.1 The external morphology of Balanoidea (*S. balanoides*) and Chthamaloidea (*C. montagui*) (Source from Hayward *et al.*, 1996)

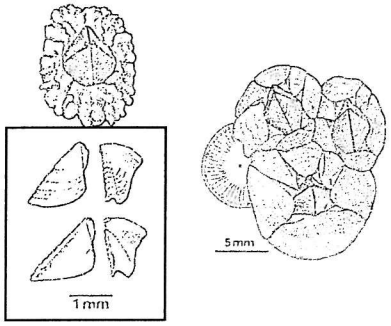
very similar in external morphology. In *B. crenatus*, however, the operculum has a more rounded shape and the carina is slightly concave towards the tip, in contrast to *B. improvisus*. These criteria can thus be used to differentiate these barnacles (see fig. 2.2), although experience is required. The patterns of lines found on their calcareous bases and the shape of the spur on the tergum can also be used to distinguish the two species (see fig. 2.2). However, in order to visualise these characteristics, organisms have to be destroyed.

2.1.2 The distribution of *B. improvisus*

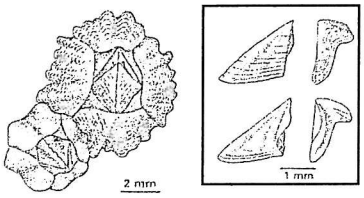
B. improvisus is a temperate euryhaline barnacle, mostly found in the sublittoral zone (Hayward *et al.*, 1996). Populations of this barnacle are widely distributed around the world (Darwin, 1854, Henry and Mclaughlin, 1975; Newman and Ross, 1976). This barnacle can be found on both east and west Atlantic coastlines. On the west Atlantic coast, the populations, found from Nova Scotia to Argentina, were reported by Darwin in 1854, whilst along the east coast of America, populations have been found from Maine to Florida (Bousfield, 1955), and also extend to Mexico, Puerto Rico, and Brazil (Henry and Mclaughlin, 1975). On the eastern side of the Atlantic, the species occurs from the west coast of Sweden down to Africa. Its distribution extends into the Baltic (Gislén, 1950; Luther, 1950 cited in Furman, 1989a), Mediterranean, Black sea, Caspian Sea (Barnes and Barnes, 1961 and 1966), and Red Sea (Newman and Ross, 1976). This species has also been found at the Cape of Good Hope on the west coast of South Africa (Newman and Ross, 1976) and in Zaire (Bishop, 1951). The distributions of populations are, however discontinuous and mostly found in estuaries, brackish water lagoons, and harbours (Barnes and Barnes, 1961). Populations of *B. improvisus* in the Pacific have also been identified from the north-west American coastline down to Ecuador or Peru (Carlton and Zullo, 1969; Newman and Ross 1976;



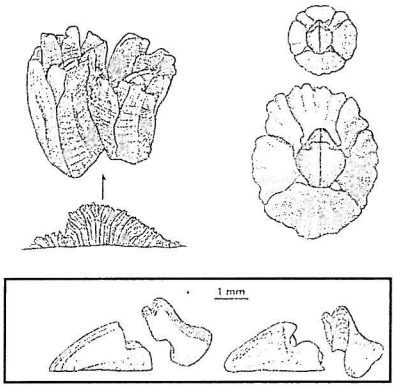
Balanus improvisus



Balanus crenatus



Elminius modestus



Semibalanus balanoides

Figure 2.2 Shell and opercular [tergum and scutum (in box)] morphologies of common barnacles in British estuaries (Source from Hayward *et al.*, 1996)

Henry and Mclaughlin, 1975). The species has also been found in Japan (Utinomi, 1970), New Zealand (Foster and Willan, 1979), and Australia (Newman and Ross, 1976; Bishop, 1951).

Turning to north-west Europe, *B. improvisus* is the most common barnacle species found in the Baltic. The first record of the species in this region was in 1844 (Gislén, 1950, cited in Furman, 1989a) and its distribution spread rapidly to the Gulf of Finland and to the Gulf of Bothnia. The most recent study has found that the distribution has extended to the north of the Gulf of Bothnia (Vaasa and Umeå) and to the east of the Gulf of Finland (Virolahti) (Furman, 1989a). The distribution of *B. improvisus* populations in big estuaries (i.e. around the Baltic) appears to be continuous within the estuaries (Furman, 1989a).

Populations of *B. improvisus* are also found around the British Isles, mainly in or the upper reaches of estuaries where a wide range of salinity fluctuations occur. It was the most common barnacle in Kentish oysterbanks (Darwin, 1854) and was later reported to be a common barnacle in the British estuaries, the River Thames (Andrews *et al.*, 1982), the River Tamar (Percival, 1929), the Conwy estuary, (Davenport, 1976), and the Milford Haven estuary (Crothers, 1966). The latest survey of the British populations was carried out by E. Furman in 1986-1989 (summarised in table 2.1 and fig. 2.3).

2.1.3 Mating system in barnacles

Barnacles are hermaphroditic species. During the breeding season, individuals acting as males copulate adjacent individuals acting as females by the extension of the penis to fertilise egg masses in the mantle cavity of the neighbouring females. In

Table 2.1 Record of *B. improvisus* populations around the British Isles (Furman, 1989a)

Number	Sites	Record (Furman, 1989a)
1	River Clyde, Clyde Bridge, Dumbarton	Absent (03/09/1986)
2	Solway Firth, Bowness, Silloth, Beaumont	Absent (07/09/1986)
3	River Ribble, Preston Docks	Large dead individuals found (10/02/1985)
4	River Mersey, Garston Rocks	Dead individuals found (15/05/1988)
5	River Dee, Connahs Quay River Dee, Queensferry Bridge	Abundant (21/11/1987) Sparse (21/11/1987)
6	River Clwyd, Rhyl	Absent (17/05/1988)
7	Conwy estuary	Sparse, individuals isolated from each other (1985-1988)
8	Caernarfon	Absent (16/05/1988)
9	River Mawddach, Barmouth	Absent (21/08/1986)
10	Milford Haven, Black Tar Point	Only a few individuals found Several empty shells (21/03/1986)
11	River Tamar	Absent (12/03/1986)
12	River Dart, Totnes	Some dead individuals found (11/03/1986)
13	The Thames, Greenhithe	Present and abundant (1985-1986) The population decreased dramatically during the winter
14	River Humber, New Holland	Very abundant (07/08/1986 and 29/08/1988)
15	Firth of Forth, Kincardine Bridge	Present and abundant (06/09/1986)
16	River Eden, Guardbridge, Coble shore	Absent (04/09/1986)
17	Firth of Tay, Newburgh, Balmerino, Newport	Absent (05/09/1986)

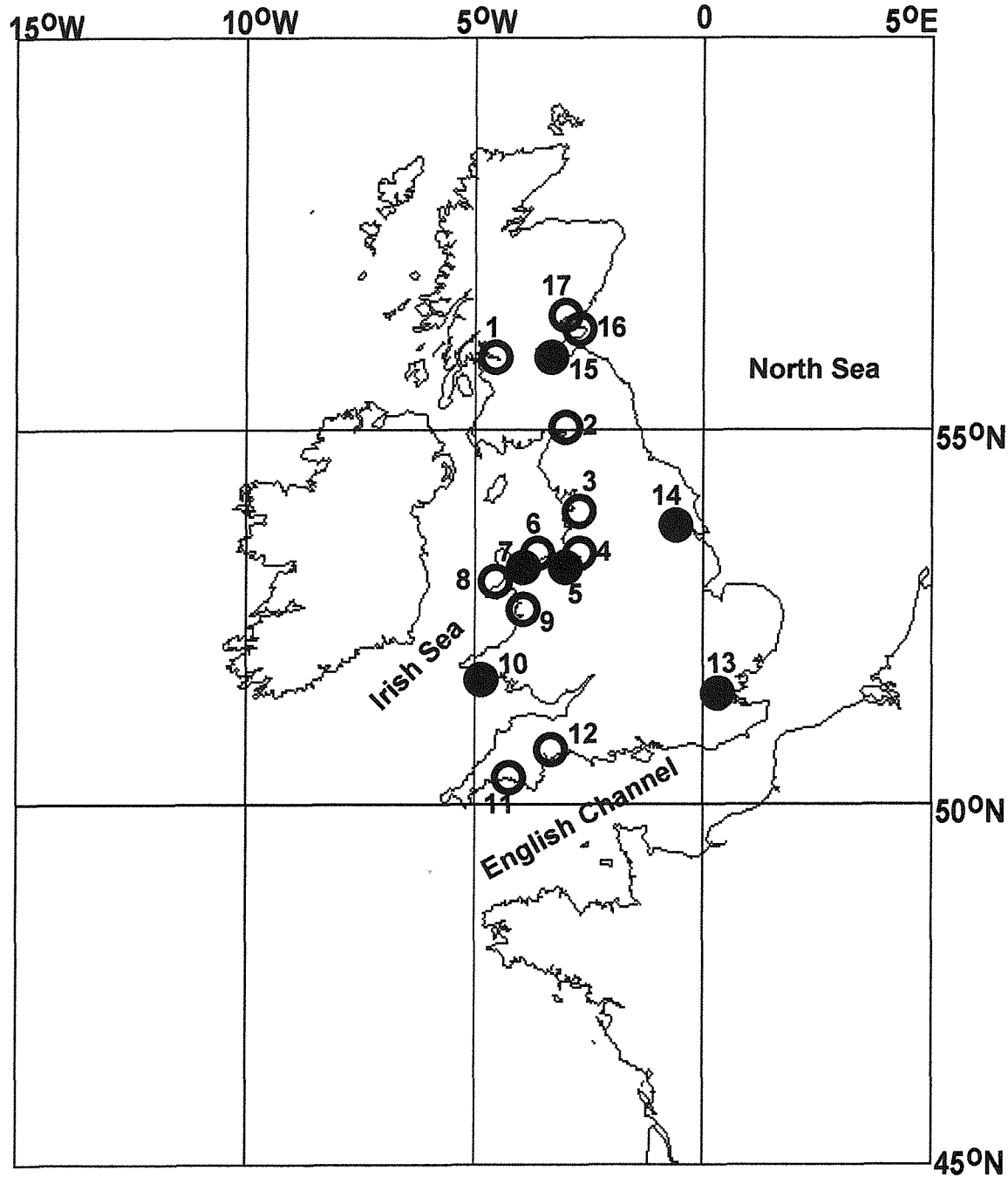


Figure 2.3 The presence of *B. improvisus* population around the British Isles using data acquired by Furman 1989a (The map numbering corresponds to that using in table 2.1) (● Present ○ Absent)

S. balanoides, males lose their penis after the fertilisation and it then gradually regenerates to maturity in preparation for the next breeding season (Stubbings, 1975). This phenomenon has not been reported in other species of barnacles.

On present evidence most barnacles appear to be obligate cross-fertilising species; examples include *S. balanoides*, *B. crenatus*, *E. modestus*, and *B. balanus* (Barnes and Crisp, 1956). However, there are some reports of self-fertilisation behaviour in isolated individuals of *B. perforatus*, *Chthamalus spp. Verruca stroemia* and *B. improvisus* (Barnes and Crisp, 1956; Furman and Yule, 1990). After fertilisation, eggs develop inside the mantle cavity until they reach the "*nauplii stage I*". This development process is termed "*brooding*". Once stage I has been reached, the lavae are released into the water column, at which point they are called planktonic larvae.

The number of broods produced per year varies in different species of barnacles. *S. balanoides* has only one brood per year (Barnes and Barnes, 1968; Stubbing, 1975). In contrast, there are several broods per year in *C. stellatus* and *C. montagui* (Crisp, 1950; Burrows, 1988; Burrows *et al.*, 1992) and it has been reported that *E. modestus* can breed all year round (Crisp and Davies, 1955; Bassindale, 1964). As a consequence of this reproductive output in *E. modestus*, the species has been able to rapidly spread and become a common species around the British Isles since it was first discovered in Chichester Harbour by Bishop (1947). In *B. improvisus*, several broods are produced in a year (Crisp, 1954). Variation in the number of broods per year at the intra-specific level has been investigated in *C. stellatus* and *C. montagui*. It was found that more broods were produced at lower shore levels (O'Riordan *et al.*, 1992). This could be the result of increased available food supplies at lower shore levels which would in turn provide more energy for growth and reproduction (Bertness *et al.*, 1991).

2.1.4 Breeding and settlement season of barnacles

Generally, barnacles release their planktonic larvae into the water column. The larvae then feed and develop in the water column, until reaching a non-feeding settlement larval stage, at which point they are called cyprids. These larvae settle on hard substrata and metamorphose to become juvenile barnacles. Adult barnacles release their larvae at different times, depending on the species. There is a unique mechanism controlling larval release found in *S. balanoides*. The larval release (hatching) is stimulated by a prostaglandin-like compound (Clare *et al.*, 1982; 1985) and the releasing of this compound is probably induced by the feeding activity of the adult barnacles after winter starvation. *Semibalanus balanoides* generally releases larvae in early spring (Crisp, 1959), and their settlement occurs from April onward (Kendall *et al.*, 1985; Hawkins and Hartnoll, 1982). The larvae of *C. stellatus* and *C. montagui* are released from early May to September (Burrows *et al.*, 1992), settling from July to September in Southern England. The planktonic larvae of *E. modestus* can be found all year round, but the peak of larval occurrence is from May to July (Crisp and Davies, 1955) and the peak of larval settlement is between June and July (Bassindales, 1964). *B. improvisus* is a warm-temperate barnacle. In tropical regions, it has been reported that it is capable of breeding all year round, but the peaks of its settlement are from March to April and from September to October (Weiss, 1948; Moore and Frue, 1959). In colder areas, such as Europe, *B. improvisus* breeding season is restricted to the summer. Planktonic larvae can be found from May to September and settlement is found between the middle of May and the end of September (Bassindale, 1964). The duration of larval development from the planktonic stage to the settlement stage is an important indicator of larval dispersal ability (Strathmann, 1974; Crisp, 1976; Furman *et al.*, 1989). It has been shown that the duration of this period can be affected by water temperature, as reported in *S. balanoides* and *E. modestus* where the duration period became shorter when the water temperature increased (Harms, 1984).

2.1.5 Recruitment

The dynamics of marine invertebrate populations with planktonic larvae depends largely upon the fluctuation of recruitment (Hawkins and Hartnoll, 1982; Caffey, 1985; Gaines and Roughgarden, 1985). The recruitment process of marine sessile invertebrates generally consists of three components: 1) larval supply in the water column, 2) settlement of competent larvae, and 3) the survival of settlers to become new members of the population (Bertness *et al.*, 1992). These are the main factors responsible for the variation in the recruitment of these animals. Although all of these factors have relative effects on recruitment variation, few studies have combined these findings (Bertness *et al.*, 1992). They have focused on assumptions that both physical and biological factors after settlement play the key roles in determining the abundance of populations and the structure of intertidal communities. These factors include the availability of resources, competition, perturbation, predation and environmental conditions (Dayton, 1971; Connell, 1975; Menge, 1976; Menge and Sutherland, 1976). Conversely, it is now thought that variation in the new recruits or larval supply can have a crucial effect on the abundance of benthic marine organisms with planktonic larvae, "*Supply-Side Ecology*" (Lewin, 1986; Roughgarden *et al.*, 1987; Underwood and Fairweather, 1989; Fairweather, 1991; Raffaelli and Hawkins, 1996). Therefore, to understand the recruitment process, the variation in larval supply must be considered.

The study of recruitment processes has been called "*Supply-Side Ecology*" (Lewin, 1986). The planktonic larvae of most marine sessile organisms can disperse far away from their indigenous areas under the action of water movement and spend several weeks in the water column (Gaines and Roughgarden, 1985; Shank, 1986; Le Fevre and Bourget, 1992). For example, the development of nauplii to the cyprid stage in *B. improvisus* takes about two to three weeks (Furman *et al.*, 1989; Gakhova *et al.*, 1990). For this reason, the variation in larval supply relies largely on chance events in the

water column such as the direction of water movement (Crisp and Southward, 1958; De Wolf, 1973; Connell, 1985), wind (Barnes, 1956; Hawkins and Hartnoll, 1982; Kendall *et al.*, 1985), temperature (Southward and Crisp, 1956; Crisp and Southward, 1958), predation (Gaines and Roughgarden, 1987; Barkai and Branch, 1988; Navarrete and Wieters, 2000), and reproductive outputs (Gaines and Roughgarden, 1987). Since the recruitment of marine sessile animal populations is derived from the propagules from other areas, the resulting population of these organisms functions as an open system (Roughgarden *et al.*, 1987; Gaines and Roughgarden, 1985). Therefore, the variation in larval supply is the most important factor for intertidal populations and communities (Lewin, 1986; Roughgarden *et al.*, 1987; Underwood and Fairweather, 1989; Fairweather, 1991; Menge, 1991). For example, it has been shown that variations in the concentration of larval supply can affect settlement rate and recruitment (Gaines *et al.*, 1985) and, in turn, studies on settlement rate can indicate the structure of marine sessile invertebrate communities (Gaines and Roughgarden, 1985). Therefore, larval supply is included in recent models of the population structure and dynamics of these organisms (Gaines and Roughgarden, 1985; Lewin, 1986; Menge and Sutherland, 1986; Roughgarden *et al.*, 1987; Underwood and Fairweather, 1989; Menge, 1991).

In conclusion, the abundance and structure of the population of marine benthic organisms depends on both pre-settlement factors (the variation in larval supply), and on post-settlement factors (e.g. physical stresses, disturbance, predation, the availability of space and competition within and between species). Therefore, both sets of factors are indispensable when studying the abundance and structure of marine sessile animal populations (Miron *et al.*, 1999).

2.2 Materials and methods

2.2.1 Survey of *B. improvisus* around the British Isles

This survey was carried out based on the records of *B. improvisus* populations around the British Isles reported by Furman (1989a) (see table 2.1 and fig. 2.3). Abundant populations and those at neighbouring estuaries were chosen for this re-investigation. The survey was carried out during the spring low tides since *B. improvisus* is mainly a sublittoral species, and it focused on the populations in the south and west of the British Isles as follows:

1. Thames Estuary, Greenhithe, London (N 51°27' 04', E 0° 16' 50')
2. River Hamble, Manor Farm Country Park, Southampton (N 50° 53' 59', W 1° 17' 35')
3. River Itchen, Cobden Bridge, Southampton (N 50° 54' 48' W 1° 21' 42')
4. Severn Estuary, Clevedon (Sea front) (N 51° 26' 30', W 2° 51'19')

   Severn Estuary, Portishead (Sea front) (N51° 29' 07', W 2° 46' 04')
5. Milford Haven Estuary, Landshipping (N 51°46' 11', W 4° 52' 53')

   Milford Haven Estuary, Black Tar Point (N 51° 45' 04', W 4° 55' 09')
6. River Dee, Connahs Quay (N 53° 13' 02', W 3° 03' 44')

   River Dee, Greenfield (N 53° 17' 18', W 3° 12' 18')

   River Dee (North), Heswall (N 53°19' 45', W 3° 05' 58')
7. River Mersey, Rock Ferry, Liverpool (N 53° 22' 26', W 3° 00' 30')
8. River Ribble, Laytham St Annes (N 53° 45' 22', W 3° 02' 14')
9. River Lune, Glasson Dock (N 53° 59' 54', W 2° 51' 06')

   River Lune, Cockersand (N 53° 58' 41', W 2° 52' 27')

(See fig. 2.4)

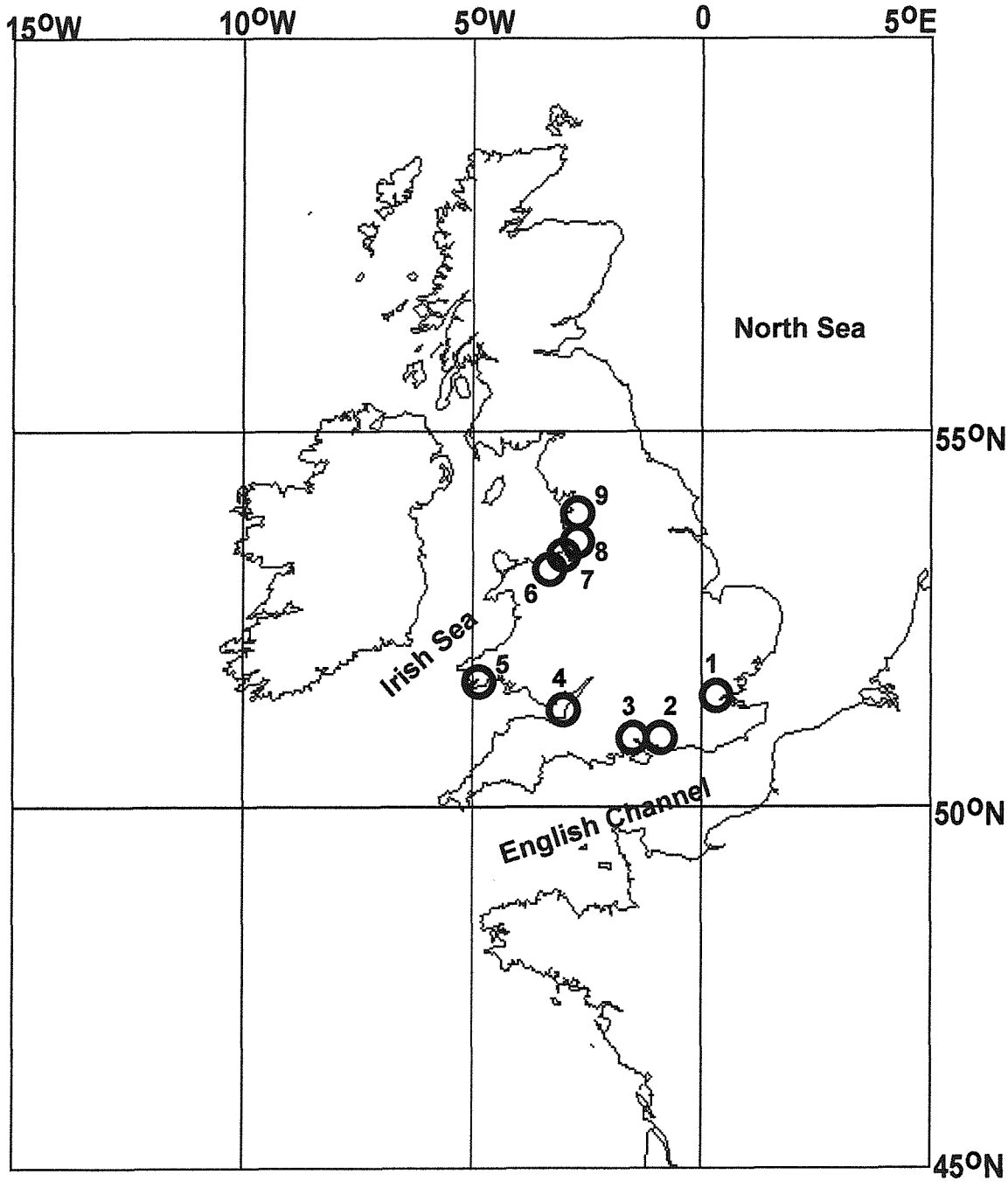


Figure 2.4 Sites investigated in this study (The numbers on this map correspond to the sites in section 2.2.1)

2.2.2 Ecological studies on *B. improvisus*

2.2.2.1 Site of study

This study was carried out at Cobden bridge, River Itchen, Southampton where the greatest numbers of *B. improvisus* occur in this river (Flavell, 1996) (see fig 2.5 and see Section 2.3.1 for a description).

2.2.2.2 The nearest distances within and between species of *B. improvisus* and *E. modestus*

The nearest distances within and between individuals chosen at random of *B. improvisus* and *E. modestus* were measured using callipers. Measurement was carried out between the opercula of neighbouring individuals.

2.2.2.3 Breeding cycles of *B. improvisus*

This survey was carried out from March 1997 to April 1998.

A. Male activity - Loss of penis

30 individuals of *B. improvisus* were examined each month during the non-breeding season and every two weeks during the breeding season (from May to October). This investigation was carried out from March 1997 to March 1998. Samples were immersed in 8% (w/v) of $MgCl_2$ in distilled water to relax the muscles. The length of the penis was then measured, using a calliper, under a dissecting microscope. The average length of the penis was then used as an indicator of male reproductive activity.

B. Female activity

30 individuals of *B. improvisus* were again examined each month during the non-breeding season and every two weeks during the breeding season (from May to

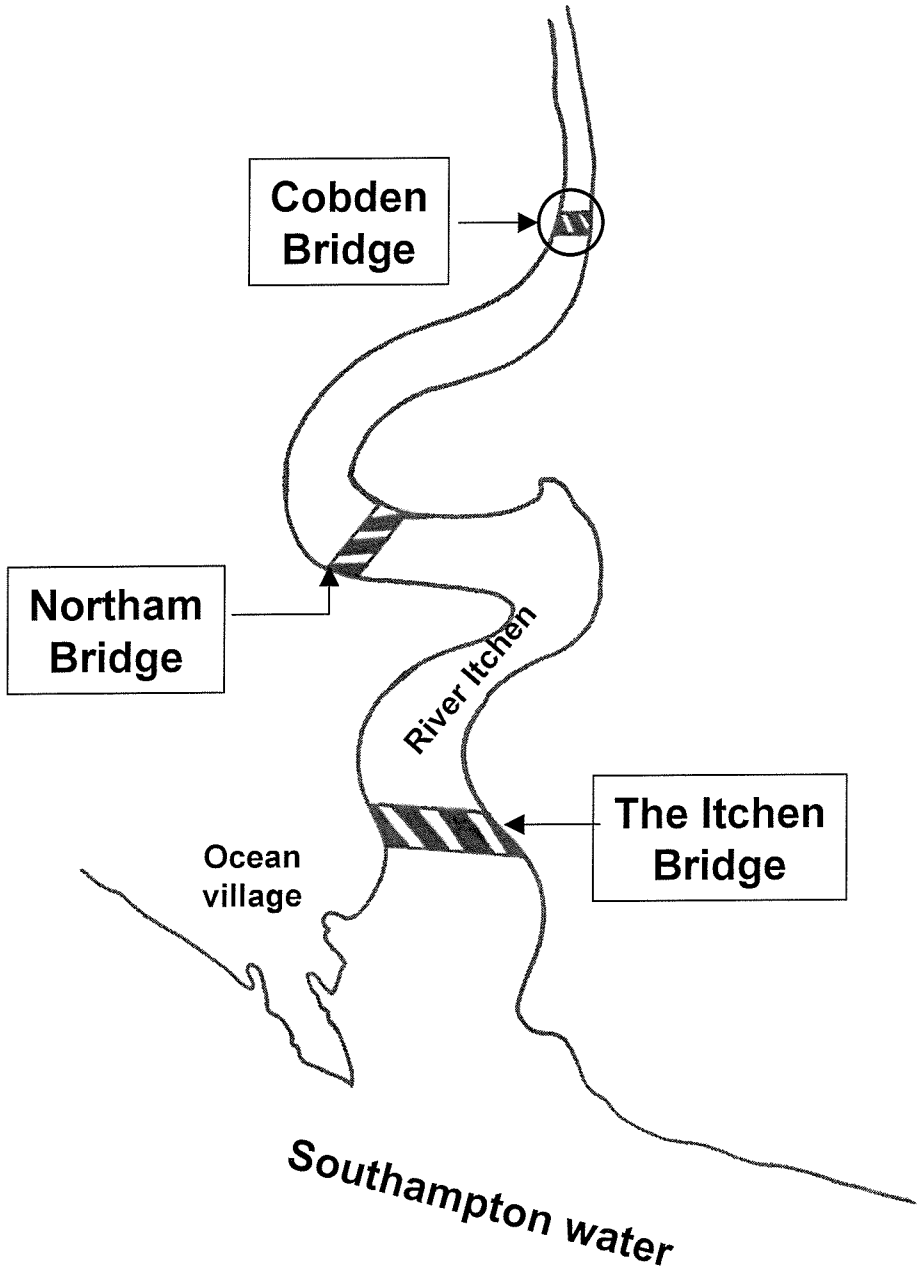


Figure 2.5 Map of the river Itchen showing the site (Cobden Bridge) used in this study

October). This investigation was carried out from March 1997 to March 1998. The samples were divided into four groups:

1. non-active individuals: i.e. specimens in which the ovary was absent or is pale in colour.
2. ovary-developed individuals: the speciman in which the ovary is well developed and yellow or orange in colour.
3. egg-mass individuals: i.e. individuals containing egg masses.
4. brooding individuals: i.e. individuals containing broods.

The above criteria were modified from O'Riordan *et al.* (1995).

2.2.2.4 Settlement and recruitment of *B. improvisus* and *E. modestus*

This study was carried out from June 1997 to May 1998. The following data were collected on settling barnacles: the population size-structure, free space, and number of barnacles at different stages.

A. The population size-structure of B. improvisus and E. modestus

The purpose of this study is to obtain data on size-classes in these two barnacle species. These data were then used to differentiate new settlers (0^+) and adult age classes (1^+, 2^+,...N^+). The opercular length and shell length of *B. improvisus* and *E. modestus* were measured using a calliper and were then applied to investigate size-structure of their populations. The frequency histograms of the opercular length and shell length were used to display the population structure of these barnacles (see section 2.3.2.3). The relationship between opercular length and shell length (rostro-carinal) was also investigated in both species. This study was carried out in March 1997.

B. Free space including and excluding new settlers (0^+)

20 small stones were randomly collected from 20 different points throughout the site of study. The percentage cover of *B. improvisus* and *E. modestus* including and excluding 0^+ was calculated by using the intersection point method with a quadrat (5x5 cm^2). The percentage of available free space was then estimated by subtracting the percentage cover of the two species from 100%. Two estimates were made:

% available free space including 0^+ = 100 - % cover of all barnacles

% available free space excluding 0^+ = 100 - %cover of all barnacles excluding 0^+

C. Density of barnacles (cm^{-2})

The following data were obtained:

1. The number of cyprids in both species (*B. improvisus* and *E. modestus*)
2. The number of individuals each species which were metamorphs
3. The number of the newly-settled spat (0^+) that settle from each species in the year 1997.
4. The number of the adults (1^+,2^+,.....,N^+ (age groups)) in each species

C.1 The criteria for separating cyprids, metamorphs, juveniles (0^+), and adults

1. Cyprids have an elliptical shape and are yellow-brown in colour (see fig. 2.6)
2. Metamorphs are individuals having newly formed shells, which are initially transparent (see fig. 2.6).
3. Juveniles (0^+) are individuals having diameter < 0.3 cm. Their shells are not transparent and clean (see data in section 2.3.2.3, see fig 2.6).
4. Adults (1^+,2^+,...N^+) have a rostro-cardinal shell diameter > 0.3 cm and their wall-plate is clean (see data in section 2.3.2.3, and fig.2.6)

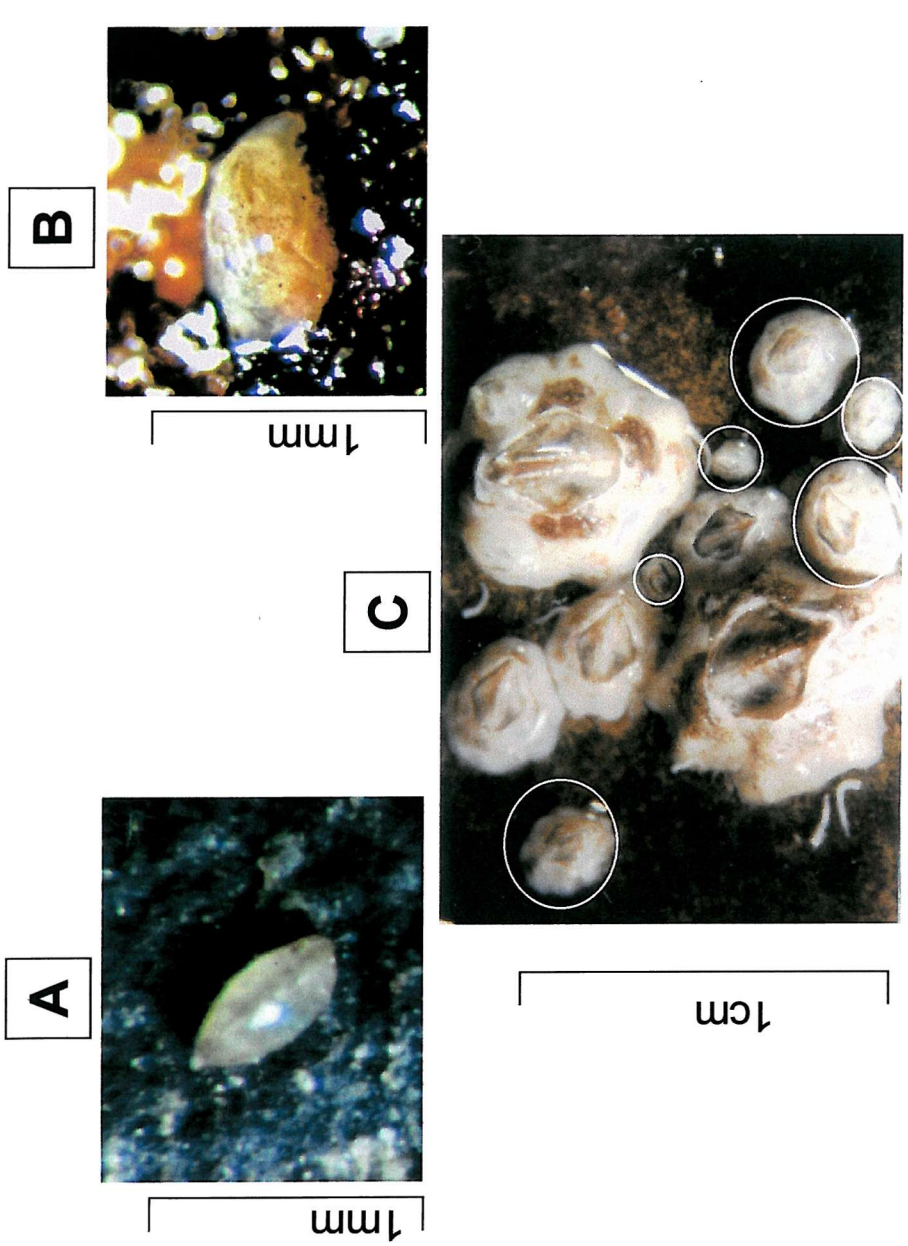


Figure 2.6 (A) a cyprid larva, (B) a metamorph, and (C) juveniles, 0+, (in the circles) and adults (all *E. modestus*)

The number of all barnacles was estimated using a 5x5 cm^2 quadrat. Only the shell length was used because it is much easier to measure shell length than opercular length on small juveniles (spat).

2.3 Results

2.3.1 Survey of the distribution of *B. improvisus* populations

Thames Estuary, Greenhithe, London

This site was investigated on 4 August 1997. Most barnacles were found on the small stones and on pier foundations made from wood. The area around the pier was very muddy. *Elminius modestus* and *B. improvisus* were both found. *Balanus improvisus* was fairly abundant at this site despite only being found in the area under the pier. In contrast, *E. modestus* was found all over the area, mostly on stones.

River Hamble, Manor Farm Country Park, Southampton

This site was investigated on 7 May 1997. It was very muddy. Sessile organisms were found on stones and on the concrete wall of a small slipway. *Balanus improvisus* was scarce at this site, and many dead individuals were found. There were a small number of *B. crenatus* individuals. The most abundant species was *E. modestus*.

River Itchen, Cobden Bridge, Southampton

This site was investigated on 4 March 1997. Most of the areas examined at this site were muddy. Only the area directly under the bridge was covered by small stones, where barnacles were abundant. The stones and the concrete wall of the bridge were suitable substrata for barnacles. There were three species of barnacles present at this site: *E. modestus*, *B. improvisus*, and *S. balanoides*. *S. balanoides* was, however,

scarce. *Elminius modestus* was the most abundant species. *Balanus improvisus* appeared only on the lower shore and was mostly found attached to small stones and to the lower part of the wall. It could be seen during the spring low tide period. *Elminus modestus* was found on both upper and lower shores where mussels also occurred.

Sea front, Severn Estuary, Clevedon

This site was investigated on 5 October 1998. The substratum were rocks and stones. Rocks were mostly covered by brown algae, and barnacles (*E. modestus*, *S. balanoides* and *B. improvisus*) were mostly found on stones. *Balanus improvisus* was fairly abundant, although each individual was settled distant from the others. A few dead inviduals of *B. improvisus* were found. *Elminius modestus* was the most abundant barnacle species at this site, and a few *S. balanoides* individuals were also found.

Sea front, Severn Estuary, Portishead

This site was investigated on 5 October 1998. Rocks and stones were the normal substratum for sessile organisms, although most of the substratum was covered by brown algae. Only *E. modestus* was found, and no *B. improvisus* was found at this site. Limpets were also found.

Milford Haven estuary, Landshipping

This site was investigated on 6 October 1998. Stones were the substrata. No *B. improvisus* was found. *Elminius modestus* was the only barnacle observed at this site.

Milford Haven estuary, Black Tar Point

This site was investigated on 6 October 1998. Rocks, stones and mussel shells were the substrata. Most of the rocks were covered by brown algae and clumps of mussels. Barnacles were mostly found on stones and mussel shells. *Balanus improvisus* was

scarce, and most of these found were dead. *Balanus crenatus* and *E. modestus* were abundant. A few *S. balanoides* individuals were also present.

River Dee, Connahs Quay

This site was investigated on 7 October 1998. The Quay wall (metal) provided a large substratum for sessile organisms. *Elminius modestus* and *B. improvisus* were both abundant. *Balanus improvisus* mostly occupied the lower area (bottom) of the wall. In contrast, *E. modestus* was mostly found on the upper parts of the wall.

River Dee, Greenfield

This site was investigated on 7 October 1998. Rocks, stones, and mussel shells were the substrata. *Elminus modestus*, *B. crenatus*, and *S. balanoides* were all abundant. A few *B. improvisus* individuals were found on mussel shells and rocks. Most of the rocks were covered by brown algae and mussels.

River Dee (North), Heswall

This site was investigated on 9 October 1998. The substrata consisted of stones and mussel shells. There were no *B. improvisus. Elminus modestus*, *S. balanoides* and mussels were all abundant.

River Mersey, Rock Ferry, Liverpool

This site was investigated on 8 October 1998. Stones, the concrete wall of a slipway, and the foundations of a pier were the substrata. A few *B. improvisus* individuals were found on stones. *Elminius modestus* and *B. crenatus* were abundant. Mussels were also abundant at this site.

River Ribble, Laytham St Annes

This site was investigated on 8 October 1998. Stones and mussel shells formed the substrata. A small population of *B. improvisus* was found. *Balanus crenatus* and *E. modestus* were abundant. Some *S. balanoides* individuals were also found.

River Lune, Glasson dock

This site was investigated on 7 October 1998. Most of the area was muddy. There were no barnacle species evident (after a quick search). However, it is likely that some barnacles were present on the dock wall. Investigations in that area could not be carried out, however, due to the muddy conditions.

River Lune, Cockersand

This site was investigated on 7 October 1998. Stones and mussel shells were the substrata for the barnacle species. *Elminius modestus* and *S. balanoides* were abundant. No *B. improvisus* was found.

From the survey of the 9 estuaries (14 sites) above, *B. improvisus* proved to be absent only in the Milford Haven Estuary and River Lune (see fig 2.7). However, there were a large number of dead individuals found at the Black Tar Point sites in the Milford Haven estuary. This could suggest that *B. improvisus* was once abundant at this site. *B. improvisus* was also found at new sites, which were not reported by Furman (1989) namely the River Itchen (Cobden Bridge), River Hamble (Manor Farm Country Park), River Severn (Clevedon (sea front), River Mersey (Rock Ferry), River Dee (Greenfield), and River Ribble (Laytham St Annes) (see table 2.2). Most of these populations were found at the upper reaches of the rivers, except for the populations found at the mouth of the Severn Estuary (Clevedon (sea front) and River Ribble (Laytham St Annes).

Common barnacles found in this survey were *E. modestus*, *B. crenatus*, *B. improvisus*, and *S. balanoides*. However, *S. balanoides* and *B. crenatus* were scarce at the upper reaches sites of the estuaries such as the River Itchen (Cobden Bridge), and river Thames (Greenhithe). It is likely that the distribution of *S. balanoides* and *B. crenatus* within estuaries is limited by the reduced salinity. *Elminius modestus* is the only barnacle species coexisting with *B. improvisus* at the top reach of the estuaries since they are both euryhaline species. *Balanus improvisus* was mostly found in the sub-littoral zone and settled on the surface of stones, concrete, mussel shells, metal, plastic, and glass.

2.3.2 Ecological studies

2.3.2.1 The nearest distance within and between *B. improvisus* and *E. modestus*.

B. improvisus and *E. modestus* were found to settle preferentially near to their conspecific species. The most frequent distance between individuals of the same species is about 0.4-0.8 cm in *B. improvisus* (see fig. 2.8.a) and 0.2-0.6 cm in *E.modestus* (see fig. 2.8b). *E. modestus* occurred closer to its conspecific species than *B. improvisus* because *E. modestus* individuals do not tend to settle more than 1.6 cm away from each other (see fig. 2.8b). *B improvisus* accounts for most of the isolated individuals seen in this study. The maximum distance between *B. improvisus* individuals was up to 3 cm. The nearest distance between these two barnacle species is about 0.4-0.6 cm, and the longest distance was up to 3 cm (see fig. 2.8c). This could be the result of *E. modestus* larvae settling without discrimination beween conspecific and non-conspecific adults (Hui and Moyse, 1982).

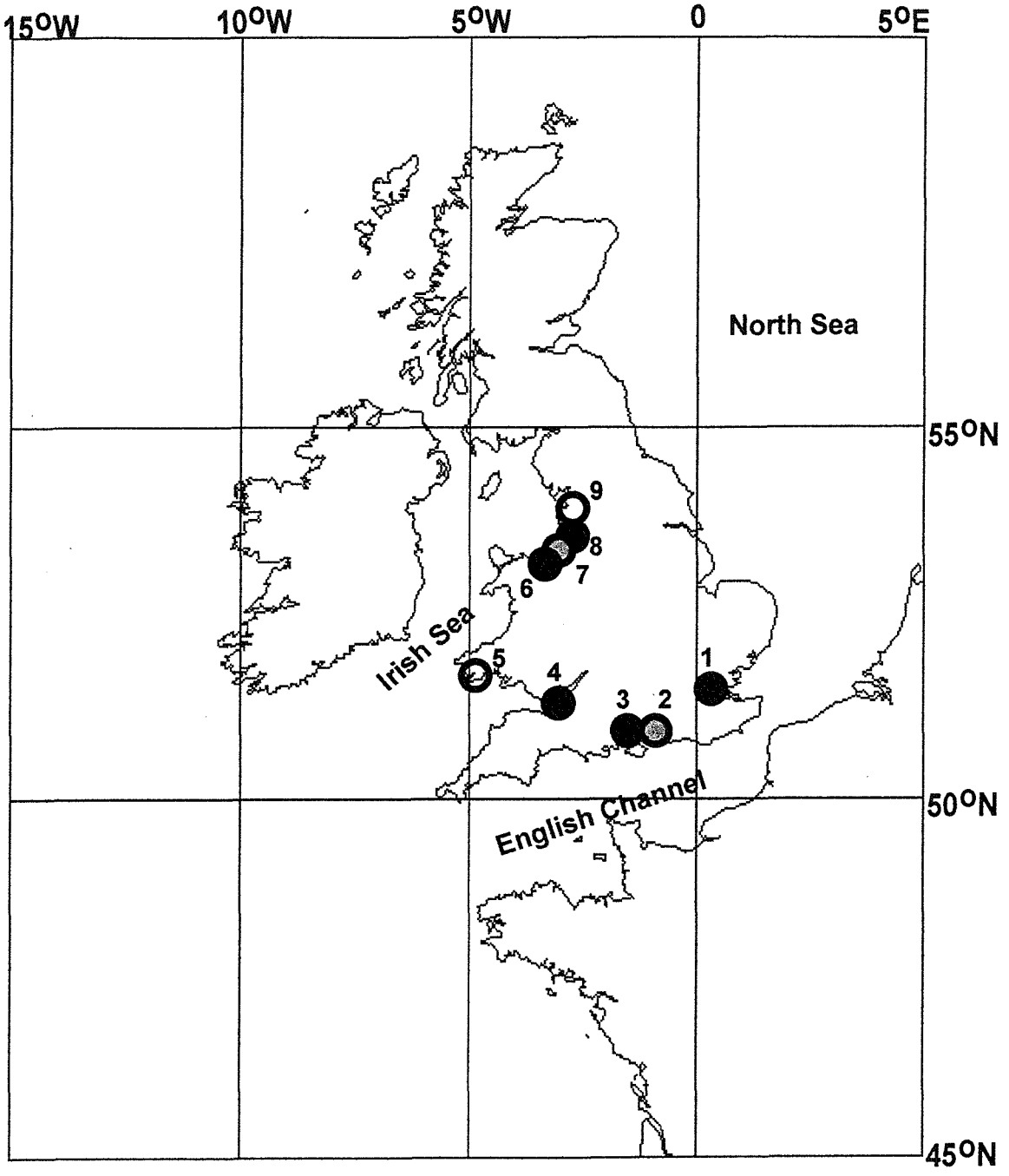


Figure 2.7 The presence of *B. improvisus* populations after reinvestigation (1997-1998) (the numbers on this map correspond to the sites listed in section 2.2.1)

Absent Very small population Abundant

Table 2.2 The results of the resurvey of *B. improvisus* populations around the British Isles in 1997-1998, compared with the records by Furman (1989a)

Sites	Original record (Furman, 1989a)	1997-1998 investigation
River Clyde, Clyde bridge, Dumbarton	Absent (03/09/1986)	Not investigated
Solway Firth, Bowness, Silloth, Beaumont	Absent (07/09/1986)	Not investigated
River Ribble, Preston Docks	Big dead individuals found (10/02/1985)	Not investigated
River Ribble, (Laytham St Annes)	Not investigated	Present (fairly abundant)
River Mersey, Garston Rocks	Dead individuals found (15/05/1988)	Not investigated
River Mersey, (Rock Ferry)	Not investigated	Present (very small population)
River Dee, Connah's quay	Abundant (21/11/1987)	Present (very abundant)
River Dee, Queensferry Bridge	Sparse (21/11/1987)	Not investigated
River Dee, Greenfield	Not investigated	Present (fairly abundant)
River Dee (North), Heswall	Not investigated	Absent
River Clwyd, Rhyl	Absent (17/05/1988)	Not investigated
Conwy estuary	Sparse, individuals isolated from each other (1985-1988)	Not investigated
Caernarfon	Absent (16/05/1988)	Not investigated
River Mawddach, Barmouth	Absent (21/08/1986)	Not investigated
Milford Haven, Black Tar Point	Only a few individuals found several empty shells (21/03/1986)	Very few found - a lot of empty shells
Milford Haven, Landshipping	Not investigated	Absent
River Tamar	Absent (12/03/1986)	Not investigated
River Dart, Totnes	Some dead individuals found (11/03/1986)	Not investigated
The Thames, Greenhithe	Present and abundant (1985-1986) The population decreased dramatically during the winter	Present (abundant)
River Humber, New Holland	Very abundant (07/08/1986 and 29/08/1988)	Not investigated
Firth of Forth, Kincardine Bridge	Present and abundant (06/09/1986)	Not investigated
River Eden, Guardbridge, Coble shore	Absent (04/09/1986)	Not investigated
Firth of Tay, Newburgh, Balmerino, Newport	Absent (05/09/1986)	Not investigated
River Itchen, Cobden bridge, Southampton	Not investigated	Present (abundant)
River Lune, Glasson Dock	Not investigated	Absent
River Lune, Cockersand	Not investigated	Absent
Severn estuary, Clevedon (sea front)	Not investigated	Present (fairly abundant)
Severn estuary, Portishead (sea front)	Not investigated	Absent
River Hamble, Manor farm country park, Southampton	Not investigated	Present (small population)

2.3.2.2 Breeding cycle of *B. improvisus*

Male activity (Loss of penis)

Based on the average penis length at different times in the year, there is no evidence for penis loss in *B. improvisus* as is seen in *S. balanoides* (Stubbings 1975). The average length of the penis is about 0.5-0.6 cm all year round (see fig. 2.9a). However, It is possible that *B. improvisus* individuals do shed their penises (but not all in the same season), since the penises of some individuals were not well-developed (see fig.2.10). Therefore, measuring the average penis length does not provide enough information to resolve this situation. Further studies into the development of the male reproductive organ are required (see O'Riordan 1995).

Female activity

The ovary of *Balanus improvisus* begins to develop in early February and individuals containing egg-masses were present by the end of February. Individuals containing broods appear in May and are most abundant from the middle of June to early of July after that their numbers start to decline. The results above indicate that the putative peak of the period when *B. improvisus* releases its planktonic larvae is from July to August. In the tropics, *B. improvisus* can breed all year round and produce several broods per year (Crisp, 1954). In this study, the breeding season of *B. improvisus* in the south of England began in March and ended in October (see fig. 2.9b). With such a long breeding season period (approximately 8 months), it is possible that this barnacle produces several broods per year. The breeding season is suspended between the end of October and the end of January (see fig. 2.9b).

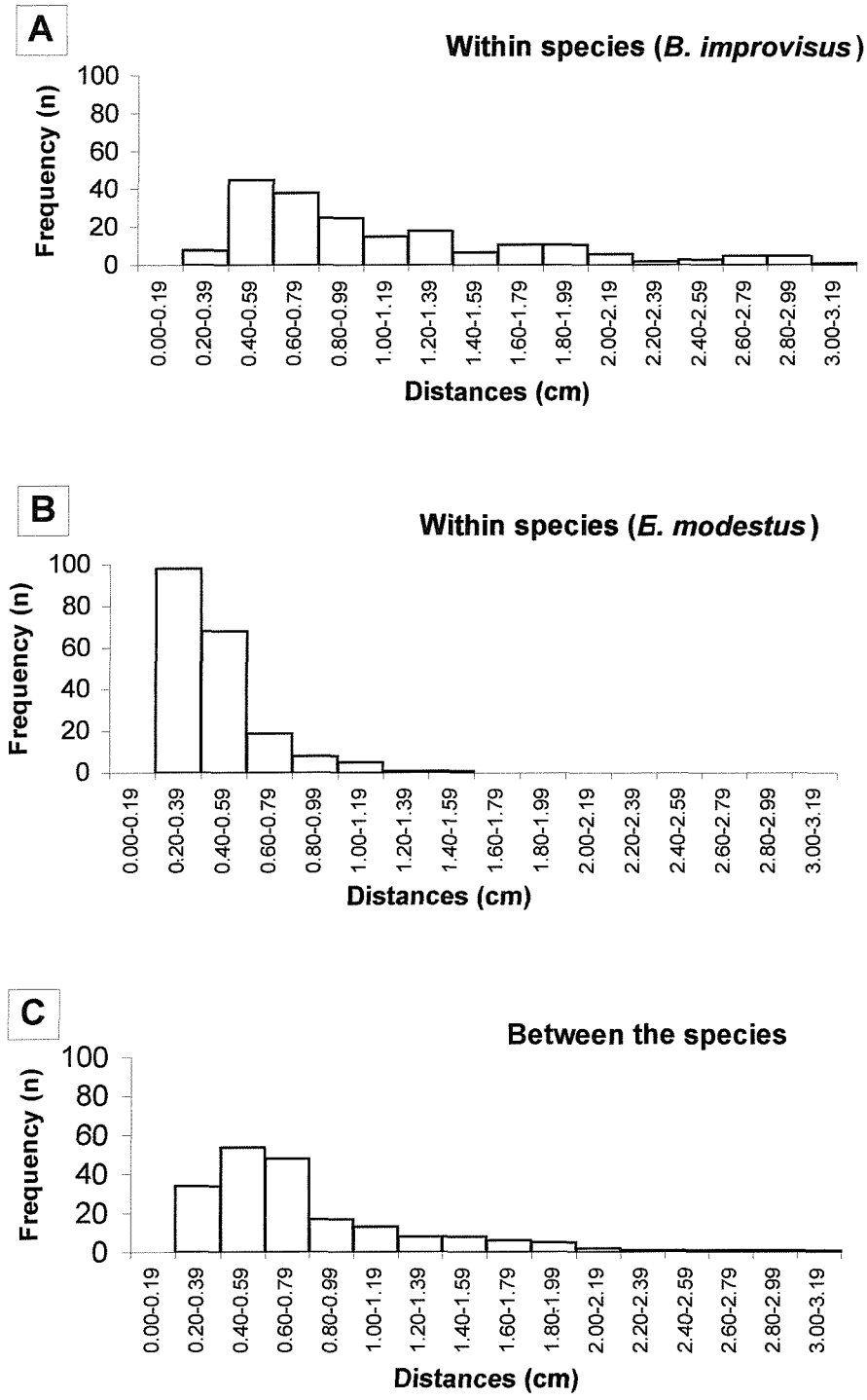


Figure 2.8 The distances between individuals: (within species of *B. improvisus (A) and E. modestus* (B) and between the species (C)) at the Cobden Bridge site, Southampton (n=200)

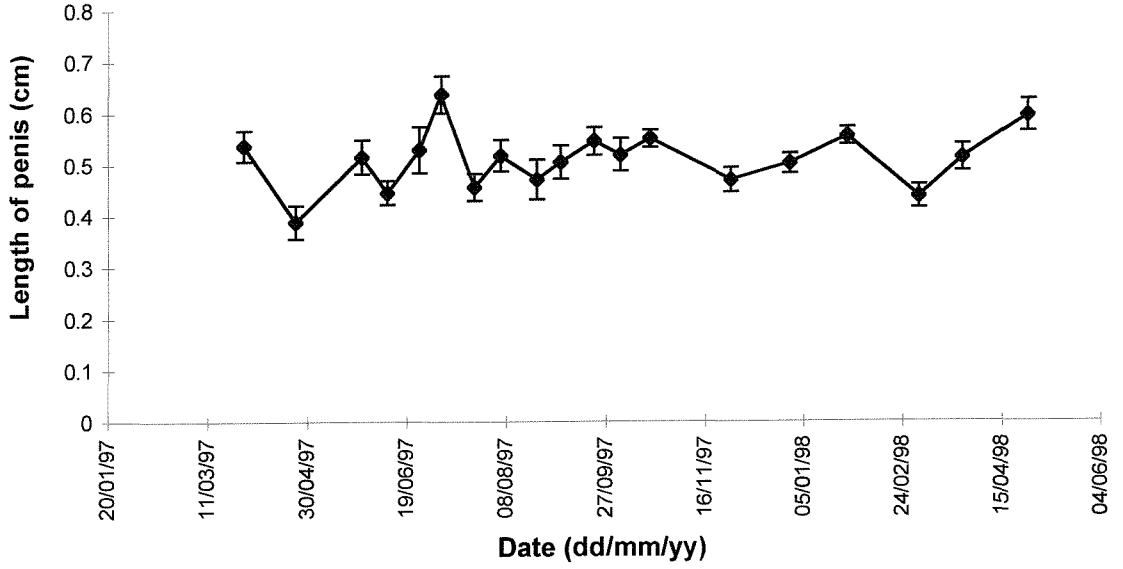


Figure 2.9a Mean penis length in *B. improvisus* with 95% limits of the mean (n=30)

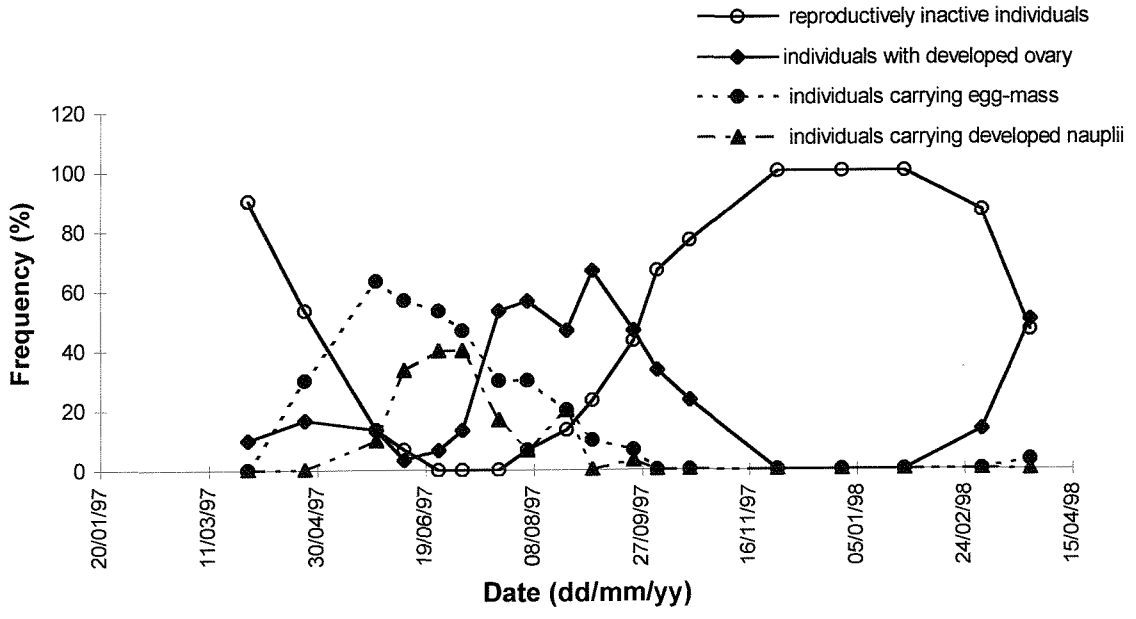


Figure 2.9b The breeding cycle (female activity) of *B. improvisus* at the Cobden Bridge site, Southampton (n=30)

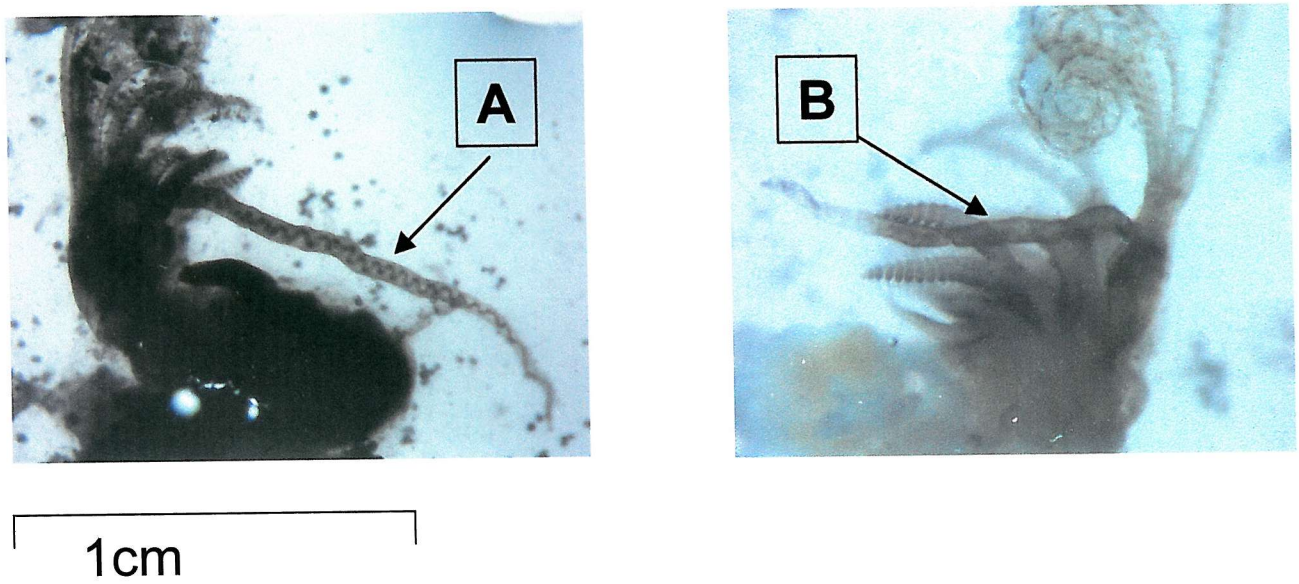


Figure 2.10 The arrows indicate (A) well developed penis and (B) poorly developed penis in *B. improvisus*

2.3.2.3 Settlement and recruitment of *B. improvisus* and *E. modestus*

Population size-structure

After measuring the shell length and opercular lengths of *B. improvisus* (n=202) and *E. modestus* (n=212), the regression analysis between opercular length and shell length showed that the shell length was about two times larger than the opercular length in both species (see figs. 2.11a and b.).

Since this investigation was carried out in March (i.e. not during the peak of settlement season for these two barnacle species) (Bassindale, 1964), the size frequency of very small juveniles was not available for incorporation into the histograms. The result showed that the opercular length frequency provided more distinct size-classes in both species compared with the results obtained from the shell length frequency analysis (see figs. 2.12a&b and 2.13a&b). However, the size-classes, 0^+ and 1^+, obtained from both sets of measurement data (shell length and opercular length) were in agreement with each other. In *B. improvisus*, the sizes of the juveniles (0^+ class) were < 0.26 cm (shell length) and < 0.15 cm (opercular length), and the sizes of the adults (1^+ class) were 0.26–0.55 cm (shell length) and 0.15–0.22 (opercular length). *In E. modestus*, the sizes of the juveniles (0^+ class) were < 0.26 cm (shell length) and < 0.11 cm (opercular length), and the size of the adults (1^+ class) were 0.26–0.50 (shell length) and 0.11–0.22 (opercular length). This information was then used to distinguish between juveniles (0^+ class) and adult (1^+, 2^+, ...N^+ classes) (see section 2.2.2.5 D).

Percentage of free space (bare rock)

The percentage of free space was around 60-70% when 0+ was excluded and 50-60% when 0^+ class was included, all year round (see fig. 2.14a). This indicated that there was a large amount of free spaces available for the settlement of larvae.

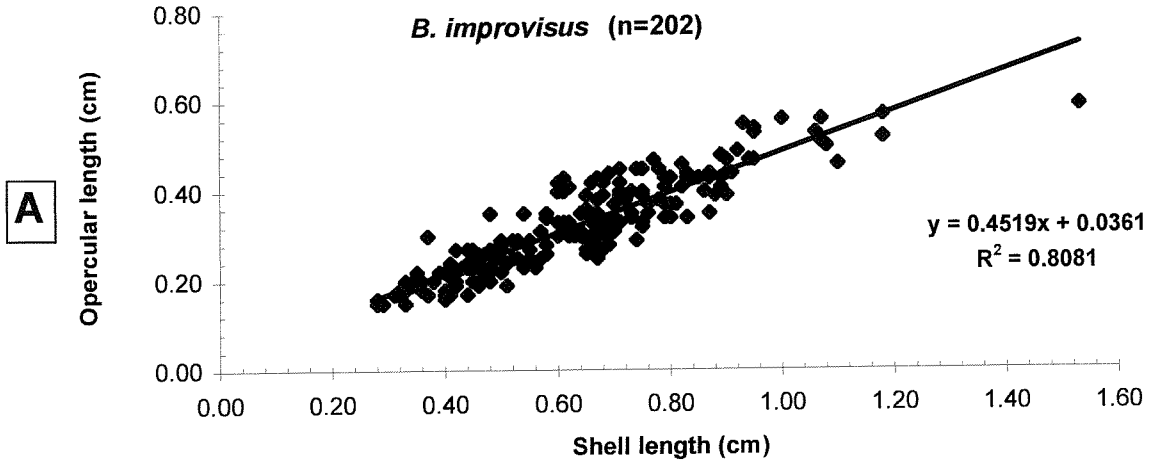


Figure 2.11a Regression analysis showing the relationship between shell and opercular length for *B. improvisus*

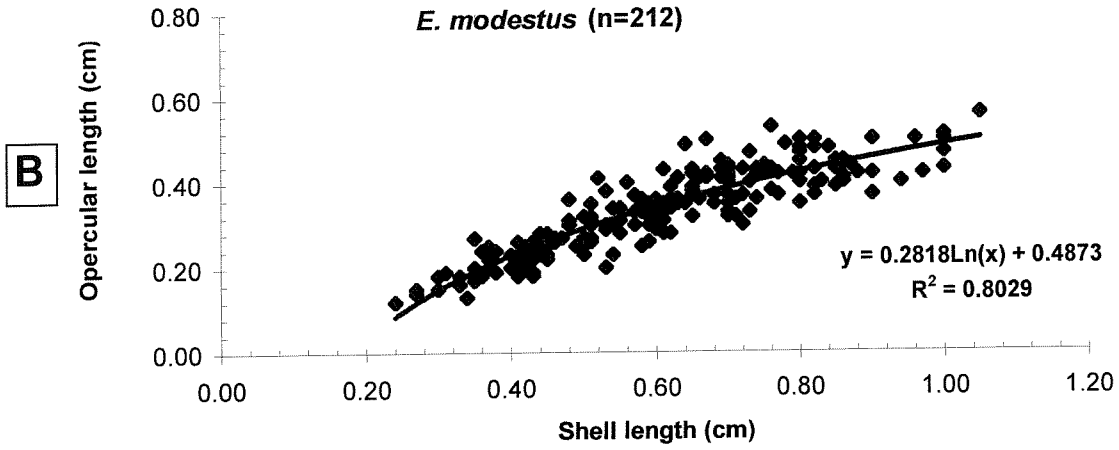


Figure 2.11b Regression analysis showing the relationship between shell and opercular length for *E. modestus*

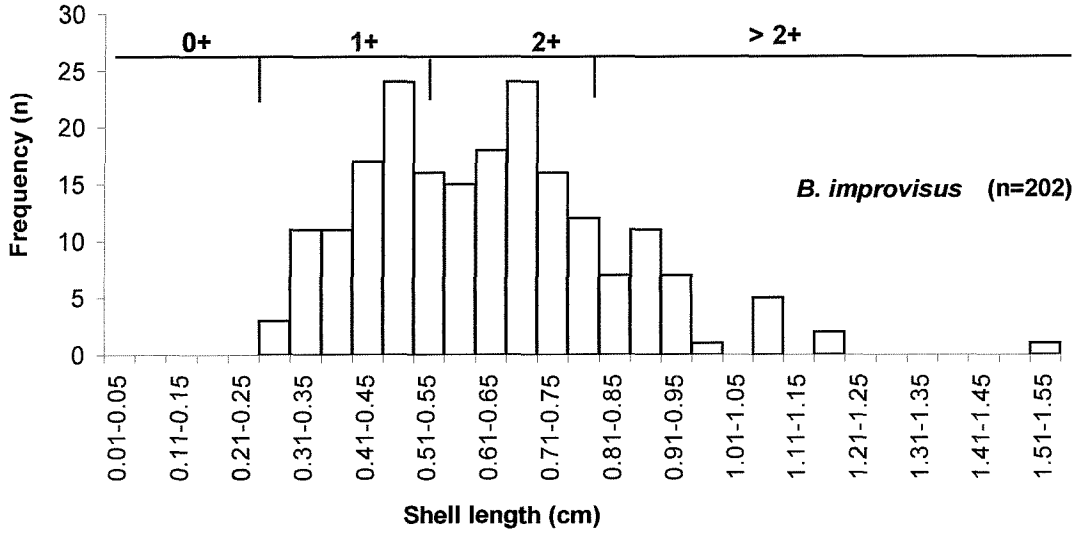


Figure 2.12a The population size-structure of *B. improvisus* at the Cobden Bridge site, Southampton using shell length data

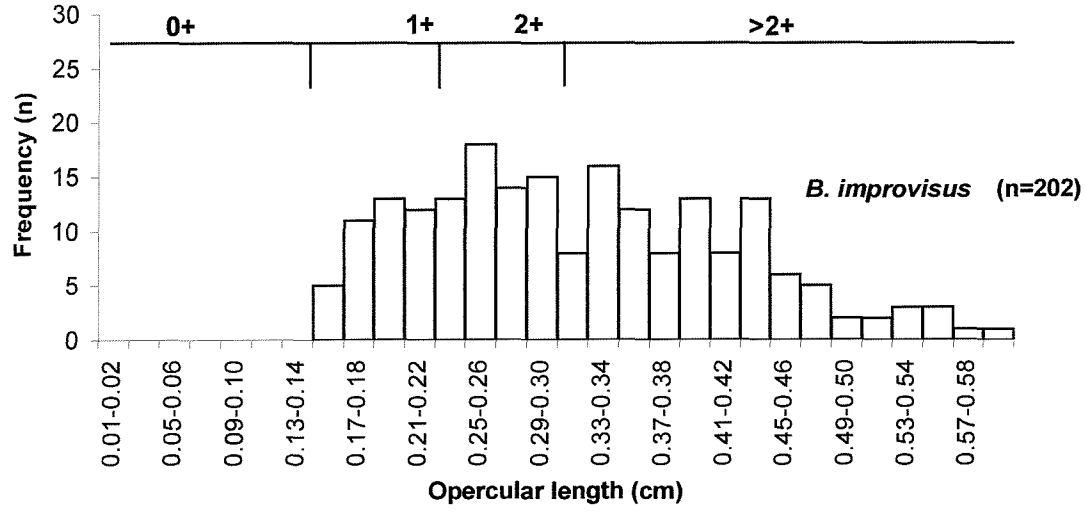


Figure 2.12b The population size-structure of *B. improvisus* at the Cobden Bridge site, Southampton using opercular length data

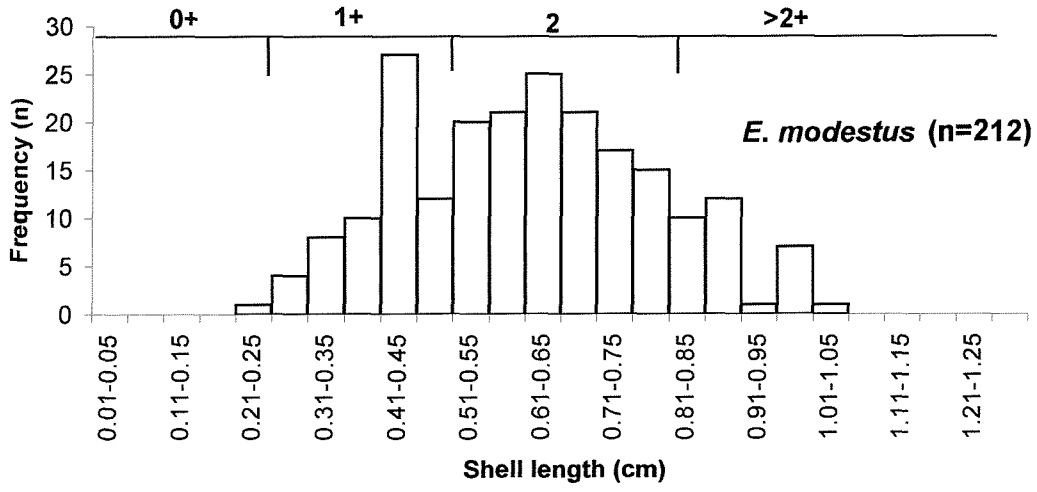


Figure 2.13a The population size-structure of *E. modestus* at the Cobden Bridge site, Southampton using shell length data

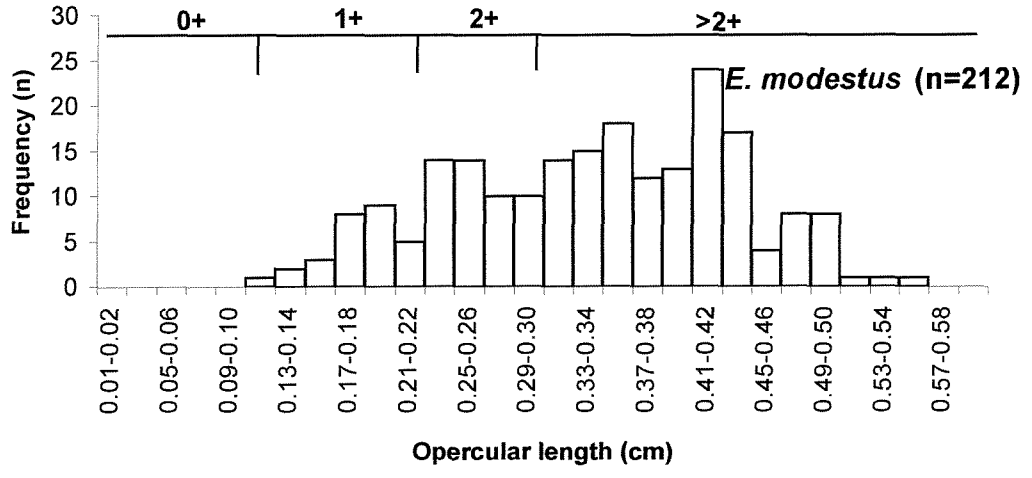


Figure 2.13b The population size-structure of *E. modestus* at the Cobden Bridge site, Southampton using opercular length data

Settlement and recruitment

Because of the difficulty in distinguishing between cyprid larvae of these barnacles, the same data set containing the overall number of cyprid larvae was used for both of these two barnacle species. Cyprids were found between late May 1997 and June 1997. The metamorphs (newly settled individuals) of *B. improvisus* were found between July 1997 and September 1997. The number of *B. improvisus* recruits (0+) was fairly constant from August 1997 to January 1998. After that, the numbers started to decline, which suggests that the settlement season of *B. improvisus* was in the summer (see fig. 2.14b).

The number of *E. modestus* metamorphs was low between the middle of August and the middle of April. This number then sharply increased from late April to late May. The numbers of *E. modestus* recruits were greatest in June and sharply declined thereafter (see fig. 2.14c). This decline might have been caused by the high mortality rate during the early stages of juvenile life. The data above reveal two main observations: 1) *E. modestus* settlements appear about 2 or 3 months earlier than those of *B. improvisus*, and 2) most of *E. modestus* settlement observed were metamorphs rather than cyprids.

2.4 Discussion

2.4.1 Distribution and temporal occurrence of *B. improvisus* populations

B. improvisus populations are widely distributed around the world (Bousfield, 1954; Carlton and Zullo, 1969; Newman and Ross, 1976), but the distribution is patchy. The patchy distribution of *B. improvisus* populations is also seen around the British Isles (Furman *et al.*, 1989). The British populations were generally found at the upper reaches of the estuaries, but in this study, populations were also found at the mouth of

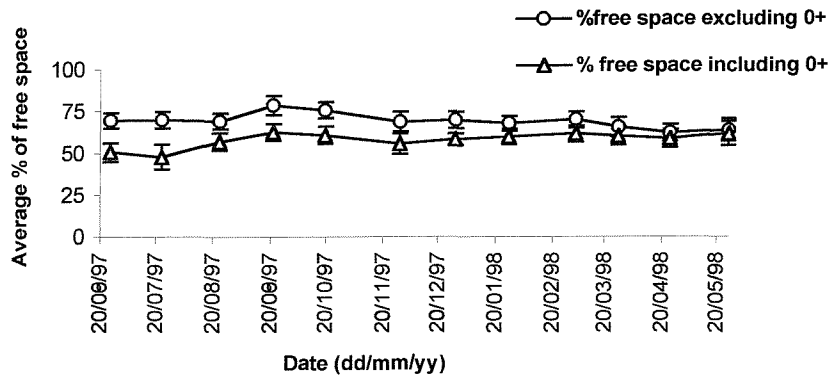


Figure 2.14a The average percentage of free space (bare rock) available at the Cobden Bridge site with 95% limits of the mean

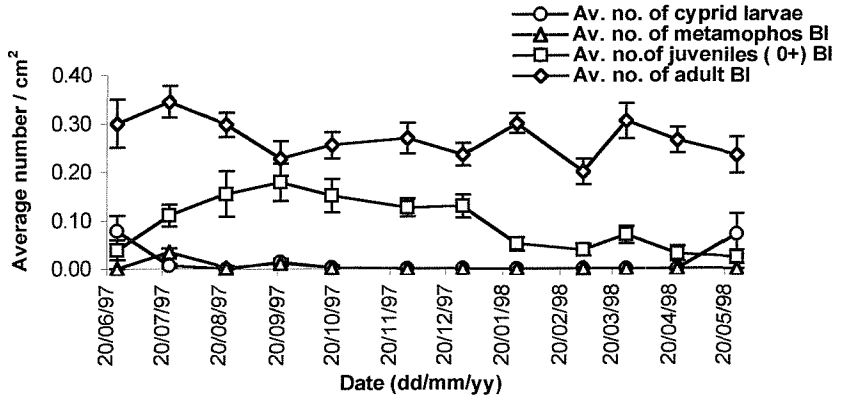


Figure 2.14b Average number of individuals (with 95% limits of the mean) poltted against time, showing the population dynamics of *B. improvisus* (BI) at the Cobden Bridge site

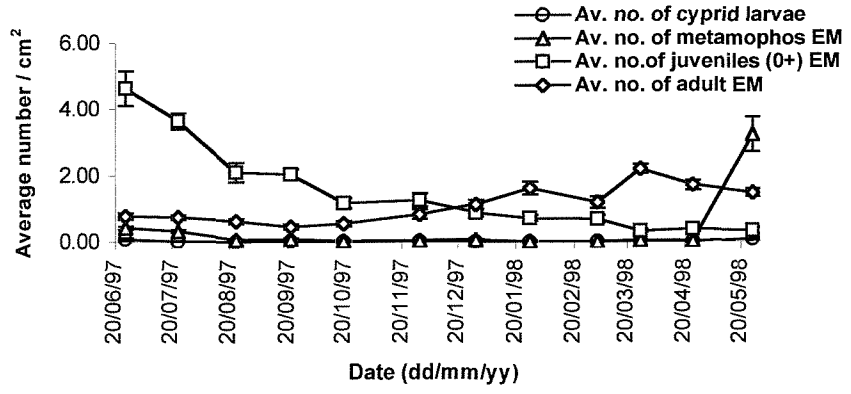


Figure 2.14c Average number of individuals (with 95% limit of the mean) plotted against time, showing population dynamics of *E. modestus* (EM) at the Cobden Bridge site

the Severn Estuary and River Ribble. This suggests that *B. improvisus* can survive in a wide range of salinity conditions as reported by Furman *et al.*, 1989. The occurrence of *B. improvisus* populations at the mouth of estuaries or rivers could be the result of the limitation of suitable habitats elsewhere in the estuaries. In general most of the areas within the estuaries are muddy, and therefore barnacle species can only occur in certain areas where hard substrata are available such as under bridges and piers.

It has been proposed that *B. improvisus* populations are unstable and temporally variable (Furman, 1989a.). This is supported by the re-emergence of the Woolwich population (Andrews *et al.*, 1982 and Furman, 1989a) after its disappearing because of pollution (Darwin, 1854; Jones and Crisp, 1953). In this study, the reinvestigation of previously abundant *B. improvisus* populations (Furman, 1989a) showed that after ten years, the populations are still present and mostly abundant, especially the population in the River Dee (Connahs Quay) and Thames (Greenhithe). However, the population in the Severn estuary (Portishead), which was previously abundant, was not found in this study. In the Milford Haven (Black Tar Point), where only a few live *B. improvisus* individuals were found during the initial investigation by Furman in 1986 and most of which has empty shells (dead). In this survey, only dead individuals were found at this site. This suggests that natal larval supply (larval retention in the estuary) (Bousfield, 1955; Flavell, 1996) could play an important role in maintaining *B. improvisus* populations, since large and abundant populations are more stable than the small and sparse populations. It is also important to consider that the disappearance of the populations can be caused by stochastic disturbances such as pollution (Darwin, 1854; Jone and Crisp, 1953). In this case, larval supplies from other populations then play an important role in the recolonisation of the population.

2.4.2 Causes of isolated individuals in *B. improvisus* populations

In this study, more isolated individuals were found of *B. improvisus* than of *E. modestus* (see fig. 2.8). There are two possible reasons for this.

Firstly, competition may exist between these two species of barnacles. As a result of higher larval supply and the 1-2 month earlier settlement found in *E. modestus* compared with *B. improvisus* (see fig. 2.14b & c), in a space-limited situation, the species that is more abundant should have an ability to occupy more space and pre-empt the space which other species could occupy, thus forcing the less competitive species to attach themselves elsewhere in more isolated environments. However, the availability of free space for settlement in this study was more than 50% all year round (See fig. 2.14a). Thus, the competition for space between these two barnacle settlements should not be the cause of isolated individuals found in *B. improvisus*.

Secondly, *B. improvisus* has been recorded as a species capable of self-fertilisation (Furman and Yule, 1990). Therefore, this barnacle species can settle far from their conspecific species without affecting the ability to breed. In contrast, cross-fertilisation in *E. modestus* is obligate (Crisp, 1954; Barnes and Crisp, 1956). Therefore, such barnacle species may prefer to settle close to their con-specific species. As far as the self-fertilisation behaviour in *B. improvisus* is concerned, the average length of penis in *B. improvisus* was about 0.5 - 0.6 cm (see fig. 2.9a), and *B. improvisus* frequently settle within the range of 0.4 –1.4 cm (see fig. 2.8). This suggests that the penis of *B. improvisus* could have an ability to extend about 2 times of its original length. In *S. balanoides*, Stubbings (1975) suggested that the penis of this barnacle could extend about 2.5 times of its body length.

The data above show that *B. improvisus* prefer to settle near their conspecific species, and as such, cross-fertilisation occurs much more frequently than self-fertilisation. Self-fertilisation would only occur when cross-fertilisation cannot take place (isolated individuals). Therefore, the ability of selfing in *B. improvisus* is likely to be the key factor accounting for the observation that more isolated individuals of *B. improvisus* are seen than of *E. modestus*

2.4.3 Recruitment

The abundance of adult barnacles could be used as an indicator for the amount of reproductive output (planktonic larvae) for their particular population, but not for larval supply, since the planktonic larvae spend several weeks in the water column and the ecosystems of most marine sessile organisms are considered to be an open system (Roughgarden *et al.*, 1987). Therefore, their larval supplies are not necessarily derived from their own indigenous populations. They can come from different sources including ships. This could mean that the abundance of recruits might not necessarily be correlated with the abundance of their adult in the population.

In estuaries or rivers, the situation might however be different, since there are several reports of larval retention within estuaries (Bousfield, 1955; de Wolf, 1973; Hedgecock, 1982). Flavell (1996) provided good evidence for *B. improvisus* in Southampton Water. Therefore, the majority of larval supply within an estuary should come from the populations themselves within the estuary. If this is the case, the abundance of adults within estuaries might be directly related to the abundance of their larval supplies, and their own larvae must therefore be the key source maintaining the population. From comparing settlement and recruitment between *B. improvisus* and *E. modestus* at Cobden bridge (river Itchen), it was seen that recruits of *E. modestus* was much higher than that of *B. improvisus*. As described above, more recruitment of *E. modestus* could

be the result of more reproductive output and larval supply produced by *E. modestus*, since *E. modestus* adults are also much more abundant than *B. improvisus* adults (see fig. 2.14b & c).

Section Two

Molecular ecological and evolutionary studies on

Balanus improvisus

Chapter Three

The development of molecular markers for an acorn barnacle, *Balanus improvisus*

3.1 Introduction

Molecular tools are now widely used and play an important role in ecological and evolutionary studies. There are now various types of genetic markers available (see section 1.4). However, different markers provide useful information in answer to different ecological and evolutionary questions. Therefore, choosing a suitable genetic marker is dependent on various factors such as the genetic information available for the organism under study, the answer required, time, and funding available (see Avise, 1994).

As far as genetic markers for barnacles are concerned, allozymes have been widely used for population genetic studies in many barnacles such as *Semibalanus balanoides* (Flowerdew, 1983; Holm and Bourget, 1994), *Tetraclita squamosa rubescens* (Ford and Mitton, 1993), *Elminius modestus* (Flowerdew, 1984), *Chthamalus spp.* (Hedgecock, 1979; Dando *et al.,* 1979; Dando and Southward 1980, Pannacciulli *et al.,* 1997) and *B. improvisus* (Furman, 1989b, 1990; Furman *et al.,* 1989). There are also a few DNA markers available for barnacles including 18S rRNA (Spear *et al.,* 1994), Cytochrome oxidase subunit I and II (Van Syoc, 1994, Power *et al.,* 1999), 12S rDNA (Mokady *et al.* 1999) and microsatellite markers for *Semibalanus balanoides* (Dufresne *et al.,* 1999).

In this chapter, the development of genetic markers (DNA markers) for *B. improvisus* will be described. As there was no genetic information nor markers available for *B. improvisus*, RAPD (see section 1.4) was the first approach tested in this study. In addition a few sets of mtDNA universal primers were also employed to produce the species-specific mtDNA marker for *B. improvisus*. Finally, microsatellite markers were also developed for the specific purpose of studying mating strategies in *B. improvisus*.

3.2 Materials and methods

3.2.1 DNA extraction

The samples were stored in absolute ethanol at –20°C. They were rinsed with sterile distilled water prior to implementing the DNA extraction procedure. Only the thorax was used for DNA extraction of *B. improvisus*. This extraction protocol for this barnacle was modified from the CTAB protocol developed by Doyle and Doyle, (1991). 50μl of 10 mg/ml proteinase K was added to the 1.5ml microcentrifuge tube containing the thorax of *B. improvisus*. The tissue was homogenised using a micropestle, and then 200μl of TEN buffer pH 8.5 with 0.5% SDS [0.1M Tris-HCl (pH 8.0), 0.05M EDTA, 0.2M NaCl, and 0.5%SDS] was added. The mixture was gently mixed, and then incubated at 55°C for 3 hours. 1 volume (250μl) of 2.5% CTAB solution (pH 8.0) [2.5% (w/v) CTAB, 1.4M NaCl, 0.2% (v/v) 2-mercaptoethanol, 0.02M EDTA, and 0.1M Tris-HCl (pH8.0)] was then added, and further incubation was carried out at 55°C for 30 minutes. After that 1 volume (500μl) of chloroform:IAA (24:1) was added and vortexed briefly. The solution was then centrifuged at 15,800g for 5 minutes. The supernatant was recovered and placed in a new 1.5ml microcentrifuge tube, and the DNA was then precipitated with 2 volumes (1ml) of ice-cold absolute ethanol and placed at -20°C for 2 hours or overnight. After that, the solution was centrifuged at 15,800g for 20 minutes to collect the DNA pellet. The supernatant was discarded and 250μl of 70% ethanol was added

for rinsing the pellet. The tube was then centrifuged at 15,800g for 10 minutes. The supernatant was discarded, and the pellet was dried under vacuum for 20-30 minutes. The DNA pellet was resuspended in 20μl of TE buffer (pH8.0) [0.01M Tris-HCl, and 1.00mM EDTA] and stored at -20°C until further use.

3.2.2 DNA quantification

The purified DNA was used, and was quantified by agarose gel electrophoresis alongside known quantities of λ phage DNA or more accurately using a fluorometer (TKO 100 (Hoefer)).

3.2.2.1 Gel electrophoresis

Rough quantification of extracted DNA was carried out using known amounts of λ DNA (e.g. 250, 125, 62.5, 31.25, 15.6ng in a total volume of 10μl). DNA samples and the λ DNA dilution in a total volume of 10μl were loaded onto a 1 % (w/v) agarose gel containing 1% of agarose in 1X TBE pH 8.3 [diluted from 10X (0.89M Tris-HCl, 0.89M boric acid, and 0.02M EDTA)], and 0.2mg/ml of ethidium bromide. The gel was run at 100 volts for 30-45 minutes, and then visualised on a transilluminator and a Polaroid photograph was taken. The concentration of the DNA was estimated by comparing the intensities of signals obtained with those of the λ DNA dilutions.

3.2.2.2 Fluorometer quantification

A TKO 100 mini-fluorometer (Hoefer) was used to quantify DNA concentration by the use of bis-benzimidazole, known as Hoechst 33258, which binds to DNA. The procedure was as described in the manufacturer's instructions.

3.2.3 Random amplified polymorphic DNA (RAPD)

20 different RAPD primers (Kit B, Operon) were tested in PCR reactions as follows: a 25μl PCR reaction was set up consisting of ~20ng of template DNA, 1x PCR buffer (Promega), 2.5mM $MgCl_2$, 0.2mM dNTPs, 8pmol of RAPD primer (Operon), and 1.5 units of *Taq* polymerase (Promega). PCR parameters routinely employed were: 95°C for 4 minutes, followed by 35 cycles of 94°C for 1 minute, 35°C for 1 minute, and 72°C for 2 minutes, and a final extension step of 1 cycle at 72°C for 7 minutes. 25μl of RAPD-PCR reactions were loaded onto a 1.5% (w/v) agarose gel containing 0.2mg/ml ethidium bromide. The gel was then run at 100 volts for 3 hours and visualised on an UV transilluminator. 200ng of 100bp DNA ladder (Gibco BRL) was employed as a size standard DNA marker and to facilitate the comparison of RAPD profiles between gels.

3.2.4 mtDNA PCR and sequencing of 16s rRNA

3.2.4.1 PCR amplification of 16s rRNA fragment

The target DNA fragment, the 16s rRNA region in the mtDNA, was successfully amplified using the universal primers which have been used in a range of animals from invertebrates to mammals (Palumbi *et al.*, 1996a). The primer sequences were as follows:

```
Forward primer: 16SAF 5′-CGC CTG TTT ATC AAA AAC AT-3′
Reverse primer: 16SBR 5′-CCG GTC TGA ACT CAG ATC ACG T-3′.
```

A 100μl PCR reaction contained 30-50ng genomic DNA, 1X PCR buffer containing 1.5mM $MgCl_2$ (Perkin Elmer), 0.1mM of each dNTP (Deoxyribonucleoside triphosphates), 10pmol of each primer, and 1.5 units of *Taq* Polymerase (Perkin Elmer). The PCR conditions consisted of 2 minutes at 95°C for initial denaturation

followed by 40 cycles of 1 minute at 94°C, 1 minute at 42°C, and 1 minute at 72°C with the final extension for 6 minutes at 72°C. 10μl of PCR product was then checked on a 1% agarose gel using a 100bp ladder (GibcoBRL) as a standard marker (about 200ng/lane).

3.2.4.2 Sequencing the PCR product (16s rRNA)

A. The preparation of single stranded DNA for sequencing

Double-stranded DNA (PCR product) was separated using streptavidin-coated magnetic beads (Dynabeads M-280, Dynal, Inc.) (Palumbi, 1996b). This protocol relies on the use of 5' biotinylated primer (16SBR) together with an unlabelled primer in the PCR reaction. PCR products will therefore have a 5'-end biotinylated strand. Biotin binds strongly to the beads. The PCR product was denatured using 0.2M NaOH and a magnetic rack (Dynal, Inc.) was used for separating DNA strands.

40μl of a streptavidin-coated magnetic bead solution was added to a 1.5ml micro-centrifuge tube. The storage buffer was removed using a magnetic rack as the magnetic beads were pulled to the bottom of the tube by magnetic force. The beads were then washed twice with 200μl of bead washing buffer (2M NaCl, 1mM EDTA, 10mM Tris-HCl pH 7.5). The beads were not allowed to become dry during any stage of the procedure. 90μl PCR product was then added to the beads, and the solution was gently mixed by rotating the tubes slowly for 25-45 minutes to allow the beads to bind to the biotin, after which, the beads were washed again using 200μl washing buffer twice. The PCR product was denatured by incubating for 10 minutes at room temperature with 20μl 0.2M NaOH. 400μl of absolute ethanol (-20°C) was added to a new 1.5 microcentrifuge tube, and then kept on ice. The 0.2M NaOH was then removed using the magnetic rack. Then, 100μl of 0.2M NaOH was added and

immediately removed. Both 20 and 100μl of 0.2M NaOH removed was then taken up into the tube containing 400 μl of -20°C absolute ethanol and kept at -20°C for 30 minutes or until needed. The beads were washed three times with 200μl 4°C sterile water to remove NaOH. After these washes, 12μl sterile water was added. The solution was then kept on ice ready for the sequencing reaction.

B. Sequencing and termination reaction

The sequencing reaction was carried out using a T7 sequencing kit (Pharmacia) following the manufacturer's instructions for single-stranded DNA sequencing. 2.5μl of four dideoxynucleotide mixes (ddATP, ddCTP, ddGTP, and ddTTP) was added to a 0.5ml microcentrifuge tubes separately and kept on ice. Primer annealing solution was then carried out by adding 11μl (1.5-2.0μg) of template, 1μl of 2.5μM primer stock, and 2μl of annealing buffer (Phamacia) [1M Tris-HCl (pH 7.6), 100 mM $MgCl_2$ and 160mM DTT] (the total volume is 14μl). The solution was gently mixed, incubated at 60°C for 10 minutes and allowed to cool down to room temperature for 10 minutes. During this time, the labelling reaction was prepared by mixing 3μl of labelling mix A (1.375μM each dCTP, dGTP, dTTP, and 333.5mM NaCl), 2.0μl of diluted T7 polymerase (diluted 1:4 in dilution buffer 20mM Tris-HCl (pH7.5), 5mM DTT 100mg BSA/ml and 5% glycerol), and 1μl of α^{35}S dATP (Amersham). The labelling reaction was then added to the annealing reaction, gently mixed and incubated at room temperature for 5 minutes. In the meantime, the microcentrifuge tubes containing each dideoxynucleotide were warmed to 37°C for at least 1 minute. Finally, 4.5μl of the labelling mixture was added to each of these tubes, incubated at 37°C for 5 minutes and stopped using 5μl of stop solution [0.3% of each bromophenol blue and xylene cyanol FF, 10mM EDTA (pH7.5) and 97.5% deionized formamide]. The beads were then removed and the solution was ready for electrophoresis.

C. Electrophoresis and autoradiography

The samples (sequencing reactions) were run on a 6% acrylamide gel. For the 21x50cm^2 gel size (Bio-Rad), 60ml of acrylamide gel solution were prepared containing 9ml of 40% (w/v) acrylamide:bis (19:1) (Appligene), 6ml of 10X TBE pH 8.3 [0.89M Tris-HCl, 0.89M boric acid, and 0.02M EDTA], 28.8 g of urea, 450μl of 10% APS, and 100μl of TEMED. [Alternatively, the ready mixed 6% acrylamide gel solution (National Diagnostics) was used to which 450μl of 10% APS was added] After the gel had set (about 90-120 minutes) the gel was pre-run for 45-60 minutes at 40w using 1X TBE as an electrophoresis buffer. Wells on the top of the gel were then cleaned with 1X TBE to remove air bubbles or urea, which can cause smearing of the sequencing bands. 2μl of each sequencing reaction was then loaded using duckbilled pipette tips after heating at 95°C foe 3 minutes. The electrophoresis was carried out at 40w for 2 hours. Then, if longer running was required (i.e. more sequence information was needed), the same set of samples was loaded and run at 40w for another 3-4 hours. The gel was fixed for 20 minutes using 10% acetic acid and 10% methanol before transferred onto Whatman 3MM filter paper, and covered with cling film, and dried at 80°C for 2 hours using a gel dryer (*Bio RAD*). The cling film was then removed and the gel was exposed to X-ray film for 2 days (or more depending on the radioactive activity). The film was then developed using Kodak developer (up to 5 minutes) and fixer 5-10 minutes, and 3% acetic acid served as stop solution (5 minutes). It was rinsed in running tap water for 20 minutes and then washed with distilled water for the final cleaning.

3.2.5. Mitochondrial DNA polymerase chain reaction-restriction fragment length polymorphism (mtDNA PCR-RFLP)

3.2.5.1 PCR of COI-COIII region

Four universal primers were initially tested: namely COI RLR, COII-Corz, CO2a, and COIII (Brown *et al.,* 1992 and Roeherdanz, 1993). Only one combination (COI RLR and COIII) produced a good amplification product. The primers were designed from *Daphnia sp.* and *Homarus sp.* sequences (COIII) and *Apis mellifera* and *Drosophila yakuba* (COI-RLR) (Brown *et al.*, 1992 and Roeherdanz, 1993 respectively). The sequence of these primers were as follows:

```
Forward primer COI-RLR 5'-TTG ATT TTT TGG TCA TCC AGA AGT-3'
Reversed primer COIII  5'-AAC ATC TCG TCA TCA TTG A-3'
```

A 25μl PCR reaction contained 10-20ng/μl template DNA, 1x PCR buffer (Advanced Biotechnologies), 3.5mM $MgCl_2$, 0.2mM dNTPs (Phamacia), 25pmol of each primer, and 1.25 units of *Taq* Polymerase (Advanced Biotechnologies). This reaction was run on a Hybaid Thermocyler (block control), 95°C for 2 minutes, followed by 30 cycles of 94°C for 30 seconds, 57°C for 1 minute and 68°C for 2 minutes and 30 seconds and the final extension at 72°C for 7 minutes.

3.2.5.2 Sequencing of COI-COIII PCR product

A. Ligation and transformation of PCR product using T-vector

25 μl PCR was loaded and run onto a 1% (w/v) agarose gel at 80 volts for 1 hour. The expected PCR band was excised and recovered using Hybaid Recovery™ DNA Purification Kit II (Hybaid). 1μl of recovered DNA mixed with 2μl of loading buffer, and

7μl of distilled water was again run on a 1% (w/v) agarose gel with known amounts of marker DNA for approximate quantification. Ligation and transformation of the PCR product was carried out by pMOS*Blue* T-vector Kit (Amersham). For the ligation step, the quantity of insert required can be calculated using the following formula (Amersham):

$$\frac{\text{Size of insert (bp)} \times \text{Amount of vector (ng)}}{\text{size of vector (bp)}} \times \frac{7.5}{1} = \text{ng of insert}$$

10μl ligation reaction contained : 1μl of 10x ligase buffer [660mM Tris-HCl (pH 7.6), 66mM $MgCl_2$, 100mM DTT, and 660μM ATP], 0.5μl of 100mM DTT, 0.5μl of 10mM ATP, 0.5μl of 50ng/μl pMOS*Blue* T-vector, 'X'μl of amplified product (depending on the concentration of recovered DNA), and 0.5 μl of 4 units/μl T4 DNA ligase. Then, the reaction was made up to 10μl using nuclease free water. The reaction was incubated at 16°C for 1 hour and kept overnight at 4°C. The recombinant vectors were then transformed into MOS*blue* competent cells (Amersham) by adding 1μl of ligation reaction into the tube containing 20μl of competent cells. The tube was left on ice for 30 minutes. Then, the cells were heat-shocked at 42°C for exactly 45 seconds. After that, the tube was placed on ice for 2 minutes. 80μl SOC medium was then added in to it. The tube was incubated at 37°C with shaking at 200-250rpm for 1 hour. 50μl of transformation solution was used for spreading onto LB-agar antibiotic plates containing [LB agar (Tryptone 10g/l, yeast extract 5g/l, NaCl 10g/l, agar 15g/l), 50μg/ml ampicillin, 50μg/ml IPTG, 50ug/ml X-gal]. The inverted plate was incubated at 37°C overnight.

B. Screening and checking recombinant plasmids

White colonies are the putative colonies containing recombinant plasmids. Colonies were picked using pipette tips and transferred into 15ml Falcon tubes containing 5ml of autoclaved LB solution (pH 7.0) [10g/l of bacto-tryptone, 5g/l of bacto-yeast extract, 10g/l of NaCl] containing 50μg/μl ampicillin. The tube was incubated at 37°C with shaking at 200-250rpm overnight. 4ml of bacteria culture were used for plasmid purification following the standard protocol of Sambrook *et al.*, (1989). The purified plasmid DNA was digested with restriction endonucleases, *Pst* I and *EcoR* I (Appligene) for checking the insert DNA (the endonuclease used depends on the cutting sites available in the plasmid used). After checking the insertion, 850μl of the culture containing the positive clone were mixed with 150μl of glycerol and kept at -20°C for further use (if required). The positive colony was recultured in 5ml of LB solution as describe above, and then purified using Wizard miniprep. (Promega) following manufacture's instructions. This purified plasmid DNA was used for sequencing.

C. Sequencing and termination reaction

The sequencing reaction was performed by a standard double-strand sequencing protocol using the T7 sequencing Kit (Phamacia). The concentration of the purified plasmid DNA was adjusted to approximately 3-4μg in 32μl of dissolved DNA solution for forward and reverse sequencing reactions (1.5-2.0μg is required per reaction). The 32μl of double stranded plasmid DNA was denatured by adding 8μl of 2M NaOH. The reaction was incubated at room temperature for 10 minutes. 7μl of 3M sodium acetate (pH 4.8) and 4μl of distilled water were added, and then the solution was gently mixed. The DNA was precipitated using 120μl of ice-cold absolute ethanol. The DNA was then pelleted by centrifugation at 15800g for 15 minutes. The supernatant was discarded. The DNA pellet was gently washed with 120μl of 70% ethanol and recollected by

centrifuging at 15800g for 10 minutes. The supernatant was then removed. The DNA pellet was dried in vacuum, and dissolved with 14μl of distilled water, and ready for the annealing reaction.

The annealing reaction consisted of 7μl of denatured template DNA (1.5-2 μg), 2μl (5-10pmol) of primer (The primer used depend on the vector), 2μl of annealing buffer (1M Tris-HCl (pH 7.6), 100mM $MgCl_2$, and 160mM DTT), and 3μl of DMSO. The annealing reaction was incubated at 37°C for 20 minutes to anneal the primer, and then placed at room temperature for at least 10 minutes. The labelling and termination reaction were then prepared as described in section 3.2.4.2 B. In this study, primer T7 and U19 were utilised first because the vector used (pMOS*Blue*) contains these sites at either end of the multiple cloning sites. Since the PCR product is approximately 2.9kb, other internal primers were required from the sequence produced by the previous primers to get further sequences. For example, primers Seq1F and Seq1R were designed from the sequences produced by T7 and U19 primer. Primer sequences are as follows:

T7	5′-TAA TAC GAC TCA CTA TAG GGA-3′
U19	5′-GTT TTC CCA GTC ACG ACG T-3′
Seq1F	5′-ACG GAG CAC AAT TTT CAT AT-3′
Seq1R	5′-CGA ATT GAG CAG CTG TTA CT-3′
Seq2F	5′-AGC AGG TAT ACC TCG TCG AT-3′
Seq2R	5′-GCA ATA TGA GCT GTG CT-3′
Seq3F	5′-CGA ACA ACC TTC CTA GT-3′
Seq3R	5′-TCT GGA GTA ATA GGT GA-3′
Seq4F	5′-ATA TTC CAC GAC CAC GC-3′

D. Electrophoresis and autoradiography

The sequencing reactions were denatured by heating at 75-80°C for 2 minutes and placed on ice before running. 2.0μl of each denatured reaction was loaded onto a 6% denaturing acrylamide gel. Sequencing gel was prepared using ready mixed polyacrylamide solution (National Diagnostics). The details of gel running and autoradiography have already been described in section 3.2.4.2 C.

E. Sequence analysis

The sequence obtained for the COI-COIII region of *B. improvisus* was used to search for any similar sequence in the GenBank database using BLAST program (available at http:\\www.ncbi.nlm.nih.gov). To identify the gene organisation of the sequence, it was aligned with known sequences using Clustal V (Higgins *et al.*, 1992) and translated to an expected amino acid sequence using OMIGA (1.1.3) computer packages.

3.2.5.3 Designing species-specific PCR primers for *B. improvisus*

Since the primer (COI-RLR and COIII) produced inconsistent PCR results for COI-COIII in *B. improvisus*, specific primers for *Balanus improvisus* were designed. Based on the *B. improvisus* COI-COIII sequence alignment with *Drosophila yakuba*, and *Daphnia pulex* sequences. A forward primer (BI-COIF) was designed in COI gene, 193bp down-stream from COI-RLR. The reverse primer (BI-ATP6R) was designed in ATPase6 gene, 166bp up-stream from COIII primer. The primer sequences and PCR conditions were as follows:

BI COIF--------5'GAT ACC CGA GCT TAT TTT AC 3'

BI ATP6R-------5'ATA CTT CTC TAG CAT ATA AAG T 3'

A 10μl PCR reaction contained 10-20ng/μl template DNA, 1x PCR buffer (Advanced Biotechnologies), 3.5mM $MgCl_2$, 0.2mM dNTPs (Phamacia), 10pmol of each primer, and 0.75 units of *Taq* Polymerase (Advanced Biotechnologies). PCR reactions were

performed on Hybaid Omnigene Thermocyler (block control) using 94°C for 2 minutes, followed by 30 cycles of 94°C for 1 minute, 51°C for 1 minute and 72°C for 2 minutes and a final extension at 72°C for 7 minutes.

3.2.5.4 Investigation of PCR-RFLP polymorphism

A. Samples

The samples used in this investigation were from:

- River Itchen (Cobden bridge), Southampton
- River Hamble, (Manor Farm Country Park), Southampton
- River Thames (Greenhithe), London

(see details in Chapter 2, section 2.3.1)

B. Digestion of PCR products

PCR products produced by BI-COIF and BI-ATP6R were tested with various restriction endonucleases. Restriction reactions consisted of 1X Buffer (depending on enzyme used), 0.5-1.0 unit of restriction enzymes, and 'X'μl (10-20ng)of PCR product. The reactions were then made up to 10μl using nuclease free water. The reaction was then incubated at the optimal temperature recommended by the manufacturer for at least 2 hours or overnight.

C. DNA fragment visualisation

2μl of loading buffer [0.25% bromophenol blue, 0.25% xylene cyanol FF, 40% (w/v) sucrose in water] was added into digestion reactions. 10μl of the reactions were loaded onto 8% polyacrylamide gels (17ml of 40% Acrylamide stock (Appligene), 8.5ml of 10xTBE buffer (as described in section 3.2.2.1), 560μl 10% APS and 65μl of TEMED) and run at 150 volts for 4 hours using Protean®II Xi Cell (Bio RAD). 1X TBE was used

as an electrophoresis buffer. For *Nde*II, the reactions were run on 4% polyacrylamide gel to produce better resolution of RFLP profiles. The profiles were visualised by sliver staining described below.

The gel was gently removed from the glass plates and immersed twice in 100ml of solution A (fixation) [10% absolute alcohol, and 0.5% acetic acid in distilled water] for 4 minutes with gentle shaking. Then, solution A was removed and 100ml of solution B [0.1% sliver nitrate in distilled water] was added and the gel was stained for 10 minutes with gentle shaking. Then the gel was washed twice (1 minute each time) with 150ml of distilled water and 150ml of solution C [1.5% (w/v) of Sodium Hydroxide, 0.0001% of Sodium Borohydride, and 0.4% (v/v) of formaldehyde in distilled water] was added. The gel was gently shaken with solution C until DNA patterns could be seen. After that the gel was washed once with 150 ml of distilled water (1 minute). Then, Solution A was added and the gel was sealed in a plastic bag for analysis.

D. Scoring RFLP profiles

RFLP polymorphism is mostly a result of a point mutation, occurring in the recognition sites of restriction endonuclease. Generally, RFLP data are scored using gains or losses of the recognition sites, which produce differences in RFLP profiles (haplotypes). The frequency of composite haplotypes (the combination of haplotypes from different restriction enzymes) was also employed in the analysis of RFLP analysis. In this study, binary matrix of gain (1) and loss (0) of cutting sites and frequencies of composite haplotypes were scored.

3.2.6 The construction of a potential genomic library enriched for (CA)n repeat DNA sequences modified from Kandpal *et al.*, 1994

3.2.6.1 The preparation of genomic DNA

60 barnacles were used to provide 40-50μg of high molecular weight genomic DNA. DNA extraction protocol employed was as described in section 3.2.1. The DNA were then pooled and a standard phenol:chloroform DNA extraction (Sambrook *et al.*, 1989) was used as an extra-step to increase the purity of the DNA. The DNA was then quantified using a TKO100 mini-fluorometer (Hoefer) following manufacturer's instructions.

3.2.6.2 *Mbo*I digestion

The digestion reaction consisted of 1x digestion buffer, 100 units of restriction enzyme (*Mbo*I), 0.1μg/μl of BSA, and ~40μg of the DNA in total volume of 300μl reaction. The reaction was incubated at 37°C for 12-15 hours. A small amount (2μl) of the reaction was run on a 1 % (w/v) agarose gel to check for smearing of the DNA as an indication of complete digestion.

3.2.6.3 Recovery of digested DNA fragments

The digested DNA was run on a 1% (w/v) agarose gel. A 100bp ladder DNA marker (Gibco BRL) was used as a standard DNA size marker. DNA fragments (200-600bp in length) were then recovered from the gel using a gel extraction kit (Qiagen) following manufacturer's instructions. The recovered DNA fragments were then quantified using a TKO100 mini-fluorometer (Hoefer) following manufacturer's instruction.

3.2.6.4 The ligation of recovered DNA fragments with MBOI adapter (Kandpal *et al.*, 1994)

A. The preparation of MBOI adapter

An MBOI adapter was prepared by annealing two PAGE purified (Genosys) oligonucleotides together. In this study, Mbol F (5'-pGAT CGC AGA ATT CGC ACG AGT ACT AC-3') and Mbol R (5'-CGT AGT ACT CGT GCG AAT TCT GC-3') (Kandpal *et al.*, 1994) were used for preparing MBOI adapter. Therefore, the oligonucleotides were dissolved with TE buffer (10 mM Tris pH 7.5 – 8.0, 1 mM EDTA) at the same molar concentrations. The annealing reaction was made of equimolar amounts of the two oligonucleotides and 50–100mM NaCl. The reaction was incubated at 80°C for 2 minutes using a PCR machine (PE 480, Perkin Elmer). Then, the machine was tuned off, and the reaction was left in the block until it cooled down to room temperature (about 40–60 minutes). The annealed oligonucleotides (MBOI adapters) were then stored at –20°C for further use.

B. Ligation of genomic DNA fragments to MBOI adapters

A 300μl ligation reaction consisted of 1x ligation buffer (Promega), 70 units of T4 DNA ligase (Promega), 1–3μg of recovered DNA fragments, and 2–6μg of MBOI adapter (3 times the amount of DNA). The reaction was incubated at 16°C for 12-16 hours in a PCR machine (PE480, Perkin Elmer). To inactivate T4 ligase, the reaction was incubated at 65°C for 10 minutes, and then the ligated DNA fragments were purified using a Qiaquick PCR purification column (Qiagen) following manufacturer's instructions.

3.2.6.5 Hybridisation of MBOI-ligated DNA fragments to biotinylated $(CA)_{15}$ probe

Since the recovered DNA fragments have been ligated to the adapter, PCR technique can be used to prepare additional amounts of DNA fragments for hybridisation. A 50μl PCR reaction was made of 1x PCR buffer (Advanced Biotechnologies), 0.2mM dNTPs, 2.5mM $MgCl_2$, 1pmol of one of oligonucleotides used to prepare the adapter, 2.5 units of *Taq* polymerase (Advanced Biotechnologies), and ~25–50ng of ligated DNA fragments. The PCR parameters were: 94°C for 4 minutes, followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 120 seconds, followed by 1 cycle at 72°C for 10 minutes. The PCR product was then purified using Qiaquick PCR purification columns (Qiagen) and quantified using a TKO 100 mini-fluorometer.

A 200μl hybridisation reaction consisted of ~2μg of denatured purified PCR product (denatured by first placing in 95°C for 10 minutes and placing on ice), 1μg of biotinylated $(CA)_{15}$, 0.5M sodium phosphate buffer pH 7.4, and 0.5% SDS. The reaction was incubated at 50°C for 16-18 hours using a PCR machine (PE 480, Perkin Elmer).

3.2.6.6 The enrichment of DNA fragments containing CA-repeated sequences

A. The preparation of salmon sperm DNA

Salmon sperm DNA was used in prehybridisation step. It helps to increase the specificity of hybridisation. 1g of salmon sperm DNA (Sigma) was dissolved in 100ml of 0.4M NaOH, in a sterile flask and the solution was stirred overnight. It was then boiled in a water bath for 45 minutes and placed on ice. The pH of the solution was adjusted to pH 4 - 7 with glacial acetic acid, while it was still on ice. The solution was then centrifuged at 4000g for 10 minutes and the supernatant was removed to a new flask. The DNA was precipitated with 2 volumes of absolute ethanol and then kept at -20°C overnight. The DNA was pelleted by centrifugation for 10 minutes. The supernatant

was removed and the DNA pellet was rinsed with 70% ethanol, desiccated, and resuspended in 50ml of TE buffer. The DNA concentration was measured using a spectrophotometer at 260nm wavelength (1 OD unit is equivalent to 50μg/ml of DNA). The concentration was adjusted to 10mg/ml and stored in aliquots at -20°C.

B. The capture of DNA fragments containing CA-repeated motif sequences using streptavidin-coated magnetic beads

Streptavidin-coated megnetic beads (Promega) were used to bind to the CA-enriched fraction of the genomic DNA through the strong nature of streptavidin-biotin binding. The beads were first washed 3 times with 300μl of buffer A [100mM tris-HCl pH 7.5 and 150mM NaCl]. They were then incubated with 300μl of buffer A and 100μg/ml denatured salmon sperm DNA at room temperature for 30-35 minutes. The beads were washed again 3 times with 300μl of buffer A, and then used for genomic DNA binding. The 200μl hybridisation reaction was added and mixed to the beads. The solution was then incubated at room temperature for 30-35 minutes to allow the beads to bind to biotinylated $(CA)_{15}$ probe. The beads were then washed a number of times as shown in table 3.1

Wash	Solution (200 μl)	Temperature (°C)	Duration (minutes)
1	Buffer A	room temperature	5
2	Buffer A	50	5
3	Buffer A	50	5
4	1/10 Buffer A	50	5
5	1/10 Buffer A	65	5
6	sterile water	65	5

Table 3.1 Protocol for selecting DNA fragments containing CA-repeat sequences

DNA fragments from each wash were recovered using a magnetic rack as described in section 3.2.4.2 A. Then, they were purified using Qiaquick PCR purification kit (Qiagen) and kept at –20°C for further use.

3.2.6.7 Checking enrichment using Southern blotting

A. PCR of DNA fragments for hybridisation

The purified DNA fragments from each wash were used as template DNA for PCR. The PCR conditions was as described in section 3.2.6.5, but 10μl PCR reactions were set up instead of 50μl PCR reaction and 0.75 unit of *Taq* polymerase was used instead of 2.5 units. PCR reactions were then run on a 0.8% agarose gel.

B. Hybridisation using ^{32}P-labelled CA-repeat probe

Vacublotting

The PCR products run on a 0.8% agarose gel were transferred onto a piece of nylon membrane (Hybond N+, Amersham) using a vacublot apparatus (Phamacia). Since the size of PCR product is less than 10 kb, the recommended initial depurination step is not necessary. 100ml of denaturation buffer [1.5M NaCl, and 0.5M NaOH] was poured on top of the gel for 20 minutes. The denaturation solution was then removed and replaced by neutralisation buffer [1.5M NaCl, 0.5M Tris-HCl (pH7.2), and 0.001M EDTA] for 15 minutes. The neutralisation buffer was removed and the blotting tray was filled with transfer buffer (20X SSC (pH 7.0)) [3M NaCl, and 0.3M Na_3citrate] for 1 hour and 30 minutes, making sure that the gel was immersed in 20X SSC during transferred process. The membrane was lifted off the vacublot and the DNA on the membrane was fixed by placing the membrane onto 2-3 pieces of Whatman 3MM filter paper soaked with 0.4M NaOH for 20 minutes. The membrane was then rinsed in 2X SSC for less

than 1 minute and air dried. After that the membrane was placed between 2 pieces of dry whatman 3MM filter paper and wrapped with aluminium foil, and then incubated at 50°C overnight or used directly for the next step (hybridisation).

Prehybridisation

The membrane was immersed in 2X SSC (diluted from 20X SSC stock see above) and rolled up along with a clean nylon mesh and placed in the hybridisation bottle. 100ml of 2xSSC was poured into the bottle and the membrane was unrolled onto the side of the bottle by gently rolling the bottle on a bench top. The solution was then poured out and 10ml of prehybridisation solution was then added. It contains: 0.75ml of autoclaved 20X SSPE pH 7.4 [3.6M NaCl, 0.2M Sodium phosphate, 0.02M EDTA pH 7.7], 0.5ml of 20% SDS, 0.5ml of 10% (w/v) Blotto [1g of skimmed milk powder in 10ml of sterile deionised water], 0.5ml of 10mg/ml of denatured salmon sperm DNA [boiled for 10 minutes and kept on ice], and 7.75ml of sterile deionised water. The bottle was then placed in hybridisation oven, at 65°C with gentle rotation for 3-4 hours.

^{32}P- Labelling of probe

20ng of a CA/GT polynucleotide (Phamacia) was labelled using a random primed DNA labelling kit (Boehringer Mannheim) following manufacturer's instructions and used as a probe for a 20x20 cm^2 nylon membrane.

Hybridisation

10ml of hybridisation solution was made up with 0.75ml of 20X SSPE [described in section 3.2.6.7.2.2], 0.5ml of 20% (w/v) SDS, 2ml of 50% (w/v) Dextran sulphate, 0.5ml of 10% boltto [described above], and 6.25ml of sterile deionised water and prewarmed at 65°C. The prehybridisation solution was removed from the bottle and replaced by the

hybridisation solution containing ^{32}P-labelled probe. The membrane was then incubated at 65°C with gentle rotation for 12–15 hours or overnight.

Washing membrane

The membrane was washed as follows. The hybridisation solution was removed and replaced by washing solution I containing 2X SSC (see above), and 1% SDS and left rotating at 55°C for 15 minutes. The second wash was carried out in a sandwich box at 55°C using washing solution II containing 0.5X SSC and 0.1% SDS. The membrane was checked every 3 minutes with a Geiger counter to prevent over-washing and then the membrane was sealed in a plastic bag.

Autoradiography

The sealed membrane was placed in an autoradiograph cassette with an X-ray film (Biomax, Kodak) and kept at -70°C up to 7 days depending on the radioactive signal tested when sealing the membrane. The X-ray film was developed in the dark room as described in section 3.2.4.2 C

3.2.6.8 Cloning CA-repeat enriched genomic DNA fragments into pGEM-T vector

DNA from the wash identified as the most enriched CA–repeat was used in another PCR reaction using the conditions described in section 3.2.6.5. In order to obtain maximum A-tailing to increase efficiency of cloning PCR products in to pGEM-T, an additional step of A-tailing was carried out by adding 1 unit of *Taq* polymerase and 25mM of dATP after the PCR cycle had been completed, and incubated at 72°C for 15 minutes. The PCR products were then purified using a Qiaquick PCR purification column (Qiagen) and quantified using TKO 100 mini-fluorometer (Hoefer). The purified PCR products were ligated to pGEM-T Easy vector (Promega) and transformed into JM 109 competent cells (Promega) following manufacturer's instruction. The ratio between

the amount of vector to DNA fragments used in this study was 1:6. Approximately, 100ng of vector was used in the ligation reaction (for more details see section 3.2.5.2 A).

3.2.6.9 Identification of recombinant plasmids with inserts containing CA repeats

A technique called PCR-based isolation of microsatellite arrays, PIMA, (Lunt *et al.* 1999) was used to identify CA-repeat containing plasmids. This technique is based on the use of a repeat-specific primer (5'TGT GGG CGG CCG $C(TG)_8V$ 3') as a primer along with two primers specific to the vector (M13 forward 5'GTT TTC CCA GTC ACG AC3' and reversed (5'CAC AGG AAA CAG CTA TGA C3') in PCR reactions. If colonies contained CA-repeat sequence, two PCR products will be produced (one across the insert from the two vector primers and one from the CA-repeat to the vector site).

10μl PCR reaction contained 1X PCR buffer (Advanced Biotechnologies), 2.5mM MgCl2, 0.2mM dNTPs, 1.0pmol of each primer, 0.5 unit of *Taq* polymerase (Advanced Biotechnologies) and 1μl of DNA (obtained by boiling bacterial colony with 10μl of HPLC water (Sigma)). Touch down PCR was carried out to obtain better results using an OmniGene thermocycler (Hybaid). PCR conditions were as follows: one cycle at 94°C for 5 minutes, followed by one cycle of (94°C, 30 seconds, 58°C, 30 seconds, and 72°C 30 seconds), one cycle of (94°C, 30 seconds, 56°C, 30 seconds, and 72°C 30 seconds), 28 cycles of (94°C, 30 seconds, 55°C, 30 seconds, and 72°C 30 seconds) and a final cycle at 72°C for 10 minutes.

The PCR reactions was loaded onto a 1.2% (w/v) agarose gel and run at 100 volts for 1 hour. Polaroid photographs were taken and used to identify positive colonies (see fig 3.8).

3.2.6.10 Sequencing of positive clones

Positive clones were purified for sequencing using the standard protocol (Sambrook, 1989), but the DNAs were precipitated without Phenol:chroloform step, and further Purified using Qiaquick PCR purification kit (Qiagen). Using this protocol produced better and more consistent result in sequencing than using plasmid purification Kit (See fig. 3.9)

Sequencing reactions were carried out using the "BigDye" cycle sequencing protocol (Perkin Elmer) following manufacturer's instruction. Briefly, the sequencing reactions contained 4μl of terminator ready mix (Perkin Elmer), ~100-250ng of purified plasmid DNA, 1.6pmol of primer (M13 forward or M13 reverse). The reactions were made up to 10μl using sterile water and then run on PE 2400 PCR machine (Perkin Elmer). PCR condition was as follows: 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. After that, the reactions were made up to 20μl with sterile water and precipitated following manufacturer's instruction and run on ABIPrism 377 automatic sequencer (Perkin Elmer).

3.2.7 Microsatellite locus PCR optimisation

3.2.7.1 Primer design

Primers were manually designed to recognise suitable microsatellite flanking regions and the melting temperature of the primers, possibility of primer dimer formation and annealing to each other were checked by primer calculator available on http://williamstone.com/primer/index.html or http://www.genosys.co.uk/cgi-win/oligo_calconly.ex

3.2.7.2 PCR conditions

A range of conditions varying in $MgCl_2$ concentration and annealing temperature regimes was tested for each locus. Broadly, 10μl PCR reaction contained 1X PCR buffer (Qiagen), 1X Q solution (Qiagen), XmM $MgCl_2$, 10pmol of primers, 0.25 unit of Hot start *Taq* polymerase (Qiagen), and 5–10ng of DNA template. The PCR conditions routinely used were 1 cycle of 95°C for 15 minutes, 30-35 cycles of 94°C 30 seconds, X°C annealing temperature for 30 seconds, and 72°C for 30 seconds, followed by 1 cycle of 72°C for 15 minutes (see table 3.4 for further details)

3.2.7.3 Electophoresis on ABI 377 automated sequencer

Once a specific PCR product was obtained upon PCR optimisation, microsatellite PCR were carried out using one of the primer 5'end fluorescent-labeled (TET, HEX, or FAM) (MWG Biotechnologies) and run on ABI prism 377 automatic sequencer (Perkin Elmer). Tammar 350 size marker (Perkin Elmer) was used as a standard DNA size marker to facilitate the comparison of the size of DNA fragments within and between gels. GeneScan computer program (Perkin Elmer) was used for data analysis.

3.3 Results

3.3.1 DNA extraction

Using the whole body of *B. improvisus* for DNA extraction resulted in the degradation of the DNA, probably due to the presence of high levels of nucleases in the body of *B. improvisus*. However, this phenomenon did not occur in other barnacle species such as *C. stellatus*, *C. montagui*, and *E. modestus*. To solve this problem, the amounts of Proteinase K and SDS were increased, and different parts of the body were used in the extraction procedure in order to optimise DNA quality. The best quality and quantity of

1-2 μg of DNA was obtained by using the thorax alone with increased amounts of Proteinase K.

3.3.2 RAPD-PCR

To carry out a population genetic study on *B. improvisus*, a species for which no genetic information was available prior to this study, we initiated an investigation using RAPD-PCR. 20 RAPD primers (Kit B (Operon)) were tested with 10 samples of *B. improvisus* from Cobden bridge, River Itchen, Southampton. No RAPD primer produced consistent results and the variation in RAPD profiles was observed with the same DNA sample on separate PCR reactions. The technique was rejected as a possible way to study the population genetics of this species and other techniques were investigated such as mtDNA markers.

3.3.3 mtDNA sequencing of 16s rRNA gene

The universal primers (16SAF and 16SBR) produced a specific PCR product about 550bp in length for *B. improvisus* and *E. modestus*. Since this set of primers can be used to amplify the 16s rRNA gene sequence from a wide range of animals (invertebrates to mamals), human DNA was used as a control to confirm that there was no contamination of human DNA in the PCR reaction. A PCR product of about 600bp was amplified from human DNA which could be easily distinguish from the *B. improvisus* PCR product on agarose gel (see fig. 3.1 A).

Partial sequencing of the PCR product was obtained for *B. improvisus and E. modestus* (202 bp and 187 bp respectively). Both sequences were similar to 16s rRNA gene sequences of insects and copepods after a Blast search, confirming that the sequences were indeed 16s rRNA gene of the barnacles. The sequences consist of 69.8% of A+T and 30.2% of G+C content for *B. improvisus* and 68.5% of A+T and

31.5% of G+C content for *E. modestus*. By aligning these two sequences, it can be seen that there are many positions of base substitution indicating inter-specific genetic variation (see fig. 3.2). However, Intra-specific variation was not investigated since rRNA genes are mostly conserved because of functional and structural constraints (Simon *et al.*, 1994).

3.3.4 Mitochondrial DNA polymerase chain reaction-restriction fragment length polymorphism (mtDNA PCR-RFLP)

3.3.4.1 PCR of COI-COIII region of *B. improvisus*

Since The PCR product used in RFLP analysis should be greater than 1kb in length to provide a sufficiently large number of polymorphic restriction sites to be detected, COI-COIII genes of *B. improvisus* were amplified using the primers for insects and copepods (COI RLR and COIII respectively) see section 3.2.5.1. They produced a specific PCR amplification product, about 3kb in length (see fig. 3.1 B). However, this set of primers produced inconsistent results. Therefore, the PCR product was sequenced and species specific primers were designed.

3.3.4.2. The sequence of the COI-COIII PCR product and its gene organisation

The PCR product was cloned and 2987 bp of sequence obtained. It consisted of 965 A, 1161 T, 464 C, and 397 G (about 71.18% of AT and 28.82% of GC content). The gene organisation of this region was determined, by aligning the sequence with COI-COIII sequences of *Drosophila yakuba*, *A. mellifera*, and *Daphnia pulex* (Clary and Wolstenholme 1985, Crozier and Crozier 1993, Crease and Little 1997 respectively), and translating the DNA sequence into an amino acid sequence using codon information of *D. yakuba* mtDNA. The PCR product consists of a partial sequence of the COI gene, unidentified

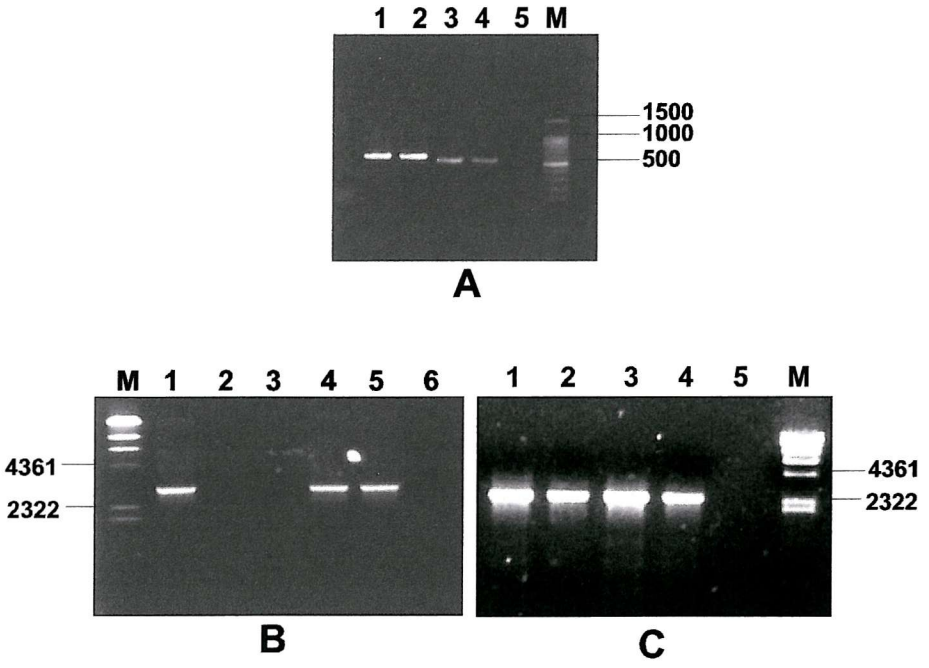


Figure 3.1 Photographs of 1% agarose gels showing: (A) amplification of 16s rRNA gene [human (lanes 1&2), *B. improvisus* (lane 3&4), negative control (lane 5), 100bp DNA ladder (Promega) (lane M)]. (B) inconsistent amplification of COI-COIII genes produced by the primers COI-RLR and COIII [*B. improvisus* (lanes 1-5), negative control (lane 6), λ *Hind*III DNA marker (lane M)]. (C) consistent amplification of COI-ATPase6 genes produced by the species-specific primers BI-COIF and BI-ATP6R [*B. improvisus* (lanes 1-4), negative control (lane 5), λ *Hind*III DNA marker (lane M)].

```
B.improvisus    TAAGATATTTTGTTGGGGCGACATTAAGATATAAAAAACTCTTTATTTTAAAAATTTTAA
E.modestus      ---------------GGGCGACATTAAGATAAAAAAAACTCTTTATTTTTTAAACTTTTA
                               *************** ***************  *** *** *

B.improvisus    TATAAATATAATTTGATCCTCTAAAAGAGATCAAAAGAAAAAATTACCTTAGGGATAACA
E.modestus      TAAAAGCTTAGCTTGATCCTTTAAAAAAGATCATAAGAAAAAATTACCTTAGGGATAACA
                ** **   **  ******** ***** ****** **************************

B.improvisus    GCGTAATCTTTTTTGAGAGTTCTAATCGACAAAAAGGTTTGCGACCTCGATGTTGGACTA
E.modestus      GNGTAATCTTTTTTGAGAGTTCTAATCGACAAAAAGGTTTGCGACCTCGATGTTGGACTA
                * ************************************************************

B.improvisus    AAATTTAGGCAGGGTGCAGCAG
E.modestus      AAATTTAGGCAAGGTGCAGCAG
                *********** **********
```

Figure 3.2 The alignment of 16s rRNA gene sequences of *B. improvisus* and *E. modestus* (* indicate the similarity between the two sequences)

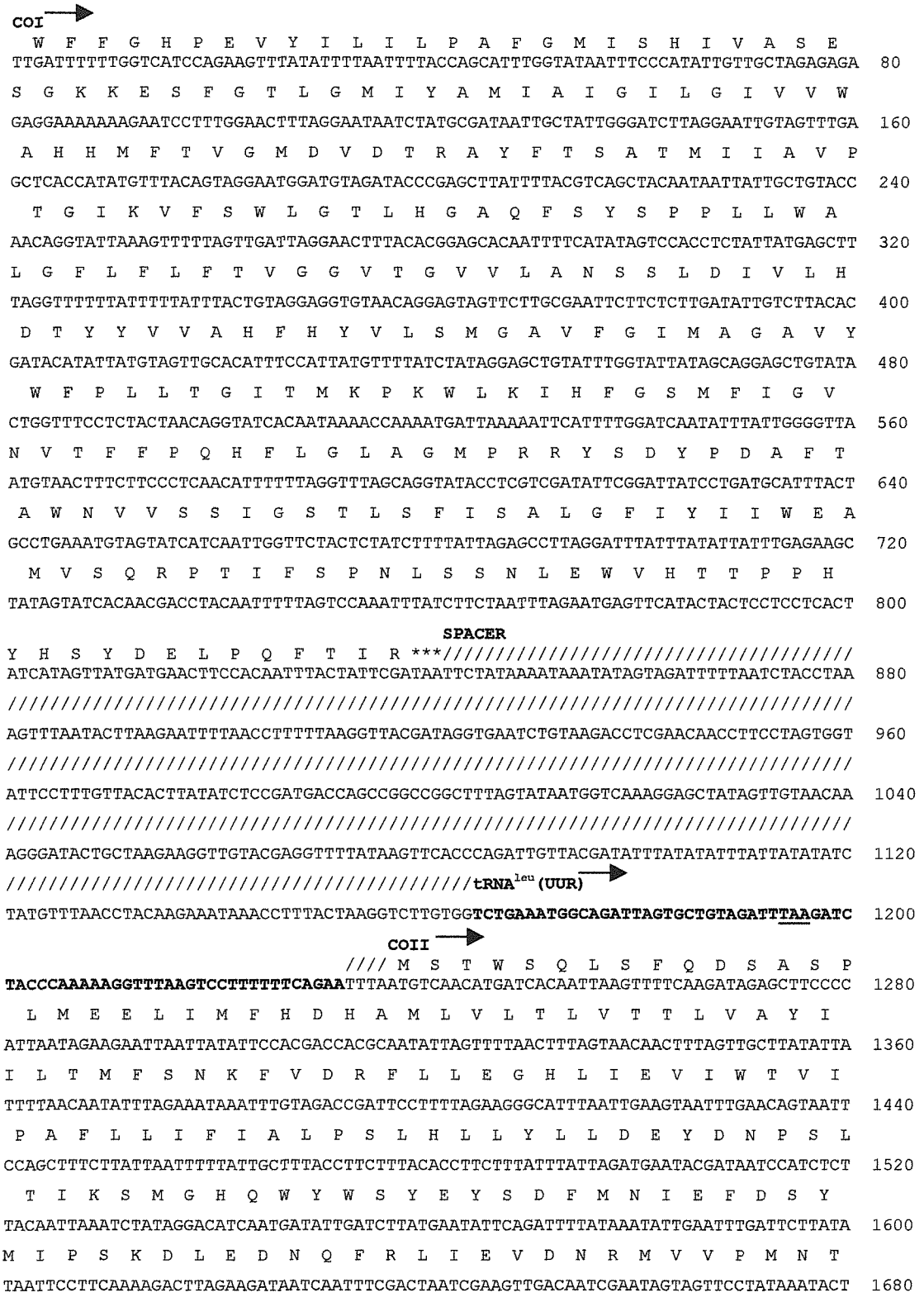


Figure 3.3 The COI-COIII sequence of *B. improvisus*. Each gene sequence is indicated by arrow. Transfer RNA genes are in bold letters and the anti-codon sequence of each tRNA is underlined.

```
 Y  I  R  I  L  V  S  S  T  D  V  I  H  A  W  T  V  P  A  L  S  V  K  A  D  A  I
TATATTCGAATCTTAGTGTCATCAACTGATGTAATCCATGCTTGAACAGTTCCAGCTCTAAGAGTAAAAGCTGATGCAAT  1760
  P  G  R  L  N  Q  L  T  F  L  V  N  R  P  G  L  F  F  G  Q  C  S  E  I  C  G
CCCAGGACGATTAAACCAATTAACTTTCCTAGTTAATCGACCAGGTTTATTTTTCGGTCAATGTTCTGAGATTTGTGGAG  1840
A  N  H  S  F  V  P  I  V  V  E  S  I  S  M  N  S  F  L  N  W  V  N  N  F  E  W
CTAATCATAGTTTTATACCTATTGTTGTAGAAAGAATTTCTATAAATTCATTCTTAAACTGAGTCAATAATTTTGAGTGA  1920
tRNA asp                                                                    ATPase8 (URFA6L)
 K  F  S ***                                                                 I  P  H  M  A
AAATTTAGTTAAATTTTATAACATTAGCTTGTCAAGCTGAAGTTACTTTAAGAAGTAATTTTCTATTCCTCACATAGCAC  2000
P  I  M  W  A  V  I  M  F  M  T  F  T  L  I  L  V  L  L  T  M  I  Y  F  G  N  S
CAATTATATGAGCAGTAATTATATTTATAACTTTTACTTTAATCTTAGTATTATTAACAATAATCTACTTTGGAAATTCA  2080
                                                                      ATPase6
 P  I  T  P  E  S  K  K  I  S  K  E  S  F  S  S  N  W  L  W ***
                                                                M  M  T  N  L  F  S  S
CCTATTACTCCAGAATCAAAAAAAATTTCTAAAGAAAGTTTTTCTTCAAACTGACTATGATAACAAATTTATTTTCATCA  2160
 F  D  P  M  S  S  T  L  N  M  Q  L  N  W  T  A  M  F  L  F  L  I  V  F  F  P  L
TTTGATCCTATATCATCAACATTGAATATACAATTAAACTGAACAGCAATATTTTTATTTCTAATTGTATTTTTTCCTTT  2240
  Y  W  I  T  N  S  K  S  S  I  L  Y  S  E  L  T  A  Y  I  T  K  V  F  L  P  L
ATACTGAATTACGAATTCTAAATCATCAATTCTATATTCAGAATTAACAGCTTATATTACAAAAGAATTCTTACCTTTAT  2320
F  K  S  Y  K  N  I  I  F  F  N  V  L  F  M  F  I  L  I  N  N  I  F  G  L  M  P
TTAAAAGTTACAAAAATATTATTTTTTTTAATGTTCTATTTATATTTATTTTAATTAATAATATTTTTGGATTAATACCA  2400
 Y  T  F  T  S  T  A  H  I  A  M  T  L  S  M  A  L  T  I  W  L  I  F  M  L  Y  G
TATACATTTACAAGCACAGCTCATATTGCTATAACTCTATCTATAGCACTAACAATTTGATTAATTTTTATGTTATATGG  2480
  W  I  N  N  T  N  H  M  F  A  H  L  V  P  L  G  T  P  I  V  L  M  P  F  M  V
TTGAATTAATAATACTAACCACATATTTGCTCATTTAGTTCCCTTAGGAACTCCTATTGTACTAATACCTTTTATAGTGT  2560
L  I  E  S  I  S  N  I  I  R  P  I  T  L  S  V  R  L  A  A  N  L  T  A  G  H  L
TAATTGAATCTATTAGAAATATTATTCGTCCTATTACTCTTTCAGTTCGGCTTGCAGCTAATTTAACAGCAGGTCACTTA  2640
 L  L  I  L  L  G  E  S  M  M  N  N  S  I  L  I  I  I  T  V  T  A  A  Q  F  A  L
TTACTAATTTTACTTGGAGAAAGTATAGTAAATAATAGAATTCTAATTATTATTACAGTAACAGCTGCTCAATTCGCCTT  2720
  M  T  L  E  A  A  V  A  V  I  Q  A  Y  V  F  A  T  L  S  T  L  Y  A  S  E  V *
AATAACTCTTGAAGCAGCAGTAGCAGTAATTCAAGCTTATGTATTTGCAACTCTTTCTACTTTATATGCTAGAGAAGTAT  2800
COIII
  M  T  T  H  S  H  H  P  F  H  L  V  D  M  S  P  W  P  L  T  A  S  I  G  A  L
**
AATGACAACTCATTCACATCATCCTTTCCATTTAGTTGATATAAGTCCTTGACCTTTAACTGCATCAATTGGTGCTTTAA  2880
T  M  T  S  G  L  S  Y  W  F  H  Y  N  S  M  T  I  L  S  L  G  L  F  I  I  I  I
CTATAACATCTGGCCTATCCTATTGATTCCACTATAATTCTATAACAATTCTTTCTTTAGGTCTATTTATTATTATTATT  2960
 S  S  Y  Q  W  W  R  D  V
TCCTCATATCAATGATGACGAGATGTT                                                  2987
```

Figure 3.3 Continued...

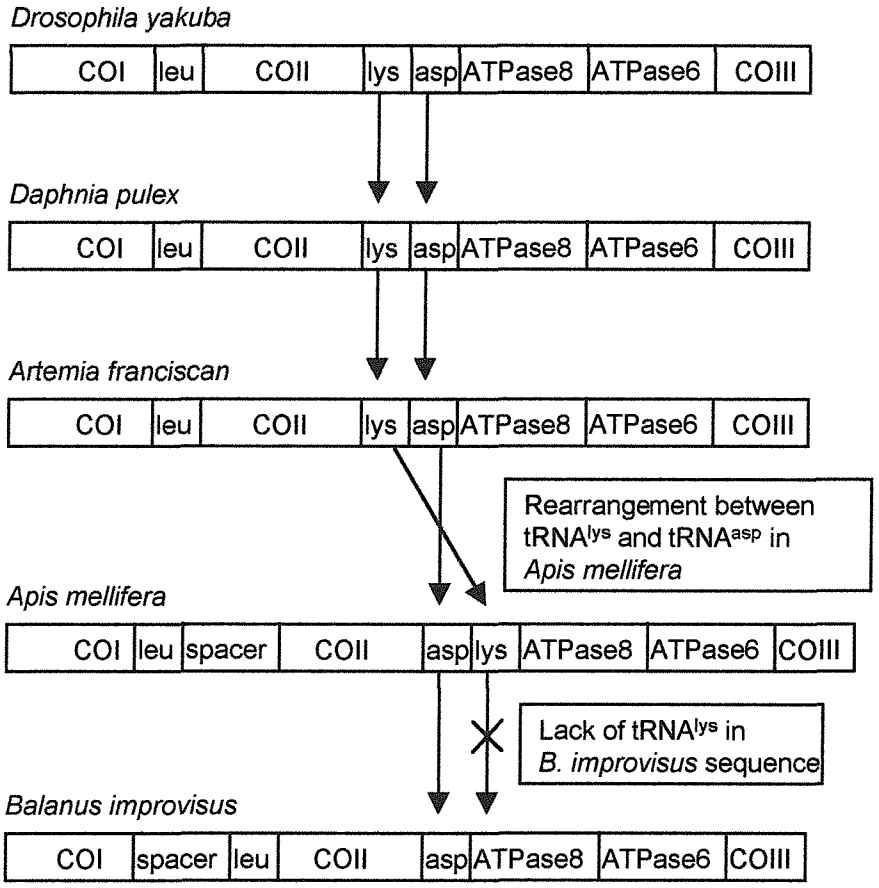


Figure 3.4 A linear diagram of the COI-COIII gene organisation and gene rearrangement in *B. improvisus* compared with other crustaceans and insects

sequence (about 300 bp), complete sequences of $tRNA^{leu}$, COII, $tRNA^{asp}$, ATPase8 and ATPase6, and partial sequence of the COIII gene (see fig. 3.3 and 3.4)

3.3.4.3 *B. improvisus* specific PCR primers

The species-specific PCR primers for *B. improvisus* were designed, based on COI-COIII sequence obtained. This new set of primers proved consistent in producing a 2628bp PCR product (see fig. 3.1 C), which was used as a genetic marker for the investigation of RFLP polymorphisms.

3.3.4.4 The investigation of PCR-RFLP polymorphisms

In order to select suitable restriction enzymes for RFLP analysis, 18 individuals from the River Itchen, Cobden bridge, Southampton were initially tested with various restriction enzymes (see Table 3.2). Six enzymes produced polymorphic RFLP profiles, namely *Hinf*I, *Vsp*I, *Taq*I, *Dde*I, *Nde*II and *EcoR*I (see fig. 3.5). They were also used to screen more individuals from River Thames, London and River Hamble, Southampton.

After the screening, 25 composite haplotypes were assembled and their frequencies were determined. The AAAAAA haplotype was the most common occurring in all populations. However, there were also some other distinct composite haplotypes in each population (see fig. 3.6).

3.3.5 Development of CA-repeat microsatellite markers

Checking the CA-motif enrichment using DNA-DNA hybridisation showed that the most CA-enriched DNA fragments were in the fifth wash (see fig. 3.7). After the construction of a enriched partial genomic library, 282 white bacterial colonies were screened using the PIMA technique. 68 colonies showed that they might contain plasmids with the inserts having CA-repeat motif sequences. Upon sequencing 57 positive clones, 32

Table 3.2 Restriction endonucleases, which were tested for RFLP analysis with recognition sites and results obtained.

Restriction Endonuclease	Recognition sites	Polymorphisms
*Rsa*I	GT'AC CA'TG	Monomorphic
*EcoR*I	G'AATTC C TTAA'G	Polymorphic
*Hinf*I	G'ANT C C TNA'G	Polymorphic
*Taq*I	T'CG A A GC'T	Polymorphic
*Vsp*I	AT'TAA T TA ATT'A	Polymorphic
*Dde*I	C'TNA G G ANT'C	Polymorphic
*Nde*II	'GATC CTAG'	Polymorphic
*Hha*I	G CG'C C'GC G	Ambiguous (smeared)
*Pvu*II	CAG'CTG GTC'GAC	Monomorphic
*Hae*III	GG'CC CC'GG	Ambiguous (smeared)
*Msp*I	C'CG G G GC'C	Ambiguous (smeared)
*Alu*I	AG'CT TC'GA	Ambiguous (difficult to score)
*Tru9*I	T'TAA AAT'T	Ambiguous (difficult to score)
*Hsp92*I	G(A/C)'CG (T/C)C C(T/G) GC'(A/G)G	Ambiguous (smeared)
*ScrF*I	CC'NG G GG NC'C	Monomorphic
*Bfa*I	C'TA G G AT'C	Monomorphic
*Sac*I	GAGCT'C C'TCGAG	Monomorphic

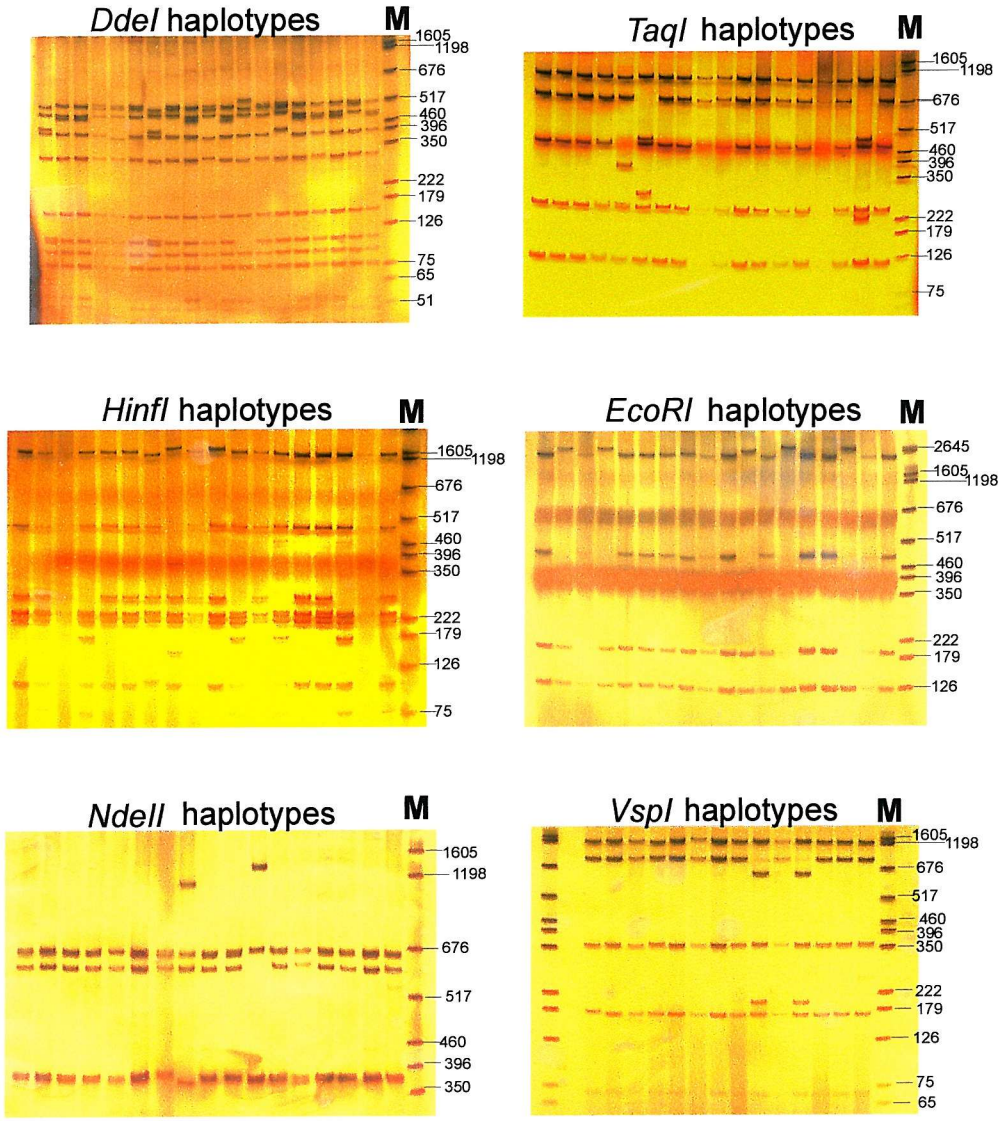


Figure3.5 8% acrylamide gels (4% for *NdeII*) showing RFLP profiles with six restriction endonucleases. Sliver stanining was employed to visualise the profiles. M indicates pGEM DNA marker (Promega)

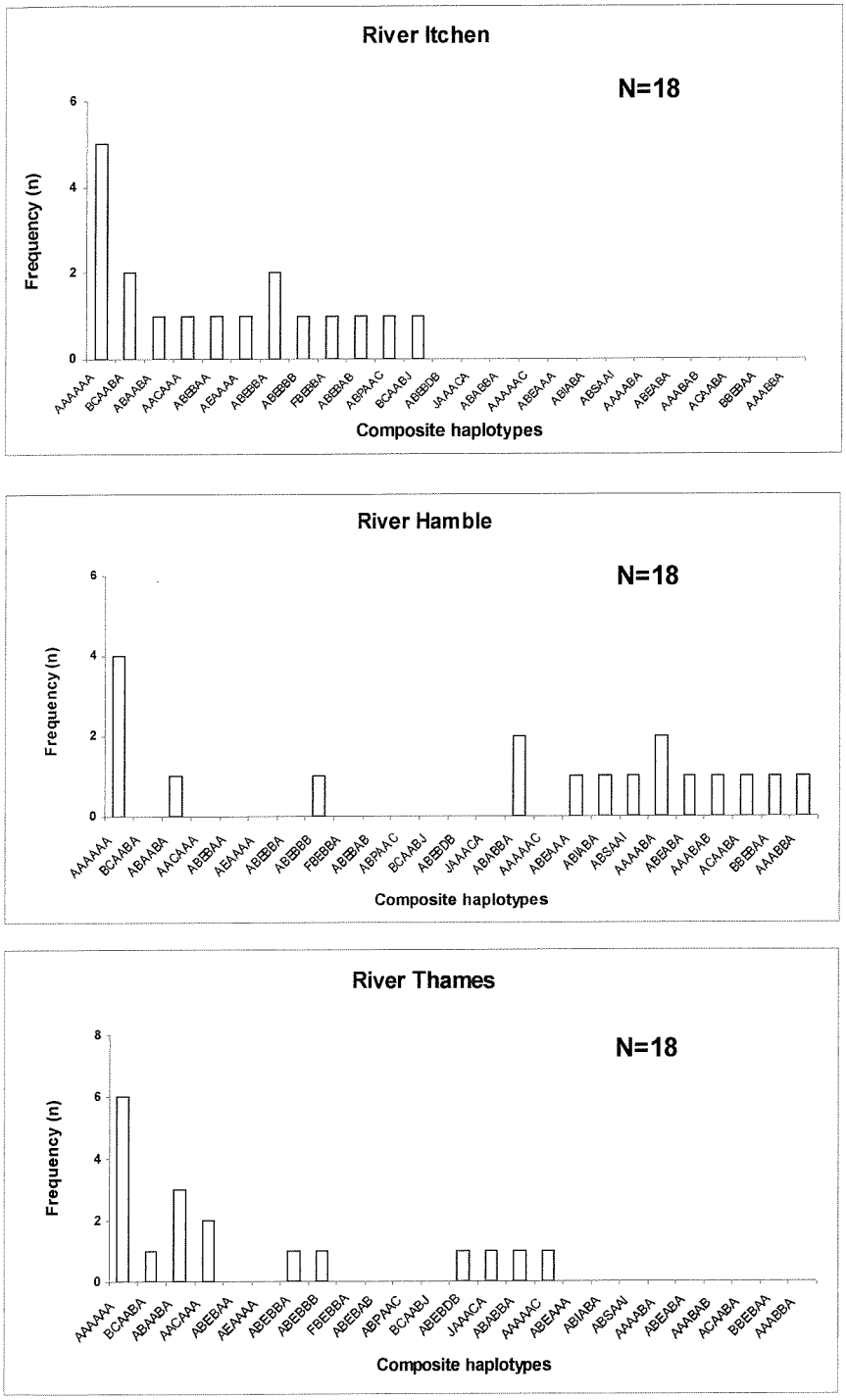


Figure 3.6 The frequency of composite haplotypes generated by the combination of *TaqI*, *EcoRI*, *DdeI*, *NdeII*, *HinfI*, and *VspI* haplotypes.

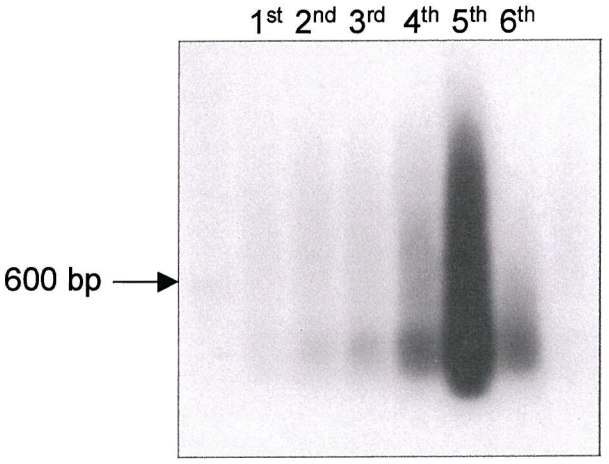


Figure 3.7 The autoradiograph of southern blot hybridised with a 32P labelled CA/GT polynucleotide. The fifth wash was found to be the most enriched of CA-repeat sequence.

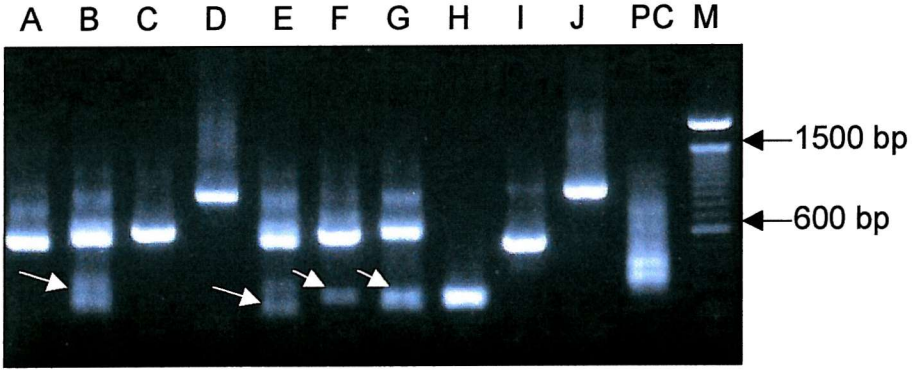


Figure 3.8 Photogragh of 1.2 % agarose gel showing PIMA profiles (lane B, E, F, and G are the positive clones containing CA-repeat sequence (with addition bands indicated by arrows), lane A, C, D, H, I,and J are negative clones, lane PC is positive control, and lane M is a 100bp ladder DNA marker.).

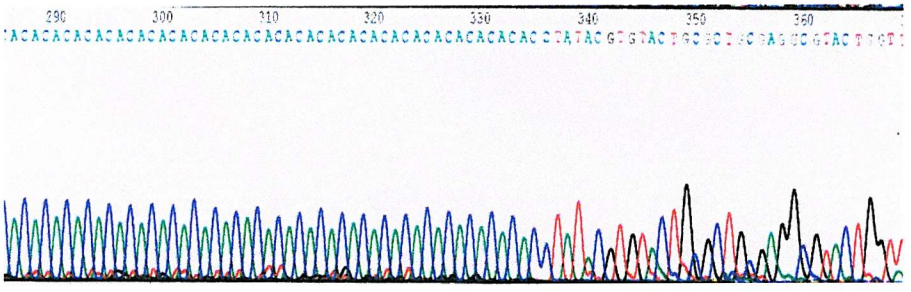


Figure 3.9 Chromatogram of a DNA sequence containing a CA-repeat motif

Table 3.3 The positive clones with repeat sequences present, and PCR optimisation results of each locus

Identified Clone	Repeat Unit Found			No. of Clones	PCR results
	Perfect	Imperfect	Compound		
BI45	(GT)13	-	-	1	Could not design the primers
BI57, BI14.2, BI15.2, BI74	(CA)12	-	-	4	Could not design the primers
BI33	(GT)12	-	-	1	Smeared PCR product
BI61, BI11.1	-	(C)8(CA)7CG(AC)2 GCATA(CA)11AA (CA)7CG((CA)3CG)2 (CA)4CG(CA)3CG (CA)5CG(CT)4	-	2	Non-specific amplification
BI85, BI76, BI77	-	(CA)5TA(CA)13 (CGCA)3	-	3	Non-specific amplification
BI4	-	(GTCA)3(GT)3 GG(GT)11	-	1	Smeared PCR product
BI54	(CA)10	-	-	1	Specific amplification
BI88, BI7, BI2.2,	-	(GT)9GC(GT)4 CT(GT)2	-	3	Specific amplification
BI3341	-	(GC)4(GT)7(CA)2 GTGCACTGC (GT)7(AC)2(TG)2 CACTGC(GT)4	-	1	Non-specific amplification
BI58, BI59, BI31	-	(CA)2CC ((CA)3CC)2(CA)4	-	1	Smeared PCR product
BI47, BI2.1, BI79	(CA)12*	-	-	3	Inconsistent amplification
BI35	(GT)27	-	-	1	Specific amplification
BI39	-	(GT)6CG ((GT)3CG)3 (TGCG)3(TG)6	-	1	Inconsistent amplification
BI41, BI4.2	(CA)41	-	-	2	Non-specific amplification
BI46.2	-	-	(GA)2(GC)6 (AC)11	1	Inconsistent amplification
BI22.2	-	(GT)6GA(GT)8	-	1	Smeared PCR product
BI107.2	-	-	(CAAA)5(CA) CAAA	1	Smeared PCR product
BI44.1	-	-	(GGGGC)4(G)4	1	Not tested
BI26.2	-	(CA)7GA(CA)5+ (CA)12+(CA)8GA (CA)4GA(CA)5	-	1	Could not design the primers
BI14.3	(CA)8	-	-	1	Not tested
BI35.3	-	(GT)3AT(GT)3 GACT(TG)5	-	1	Not tested
			TOTAL	32	

Table 3.4 Microsatellite markers for *B. improvisus* with primer sequences, PCR conditions, number and size of alleles, observed heterozygosity (H_O) and expected heterozygosity (H_E)

Locus	Repeat sequences	Primer sequences (5'-3')	Annealing Temp. (C°)	$MgCl_2$ (mM)	No. of alleles	Sizes (bp)	H_O, H_E	Individuals screened (n)
BI35	(CA)27	CGGAGTCTAACAATCGATGC GAATGTACCTCCACTGACAC	55	1.5	12	165-207	0.17, 0.89	18
BI7	(GT)9GC (GT)4CT(GT)2	GCTTGCCAGATGCCCTAGAG CAGCTGCCGAGCACTGTAAC	62	1.5	3	249-255	0.11, 0.20	18
BI54	(CA)10	CTGGTCGGCGTCTAACGAC TGAGCTGGTAGGTGATGCAG	60	1.5	1	130	0.00, 0.00	10

were found to contain CA-repeat sequences, although some of them contained the same repeat sequences and were too close to the linker to allow primer design (see table 3.3). PCR primers were designed for each microsatellite locus (see Appendix section 1). After many attempts at PCR optimisation, primers for 3 loci were found to produce a specific PCR product and 2 of these were polymorphic (see table 3.4).

3.4 Discussion

The lack of success with the RAPD technique was probably caused by the sensitivity and unreliability of the technique which has been reported by a number of researchers (e.g. Halldén *et al.,* 1996; Ellsworth *et al.,* 1993). All the PCR components are crucial factors, which can cause inconsistent results in this technique, especially primers and the quality of DNA template. In order to obtain reliable RAPD primers, a large number of primers have to be screened and there is no guarantee how many of them will be useful. After testing 20 RAPD primers, no primer was found to produce consistent results. Therefore, this technique was stopped without further investigation using such primers.

Recently, a large amount of DNA sequence information from animal mtDNA has become available, allowing researchers to use conserved regions found throughout the mtDNA genome for designing PCR primers (see Palumbi, 1996a). For this reason universal mtDNA primers were employed to provide genetic information of *B. improvisus*, and a specific marker was designed.

As far as the use of 16s rRNA as a genetic marker for population genetic study of *B. improvisus* is concerned, it is not really suitable to use for two reasons. Firstly, it is fairly conserved and used mostly in phylogenetic studies (Simon *et al.,* 1994). Secondly, only sequencing analysis can be used because the PCR product is too short (550bp). Even

though the direct sequencing technique used to produce 16s rRNA sequences in this study can speed up the process, there were two disadvantages. Firstly, It is still time consuming, labour intensive, and expensive. Secondly, only one strand produced a good quality sequence (the strand that binds to the beads see section 3.2.4.2.1), and less than 250 bp of sequence was obtained. The poor result of the other strand was probably caused by the difficulty of the neutralisation step during the precipitation of DNA. Although this could now be carried out on a automated sequencer. The expense is still far too high for this project. Therefore, the intra-specific genetic variation using this marker was not investigated.

The primers COI-RLR and COIII produced a 3kb PCR product, which mostly consisted of protein-coding genes including COI, COII, ATPase8, ATPase6, and COIII (see fig.3.4). These regions are more variable than ribosomal RNA genes since the change of the third base of the codon does not generally cause changes in the relevant amino acid because of degeneracy of the code. Interestingly there was an unidentified sequence (about 300 bp in length) unique to *B. improvisus*. This seems to be an intergenic spacer because it was located between COI and $tRNA^{leu}$. This phenomenon is very rare in the mitochondrial genome since the molecule of animal mitochondrial DNA is mostly compact and intergenic spacers are short (10-20 bp) (Brown, 1983). However, there are some known examples of this phenomenon in salamanders (McKnight and Shaffer, 1997), snakes (Kumazawa *et al.,* 1996) and the honeybee, *A. mellifera* (Cornuet *et al.,* 1991) (these details are discussed in Chapter 5). The other aspect, which is distinct in the gene organisation of this PCR product, is the lack of $tRNA^{lys}$ located between COII and $tRNA^{asp}$ as in *D. yakuba*, *D. pulex*, and *A. franciscan*. In honey bee (*A. mellifera*) $tRNA^{asp}$ and $tRNA^{lys}$ are inverted in their position (see fig.3.4). Although this character could be distinct for barnacles, the gene organisation of other barnacle must be determined. To obtain a clear answer about the location of the

gene coding for $tRNA^{lys}$ in mitochondrial genome of *B. improvisus*, the complete sequence of the genome is required. Unfortunately, this was outside the scope of the present study.

After, the investigation of RFLP polymorphisms in COI-ATPase6 genes, although the distribution of the composite haplotype frequencies (fig. 3.6) showed that all populations shared a common composite haplotype (AAAAAA), there were distinct composite haplotypes in each population, and as this PCR fragment occupies about 1/6 of the whole mitochondrial genome. Therefore, It should provide sufficient variation and information useful for the study of population genetic structure of *B. improvisus* both local and broad geographic scales. Therefore, PCR-RFLP of COI-ATPase6 genes was used as a genetic marker for the study of a population genetic structure of *B. improvisus*. This marker was employed for the study in Chapter 4.

The enrichment protocol (Kandpal *et al.,* 1994) employed for microsatellite marker isolation combined with PIMA technique (Lunt *et al.,* 1999) for positive clone selection was found to be rapid and cost-effective method of screening compared with other technique such as colony blotting followed by hybridisation. The accuracy of the PIMA technique was fairly high (32 out of 57 colonies-56%). However, the primer used in PIMA technique is available only for CA-repeated motifs. If other types of motifs are required, new primers suitable for those motifs need to be developed.

The main purpose of micosatellite marker development was to use it in the study of mating strategies in *B. improvisus*. Highly polymorphic microsatellite markers were important to allow identification at the individual level. Unfortunately, only one marker (BI35) was found to be highly polymorphic and suitable for this study (see table 3.4). It showed that the *Ho* (0.167) was much lower than the *He* (0.895) i.e. although there

were a large number of different alleles in the population, most of individuals in the population were homozygous. This suggested that self-fertilisation behaviour in *B. improvisus* (Furman, 1990) might be an explanation for the result above as reported in the freshwater snail, *Bulinus truncatus* (Viard *et al.,* 1997). However, it is difficult to draw this conclusion since only 18 individuals were screened. However, this marker (BI35) should provide useful information for the study of mating strategies in *B. improvisus* including its self-fertilisation behaviour (for further information on mating strategies see Chapter 6).

Chapter Four

The study of the population genetic structure of an acorn barnacle, *Balanus improvisus*, in north-western Europe using mtDNA PCR-RFLP analysis

4.1 Introduction

Most marine ecosystems are generally considered to be open and it is difficult to understand the population dynamics of these systems, in particular for those organisms having pelagic larval stages (Menge, 1991; Caley *et al.*, 1996). In marine sessile organisms with planktonic larvae (i.e. barnacles), the variation in larval supply (recruitment) plays an important role in their population structures (as described in section 2.1.4) (Underwood, 1979, Connell, 1985, Lewin, 1986; Roughgarden *et al.*, 1987; Fairweather, 1988; Underwood and Fairweather, 1989; Fairweather, 1991; Le Fevre and Bourget, 1992). Therefore, It is important to investigate the source of the larval supply. There are various ways to investigate the dispersal ability of the larvae such as labelling larvae with chemical substances (Levin, 1990, Jones *et al.*, 1999), the use of satellite tracking coral larvae (Willis and Oliver, 1990), or the use of planktonic stage duration (Richmond, 1987). Nevertheless, none of these methods can provide information about the integration of new recruits into their new habitats. They can only provide information concerning how far the larvae might disperse. One potential indirect approach that could provide such information is the use of genetic markers to

investigate the relationship and the degree of gene exchange (gene flow) between the populations of a particular species.

Studies on the population genetic structure of marine organisms have previously showed a relationship between genetic differentiation and dispersal ability of their planktonic larvae (Scheltema, 1971; Gooch, 1975; Crisp, 1978). However, later Hedgecock (1986) suggested that genetic differentiation found in these marine organisms is not only dependent on larval dispersal ability, but also on biological or physical barriers, the fitness of the larvae, and the differences in survival and fitness of new immigrants. In addition, there are other factors to be considered when the paradox between genetic differentiation and the dispersal ability of the larvae is concerned, such as the behaviour of planktonic larvae (Bousfield, 1955; Burton and Feldman, 1982), oceanographic circulation patterns (Benzie and Stoddart, 1992; Bertness and Gaines, 1992), and selection both during the larval phase and post-settlement (Koehn *et al.*, 1980; Karl and Avise, 1992; Holm and Bourget, 1994).

Population genetic studies in barnacles have mostly been carried out using allozyme or isozyme markers (eg. Flowerdew, 1984; Holm and Bourget, 1994; Pannacciulli *et al.*, 1997) (see also section 3.1). Genetic differentiation was found over a great geographical distance such as the differences found among *S. balanoides* from North America, the Pacific and Europe (Flowerdew and Crisp, 1975; Flowerdew, 1983). But, Holm and Bourget (1994) suggested that the genetic variation of *S. balanoides* at the *Gpi* and *Mpi* allozyme loci may have occurred on a much smaller scales (within Gulf of St. Lawrence) which might be caused by the selection associated with environmental temperatures. Two forms (Atlantic and Mediterranean) of *C. stellatus* and *C. montagui* has also been reported (Dando and Southward, 1981; Pannacciulli *et. al.*, 1997) associated with the hydrographic separation of the Atlantic and Mediterranean. In

contrast, genetic similarity has been found between *E. modestus* populations separated by long geographical distances (the Antipodean and European samples) (Flowerdew, 1984). *E. modestus* is an immigrant species migrating from Australia to Europe (Bishop, 1947). Flowerdew (1984) suggested that the colonisation of this barnacle species in Europe might have been initiated by a large number of founders from Australia. The effect of selection or genetic drift on genetic differences was thought to be limited due to the short duration of separation of the two populations (~30 years). Similar results have also been found in *B. improvisus* populations. There was no genetic differentiation among *B. improvisus* form the Baltic, British Isles and American populations using enzyme variation method (Furman, 1990).

In this study, mtDNA PCR-RFLP, as described in chapter 3, was employed to reveal population genetic structures of *B. improvisus*. Three aims of this study are: 1. to investigate broad scale population structures of *B. improvisus* (between the Baltic and British populations), 2. to investigate local population structures of this barnacle (within the British Isles and within the Baltic), 3. to compare the dispersal patterns of planktonic larvae from patchily distributed populations in isolated British estuaries with the patterns from continuously distributed populations in the Baltic.

4.2 Materials and methods

4.2.1 Sites of study

The populations of *B. improvisus* used in this study can be divided into two groups, those obtained from the British Isles and those from Baltic populations. The British Isles populations were described in section 2.3.1). Seven populations from around the British Isles were used in this study. These were obtained from the estuaries of River Ribble, Mersey, Dee, Severn, Itchen, Hamble, and Thames (see also fig. 4.1). There

were also five populations from the Baltic used in this study, including three from the Gulf of Finland provided by Dr. E. Furman [Archipelago sea (N 60°06'54' and E 21°41'38'), Sipoo (N 60°06'48' and E 25°41'65'), and Inkoo (N 60°06'10' and E 24°10'10')], one from the west coast of Sweden, Tjärnö (N 58°53'and E 11°8') provided by Dr. P. Jonsson, and one from northern Germany, the River Lippe, near Kiel provided by Dorothea Somerfeldt (see fig. 4.1).

4.2.2 Samples

All samples were preserved in absolute ethanol and kept at –20°C as soon as possible for long term use. At least 30 individuals were used as the representatives for most of the populations in this study, except for the populations from the River Mersey (11 individuals) and Hamble (18 individauls) because these populations were very small as described in section 2.3.1). The population from the west coast of Sweden was obtained from laboratory maintained samples. However, the samples were collected from the shore near Tjärnö Marine Biological Laboratory, by leaving plastic plates on the shore during the settling season. Then, the plates, having the settlements attached on them, were brought to the laboratory and the barnacles were reared in tanks and fed with *Artemia*. This sample was included in the analysis because it was the only population obtained from the area.

4.2.3 Genetic markers and data analysis

The genetic marker used in this study was mtDNA (COI-COIII) and the PCR-RFLP technique were used for detecting polymorphism as described in section 3.2.5. The matrix of the presence and absence of restriction sites was prepared using Generate and Group programs in REAP (McElroy *et al.*, 1992). The matrix was used to generate a minimum spanning network (MSN) (Prim, 1957) to investigate the distribution and the

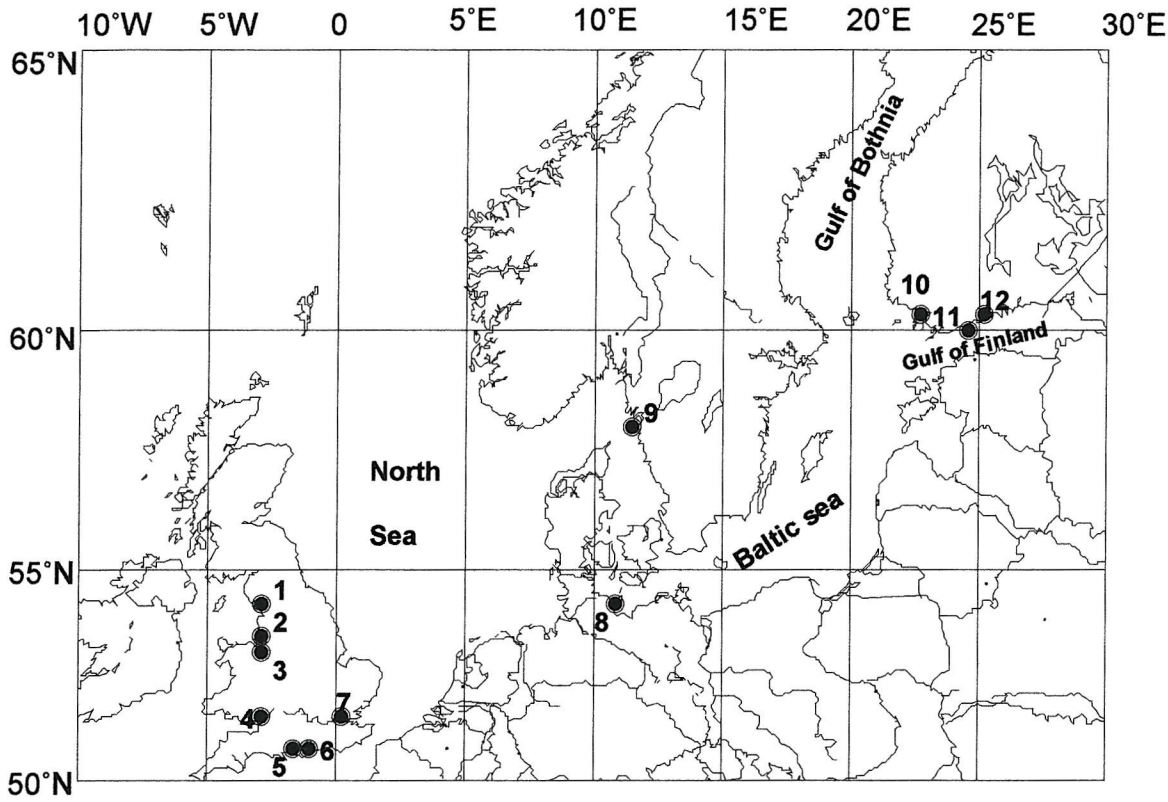


Figure 4.1 Sampling sites used in this study, indicated by black circles (1. River Ribble, 2. River Mersey, 3. River Dee, 4. River Severn, 5 River Itchen, 6. River Hamble, 7. River Thames, 8. River Lippe, 9. Tjärnö, 10. Archipelago Sea,11. Inkoo, and 12. Sipoo)

relationship of haplotypes. Haplotype diversity or average gene diversity (Nei, 1987) and average number of pairwise difference (Nei and Li, 1979, Tajima, 1983; Nei and Miller, 1990) was also estimated.

Pairwise population comparisons were carried out using Fst values. These values were then used to construct a Neighbour-joining tree (Saitou and Nei, 1987), using PAUP* beta version 4.0b4a computer software (Swofford, 1998), to investigate the relationships among the populations. The significant test of pairwise Fst values was carried out by permuting haplotypes between populations (10,000 times). Pairwise Fst values were also plotted against geographical distances and regression analysis was performed using Excel. The significant test of correlation between Fst values and geographic distance was carried out by Mantel's test (Mantel, 1967; Sokal, 1979). Geographical distance was measured using the shortest distance between sites by water.

The population genetic structure was determined using Analysis of Molecular Variance (AMOVA) (Excoffier *et al.*, 1992) available in Arlequin version 2.0 computer program (Schneider, 2000). The significant test for the AMOVA analysis was carried out by comparing observed values with random values calculated from 10,000 permutations as follows: for Fst haplotypes (individuals) were permuted across populations and groups, for Fsc, haplotypes were permuted across populations within groups, and for Fct, the populations were permuted across groups (Excoffier *et al.*, 1992). The hierarchical genetic structure analysis was carried out by both broad-scale (between the British populations and Baltic populations) and local scale comparison (within the Baltic and within British Isles). For the local comparison, the British populations were divided into two groups: southern coast (River Severn, Itchen, Hamble, and Thames) and north-west coast (River Ribble, Dee, and Mersey). The Baltic populations were

also divided into two groups: the Danish Belts (Tjänrö, and River Lippe) and the Gulf of Finland (Archipelago sea, Inkoo, and Sipoo).

4.3 Results

4.3.1 Intra-specific population variation

469 individuals of *B. improvisus* from 12 populations in Western Europe (see fig. 4.1) were screened with 6 restriction endonucleases (*EcoR*I, *Taq*I, *Hinf*I, *Dde*I, *Vsp*I, and *Nde*II). There are 104 composite haplotypes with 68 restriction sites. The most common haplotype (H1) was present in all populations (48.0% of all individuals). There are also haplotypes present in nearly all populations (H2 (5.3%), H3 (6.2%), and H18 (4.9%)) (see also Table 4.1).

The minimum spanning network was obtained and divided into four groups to provide clearer profiles of the distribution of haplotypes (see fig. 4.2). From the diversity among populations illustrated in fig. 4.3, it was expected that the populations within the regions should show patterns of grouping among haplotypes which have evolved by small mutational steps and the populations from different regions (Baltic and British) should show different patterns of grouping. The result showed that there was no such pattern and each population had its own unique haplotypes, which were not present elsewhere.

The variation within populations, haplotype diversity (h) values ranged from 0.51 (River Dee) to 0.95 (River Hamble). The populations showing low variation in haplotype diversity were from west cost of the British Isles, River Ribble (0.62), River Mersey (0.60), and River Dee (0.51)) (fig. 4.4a). Nucleotide diversity (π) values ranged from

Table 4.1 Haplotype frequency and percentage of haplotypes found in this study (RM:River Mersey (RR:River Ribble, RD:River Dee, RS:River Severn, RI:River Itchen, RH:River Hamble, RT:River Thames RL:River Lippe, Archi:Achipelago sea)

Haplotypes	BALTIC SEA					BRITISH ISLES							Total	%
	GULF OF FINLAND			DANISH BELT										
	Archi	Inkoo	Sipoo	Tjärnö	RL	RD	RR	RM	RH	RI	RS	RT	Freq.	
	(N=42)	(N=45)	(N=41)	(N=50)	(N=30)	(N=47)	(N=44)	(N=11)	(N=18)	(N=41)	(N=54)	(N=46)	(Hi)	
H1	19	25	21	15	16	33	27	7	4	18	15	25	225	47.97
H2	3	4	1	1	1	1	5	2	0	2	3	2	25	5.33
H3	1	2	0	1	0	3	2	1	1	1	12	5	29	6.18
H4	0	0	0	0	0	0	0	1	0	0	0	0	1	0.21
H5	0	0	0	0	0	0	1	0	0	0	1	0	2	0.43
H6	0	0	0	0	0	0	1	0	0	0	1	0	2	0.43
H7	0	0	1	2	0	0	1	0	0	0	1	0	5	1.07
H8	0	0	0	0	0	0	1	0	0	0	0	0	1	0.21
H9	0	0	0	0	0	0	1	0	0	0	0	0	1	0.21
H10	0	0	0	0	0	0	1	0	0	0	2	0	3	0.64
H11	0	0	0	0	0	0	1	0	0	0	0	0	1	0.21
H12	0	0	0	0	0	0	1	0	0	0	0	0	1	0.21
H13	0	0	0	0	0	0	1	0	0	0	0	0	1	0.21
H14	4	1	2	0	1	0	1	0	0	1	0	2	12	2.56
H15	0	0	1	1	0	0	0	0	0	1	0	0	3	0.64
H16	0	0	0	0	0	0	0	0	0	1	0	0	1	0.21
H17	0	0	0	0	0	0	0	0	0	1	0	0	1	0.21
H18	3	1	2	7	3	0	0	0	0	2	3	2	23	4.90
H19	0	0	0	0	0	0	0	0	0	1	0	0	1	0.21
H20	1	0	0	0	0	0	0	0	0	1	0	1	3	0.64
H21	0	0	0	0	0	0	0	0	1	2	1	1	5	1.07
H22	0	0	0	0	1	0	0	0	0	1	0	0	2	0.43
H23	0	0	0	0	0	0	0	0	0	1	0	0	1	0.21
H24	0	0	0	0	0	0	0	0	0	1	0	0	1	0.21
H25	1	0	0	0	0	0	0	0	0	1	1	0	3	0.64
H26	0	0	0	1	0	0	0	0	0	1	0	0	2	0.43
H27	0	0	0	0	0	0	0	0	0	1	1	0	2	0.43
H28	0	0	0	0	0	0	0	0	0	1	0	0	1	0.21
H29	0	0	0	0	0	0	0	0	0	1	0	0	1	0.21
H30	0	0	0	2	2	1	0	0	0	1	1	0	7	1.49
H31	0	0	0	0	0	0	0	0	0	1	0	0	1	0.21
H32	0	0	0	0	0	0	0	0	0	0	0	1	1	0.21
H33	0	0	1	0	0	0	0	0	0	0	0	1	2	0.43
H34	0	0	0	0	0	0	0	0	2	0	0	1	3	0.64
H35	0	0	0	1	0	0	0	0	0	0	0	1	2	0.43
H36	0	0	0	0	0	0	0	0	0	0	0	1	1	0.21
H37	0	0	0	0	1	0	0	0	0	0	0	1	2	0.43
H38	1	0	1	0	0	0	0	0	0	0	0	1	3	0.64
H39	0	0	0	0	0	0	0	0	0	0	0	1	1	0.21
H40	0	0	0	0	0	0	0	0	1	0	0	0	1	0.21
H41	0	0	0	2	0	0	0	0	1	0	1	0	4	0.85
H42	0	0	0	0	0	0	0	0	1	0	0	0	1	0.21
H43	0	0	0	0	0	0	0	0	2	0	1	0	3	0.64
H44	0	0	0	0	0	0	0	0	1	0	0	0	1	0.21
H45	0	0	0	0	0	0	0	0	1	0	0	0	1	0.21
H46	0	1	0	0	0	0	0	0	1	0	0	0	2	0.43
H47	0	0	0	0	0	0	0	0	1	0	0	0	1	0.21
H48	0	0	0	0	0	0	0	0	1	0	0	0	1	0.21
H49	0	0	0	0	0	0	0	0	0	0	1	0	1	0.21
H50	0	0	0	1	1	1	0	0	0	0	1	0	4	0.85
H51	0	0	0	0	0	0	0	0	0	0	1	0	1	0.21
H52	0	0	0	0	0	0	0	0	0	0	1	0	1	0.21

Table 4.1 Continued...

Haplotypes	BALTIC SEA					BRITISH ISLES							Total	%
	GULF OF FINLAND			DANISH BELTS										
	Archi (N=42)	Inkoo (N=45)	Sipoo (N=41)	Tjärnö (N=50)	RL (N=30)	RD (N=47)	RR (N=44)	RM (N=11)	RH (N=18)	RI (N=41)	RS (N=54)	RT (N=46)	Freq. (Hi)	
H53	0	0	0	0	0	0	0	0	0	0	1	0	1	0.21
H54	0	0	0	0	0	0	0	0	0	0	1	0	1	0.21
H55	0	0	0	0	0	0	0	0	0	0	1	0	1	0.21
H56	0	0	0	0	0	0	0	0	0	0	1	0	1	0.21
H57	0	0	0	0	0	0	0	0	0	0	1	0	1	0.21
H58	0	0	1	0	0	0	0	0	0	0	1	0	2	0.43
H59	0	0	0	0	0	1	0	0	0	0	0	0	1	0.21
H60	0	0	1	2	0	1	0	0	0	0	0	0	4	0.85
H61	1	1	0	0	0	1	0	0	0	0	0	0	3	0.64
H62	0	0	0	0	0	1	0	0	0	0	0	0	1	0.21
H63	2	1	0	0	0	1	0	0	0	0	0	0	4	0.85
H64	0	0	0	0	0	1	0	0	0	0	0	0	1	0.21
H65	0	0	0	0	0	1	0	0	0	0	0	0	1	0.21
H66	0	0	0	0	0	1	0	0	0	0	0	0	1	0.21
H67	1	1	0	0	0	0	0	0	0	0	0	0	2	0.43
H68	1	0	0	0	0	0	0	0	0	0	0	0	1	0.21
H69	1	0	0	0	0	0	0	0	0	0	0	0	1	0.21
H70	1	0	0	0	0	0	0	0	0	0	0	0	1	0.21
H71	1	0	0	0	0	0	0	0	0	0	0	0	1	0.21
H72	1	0	0	0	0	0	0	0	0	0	0	0	1	0.21
H73	0	1	0	0	0	0	0	0	0	0	0	0	1	0.21
H74	0	1	0	1	0	0	0	0	0	0	0	0	2	0.43
H75	0	1	1	0	0	0	0	0	0	0	0	0	2	0.43
H76	0	1	0	0	0	0	0	0	0	0	0	0	1	0.21
H77	0	1	0	0	0	0	0	0	0	0	0	0	1	0.21
H78	0	1	0	0	0	0	0	0	0	0	0	0	1	0.21
H79	0	1	0	0	0	0	0	0	0	0	0	0	1	0.21
H80	0	1	0	0	0	0	0	0	0	0	0	0	1	0.21
H81	0	0	1	0	0	0	0	0	0	0	0	0	1	0.21
H82	0	0	1	0	0	0	0	0	0	0	0	0	1	0.21
H83	0	0	1	0	0	0	0	0	0	0	0	0	1	0.21
H84	0	0	1	0	0	0	0	0	0	0	0	0	1	0.21
H85	0	0	1	0	0	0	0	0	0	0	0	0	1	0.21
H86	0	0	1	0	0	0	0	0	0	0	0	0	1	0.21
H87	0	0	1	0	0	0	0	0	0	0	0	0	1	0.21
H88	0	0	1	0	0	0	0	0	0	0	0	0	1	0.21
H89	0	0	0	1	0	0	0	0	0	0	0	0	1	0.21
H90	0	0	0	1	0	0	0	0	0	0	0	0	1	0.21
H91	0	0	0	1	0	0	0	0	0	0	0	0	1	0.21
H92	0	0	0	1	0	0	0	0	0	0	0	0	1	0.21
H93	0	0	0	1	0	0	0	0	0	0	0	0	1	0.21
H94	0	0	0	1	0	0	0	0	0	0	0	0	1	0.21
H95	0	0	0	1	0	0	0	0	0	0	0	0	1	0.21
H96	0	0	0	1	0	0	0	0	0	0	0	0	1	0.21
H97	0	0	0	1	0	0	0	0	0	0	0	0	1	0.21
H98	0	0	0	1	0	0	0	0	0	0	0	0	1	0.21
H99	0	0	0	1	0	0	0	0	0	0	0	0	1	0.21
H100	0	0	0	1	0	0	0	0	0	0	0	0	1	0.21
H101	0	0	0	1	0	0	0	0	0	0	0	0	1	0.21
H102	0	0	0	0	2	0	0	0	0	0	0	0	2	0.43
H103	0	0	0	0	1	0	0	0	0	0	0	0	1	0.21
H104	0	0	0	0	1	0	0	0	0	0	0	0	1	0.21

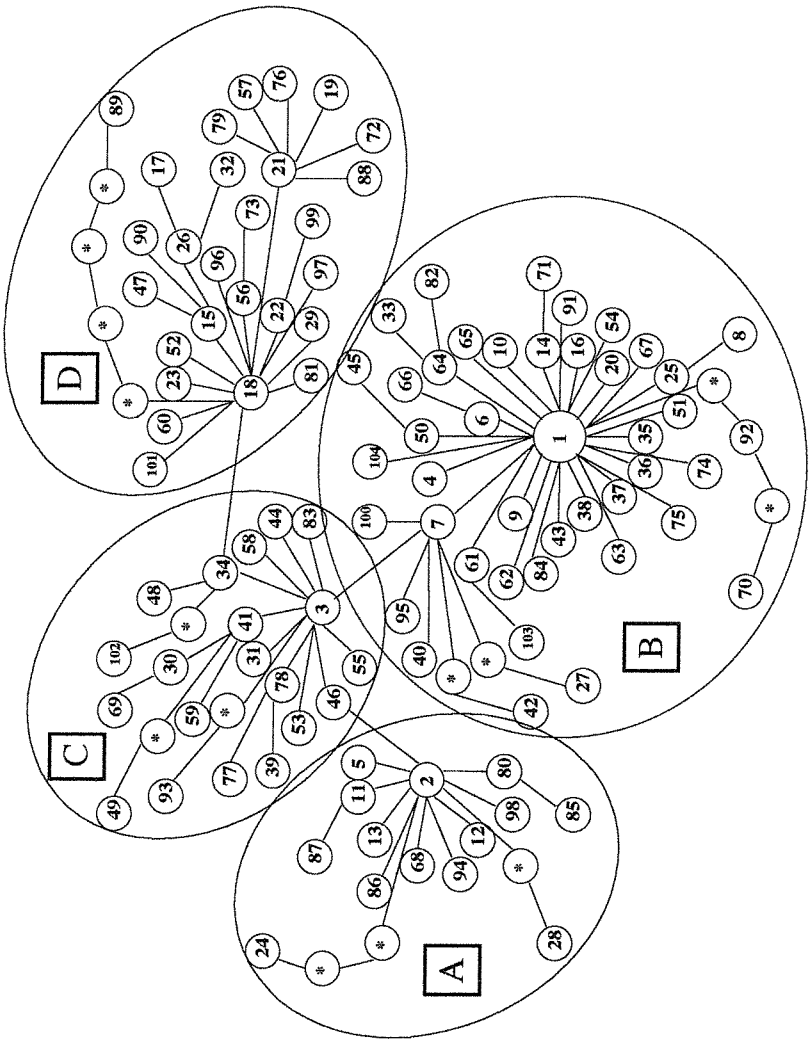


Figure 4.2 Minimum spanning network of 104 haplotypes found among 12 populations. The asterisks indicate haplotypes that were not found in this study. The most common haplotype (H1) has been enlarged for clear recognition.

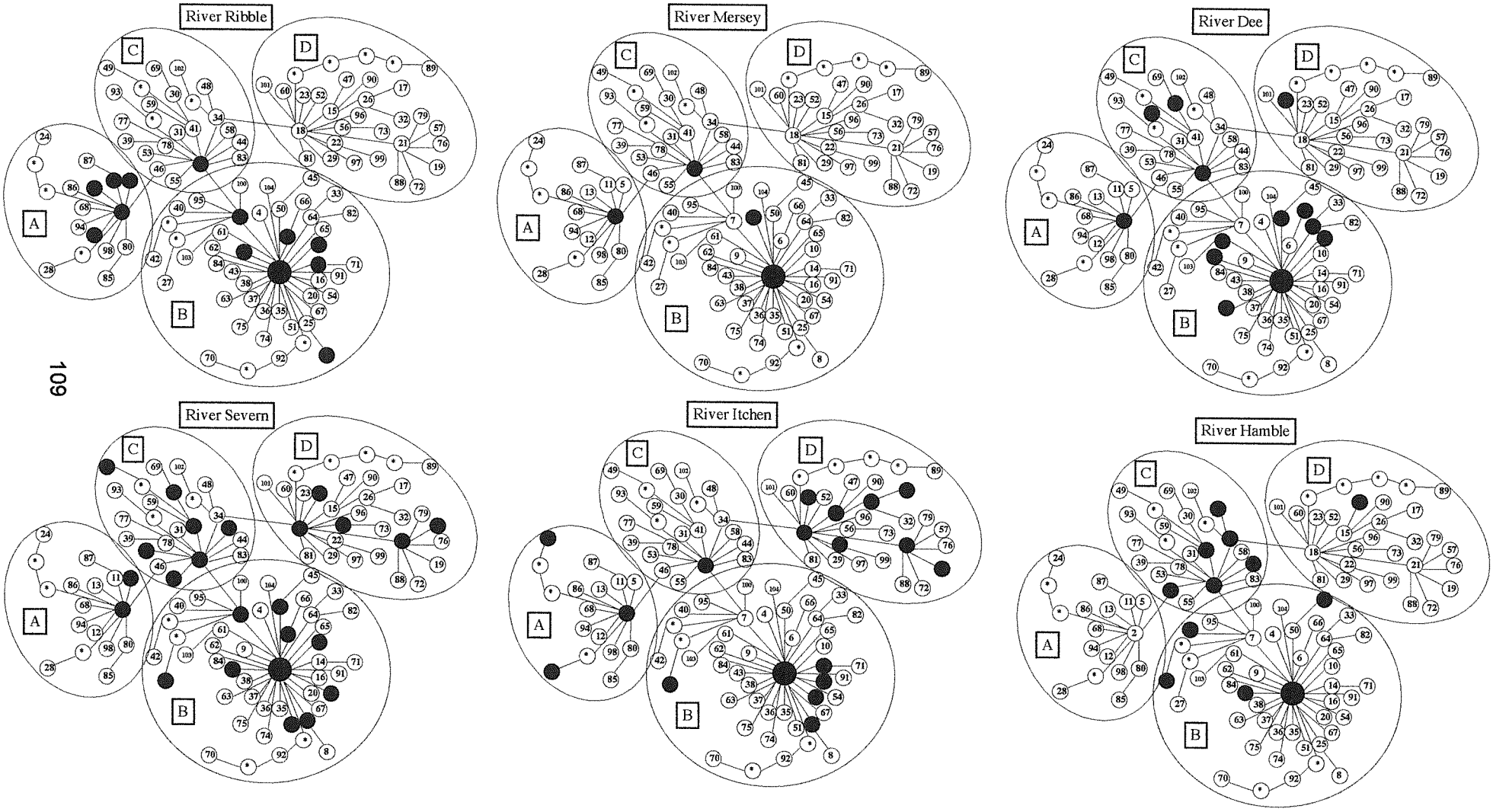


Figure 4.3 Haplotype diversity of each population in this study. Haplotypes found in each population are indicated by black circles.

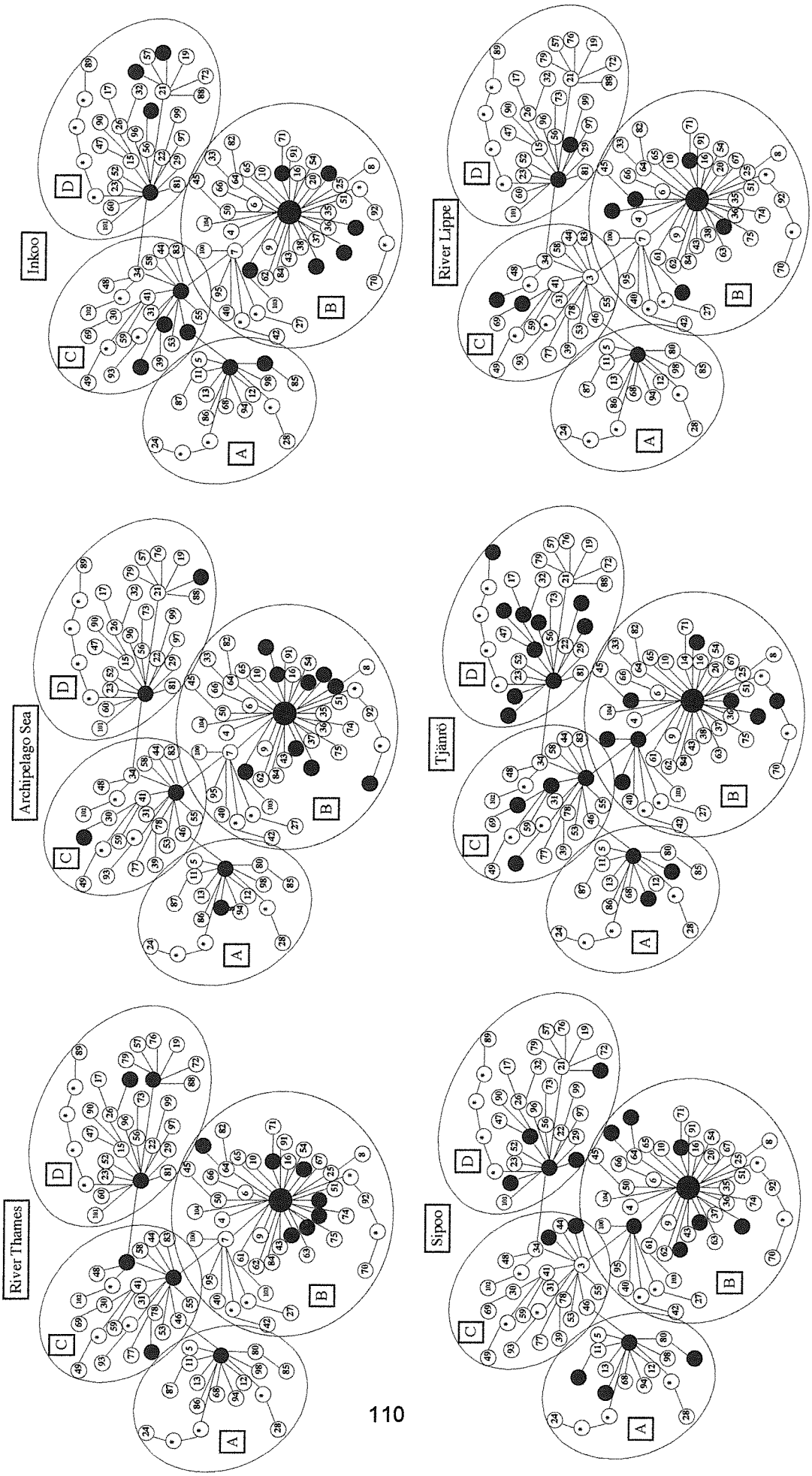


Figure 4.3 Continued...

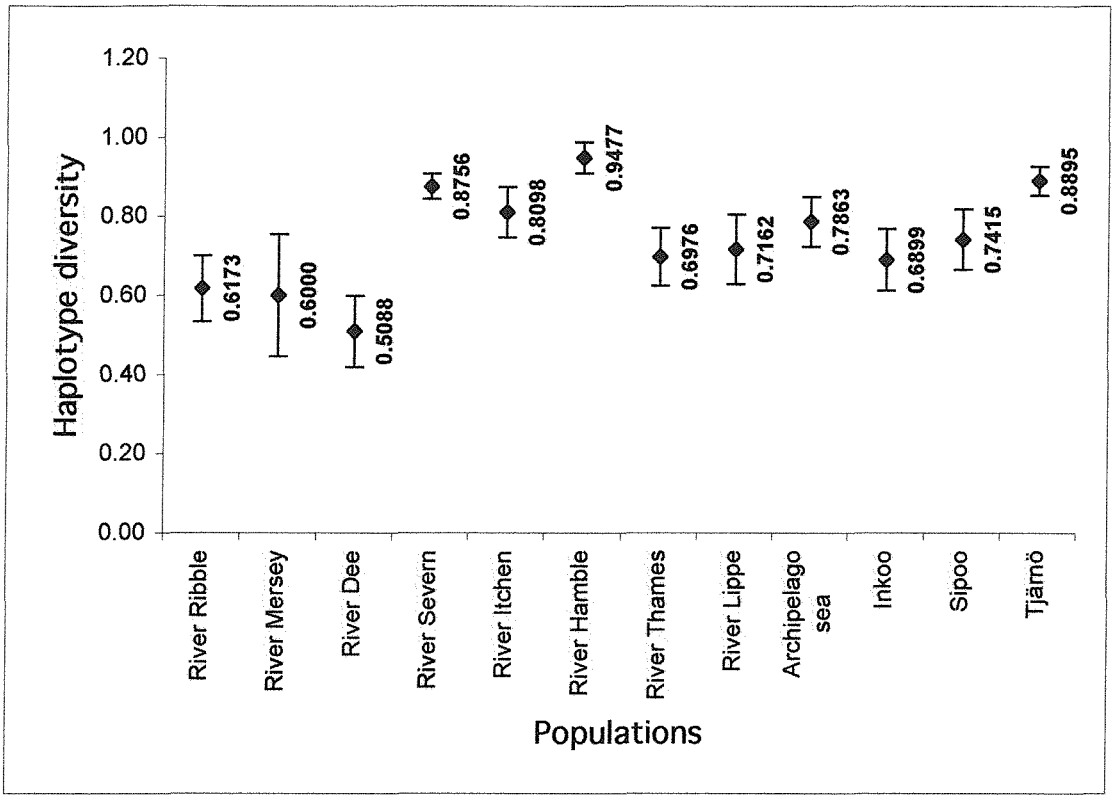


Figure 4.4a Haplotype diversity (h) of each population and their standard error

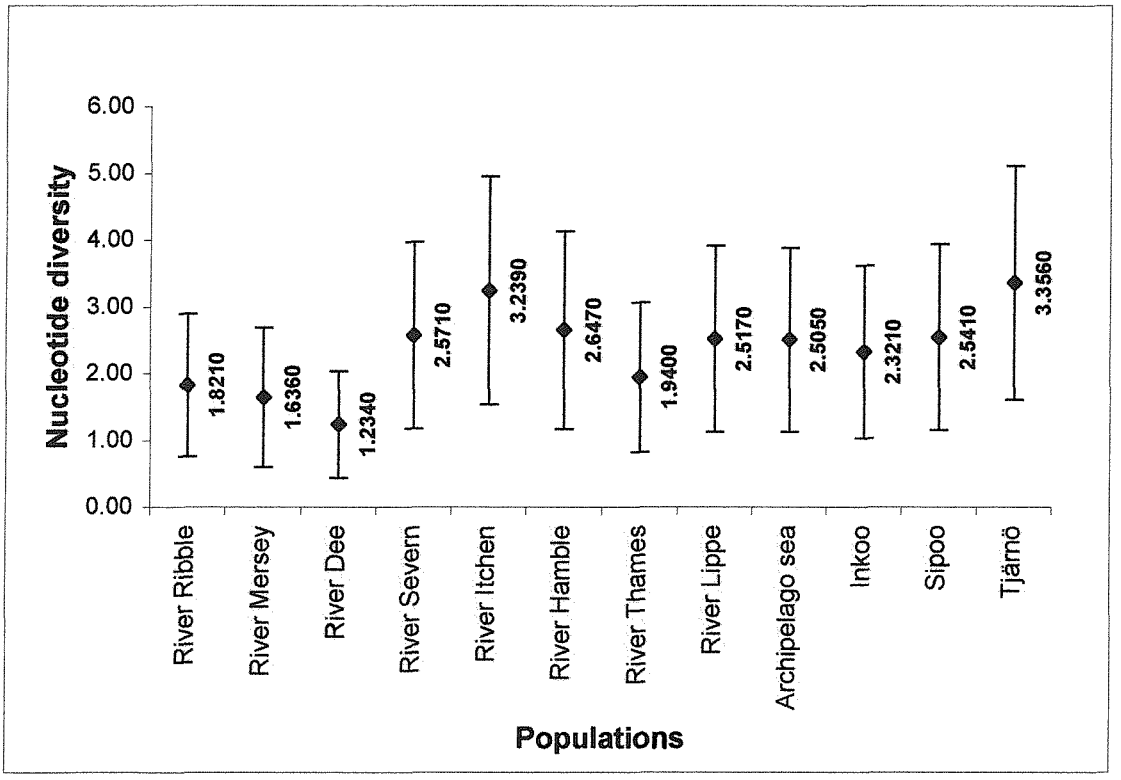


Figure 4.4b Nucleotide diversity (π) or mean number of pairwise difference of each population and their standard error

1.23 (River Dee) to 3.36 (Tjänrö) and low value of nucleotide diversity were also found in west coast populations from the British Isles, River Ribble (1.82), River Mersey (1.64) and River Dee (1.23) (see fig 4.4b).

4.3.2 Inter-specific populations variation

4.3.2.1 Population comparison

Pairwise comparisons between populations including the cladogram produced from the Fst matrix, showed no systematic grouping, when all pairs of populations were analysed (the British Isles and Baltic Sea) (see fig. 4.5). There was also no pattern in grouping of the populations within the British Isles. However, in the Baltic, the population from the west coast of Sweden showed significant genetic differentiation to the rest of the samples (populations), while there was no genetic differentiation between the other populations in the Baltic and sites (see table. 4.2a, b &c and fig. 4.5). Thus, there were indications of separation between Danish Belts and Gulf of Finland regions (see fig 4.5).

4.3.2.2 Population Structures

The AMOVA showed that there was no genetic differentiation between the British and Baltic groups (Fct = -0.0070, P value = 0.7189). Most of the variation was within populations (96%) and among populations within groups (4.69%). There were significant differences among populations within groups (Fsc = 0.0466, P value < 0.00001) and among populations (Fst = 0.0398, P < 0.00001) (see also Table 4.3).

The results from populations around the British Isles showed no significant difference between samples from the north-west coast and the south of the British Isles (Fct = 0.058, P value = 0.088). However, it showed significant differences among the

populations and among populations within the groups (see table 4.4). Therefore, there appeared to be no clear genetic pattern among the British populations.

Genetic structure within the Baltic populations showed slightly different results when the populations were divided into two groups (Danish Belts and Gulf of Finland). It was seen that there were no significant differences among the groups (Fct = 0.0378, P value = 0.101) and among populations within the groups (Fsc = 0.0075, P value = 0.1712). The significant differences among the populations are still present (see also table 4.5). This difference is potentially caused by the variation from the Swedish west coast population since the pairwise comparison showed significant difference between this population to the other Baltic populations (see table 4.5) and by excluding the Swedish population, the differences among the Baltic population were not significant (see table 4.6).

The regression analysis and Mantel's test showed no correlation between genetic distances (Fst) and geographical distances when all pairs of populations were tested (rY1 = -1.3924 (P = 0.8823)). There is also no correlation within the British Isles populations (rY1 = 0.243019 (P = 0.1184)). However, there was slight nearly significant correlation within the Baltic populations (rY1 = 0.638077 (P = 0.0580)) (see fig. 4.6).

4.4 Discussion

4.4.1 Broad-scale structure

The lack of genetic structure between the British and Baltic populations in this study was also observed in the previous study of broad-scale geographic variation of *B. improvisus* using isozyme and morphometric studies (Furman, 1990). The isozyme

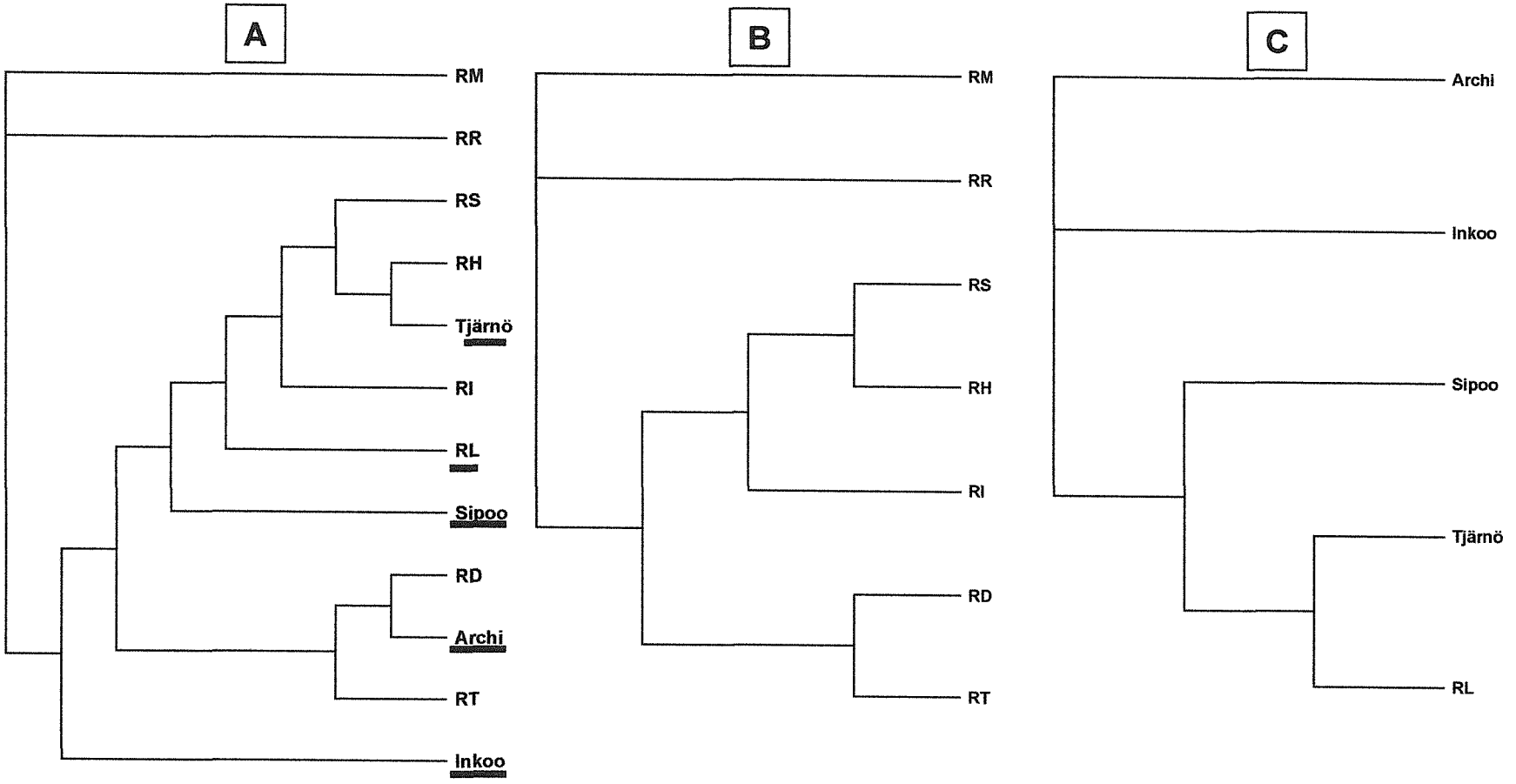


Figure 4.5 Neighbour jointing trees, generated from pairwise Fst data, showing the relationship among populations of *B. improvisus* : A) all pairs of population were analysed (Baltic populations are underlined), B) only British populations were analysed, and C) only Baltic populations Were analysed. (RM:River Mersey, RR:River Ribble, RD:River Dee, RS:River Severn, RI:River Itchen, RH:River Hamble, RT:River Thames, RL:River Lippe, Archi:Achipelago sea)

Table 4.2a Pairwise Fst matrix among populations of *B. improvisus* used in this study

River Mersey	0.00000											
River Ribble	-0.05218	0.00000										
River Severn	0.04955	0.09232	0.00000									
River Dee	0.04169	0.04810	0.14773	0.00000								
River Itchen	0.03453	0.07884	0.01583	0.11402	0.00000							
River Hamble	0.09506	0.14381	-0.01229	0.20856	-0.01522	0.00000						
River Thames	-0.00873	0.02140	0.05578	0.01585	0.03284	0.07292	0.00000					
Archipelago sea	-0.02221	0.00473	0.06506	0.01405	0.03396	0.07591	-0.01018	0.00000				
Inkoo	-0.03562	0.00185	0.03676	0.03847	0.01805	0.04838	-0.00876	-0.00969	0.00000			
Sipoo	-0.01618	0.01729	0.05116	0.02720	0.01809	0.05082	-0.01270	-0.01074	-0.01047	0.00000		
Tjärnö	0.08671	0.13736	0.02383	0.17399	0.00010	-0.02324	0.08262	0.08302	0.06490	0.06018	0.00000	
River Lippe	0.01699	0.05177	0.03635	0.03915	0.00575	0.02288	-0.00365	-0.00320	-0.00045	-0.00330	0.03967	0.00000

Table 4.2b Matrix of Fst P-Value after permuting haplotypes between populations (10100 times)

River Mersey	*											
River Ribble	0.98287+-0.0014	*										
River Severn	0.08049+-0.0029	0.00059+-0.0002	*									
River Dee	0.11128+-0.0033	0.02564+-0.0016	0.00000+-0.0000	*								
River Itchen	0.15008+-0.0040	0.00337+-0.0005	0.10623+-0.0031	0.00040+-0.0002	*							
River Hamble	0.03455+-0.0020	0.00129+-0.0004	0.65875+-0.0049	0.00020+-0.0001	0.69736+-0.0048	*						
River Thames	0.43382+-0.0047	0.09801+-0.0031	0.00663+-0.0008	0.11504+-0.0030	0.04584+-0.0021	0.01970+-0.0014	*					
Archipelago sea	0.68775+-0.0048	0.26294+-0.0041	0.00317+-0.0005	0.11553+-0.0031	0.04069+-0.0021	0.01376+-0.0010	0.76804+-0.0041	*				
Inkoo	0.83457+-0.0041	0.30819+-0.0045	0.02435+-0.0015	0.02831+-0.0016	0.12048+-0.0029	0.05534+-0.0023	0.65538+-0.0048	0.70260+-0.0048	*			
Sipoo	0.59430+-0.0049	0.12227+-0.0030	0.00931+-0.0010	0.04475+-0.0021	0.10969+-0.0033	0.03881+-0.0018	0.86645+-0.0037	0.78794+-0.0042	0.73349+-0.0040	*		
Tjärnö	0.03010+-0.0016	0.00010+-0.0001(	0.04782+-0.0020	0.00000+-0.0000	0.35680+-0.0043	0.93941+-0.0023	0.00089+-0.0003	0.00099+-0.0003	0.00495+-0.0007	0.00545+-0.0007	*	
River Lippe	0.24760+-0.0042	0.03099+-0.0019	0.04722+-0.0021	0.04168+-0.0018	0.26562+-0.0042	0.16206+-0.0037	0.44372+-0.0051	0.45075+-0.0053	0.36709+-0.0044	0.43718+-0.0057	0.03703+-0.0020	*

Table 4.2c Matrix showing significant pairwise Fst values, significance level = 0.05 (+ indicates significant Fst values, - not significant)

River Mersey	*											
River Ribble	-	*										
River Severn	-	+	*									
River Dee	-	+	+	*								
River Itchen	-	+	-	+	*							
River Hamble	+	+	-	+	-	*						
River Thames	-	-	+	-	+	+	*					
Archipelago sea	-	-	+	-	+	+	-	*				
Inkoo	-	-	+	+	-	-	-	-	*			
Sipoo	-	-	+	+	-	+	-	-	-	*		
Tjärnö	+	+	+	+	-	-	+	+	+	+	*	
River Lippe	-	+	+	+	-	-	-	-	-	-	+	*

Table 4.3 Results of hierarchical analysis of variance to test population structure between British and Baltic populations

Source of variation	d.f.	Sum of squares	Variance component	Percentage of variation	F statistics	P-Values
Among groups	1	1.674	-0.00878 va	-0.7	Fct = -0.00705	0.72038
Among populations within group	10	34.36	0.05874 Vb	4.69	Fsc = 0.04659	**<0.00001**
Within populations	457	546.759	1.19641 Vc	96.01	Fst = 0.03987	**<0.00001**
Total	468	582.793	1.24609	100.00		

Table 4.4 Results of hierarchical analysis of variance to test population structure within British populations (between south and north-western coast populations)

Source of variation	d.f.	Sum of squares	Variance component	Percentage of variation	F statistics	P-Values
Among groups	1	11.055	0.06916 Va	5.84	Fct = 0.05845	0.08871
Among populations within group	5	11.048	0.03223 Vb	2.27	Fsc = 0.02893	**0.00832**
Within populations	254	274.786	1.08184 Vc	91.43	Fst = 0.08569	**<0.00001**
Total	260	296.889	1.18323	100.00		

Table 4.5 Results of hierarchical analysis of variance to test population structure within the Baltic (between the Danish Belts and Gulf of Finland populations)

Source of variation	d.f.	Sum of squares	Variance component	Percentage of variation	F statistics	P-Values
Among groups	1	6.990	0.05299 Va	3.78	Fct = 0.03770	0.09914
Among populations within group	3	5.268	0.01017 Vb	0.72	Fsc = 0.00753	0.17257
Within populations	203	271.973	1.33977 Vc	95.50	Fst = 0.04502	**0.00452**
Total	207	284.231	1.40293	100.00		

Table 4.6 Results of hierarchical analysis of variance to test population structure among the Baltic populations exculding the population from the Swedish west coast

Source of variation	d.f.	Sum of squares	Variance component	Percentage of variation	F statistics	P-Values
Among population	3	2.696	-0.00850 Va	-0.69	Fst = -0.00695	0.74475
Within populations	154	189.753	1.23216Vb	100.69		
Total	157	192.449	1.22366	100.00		

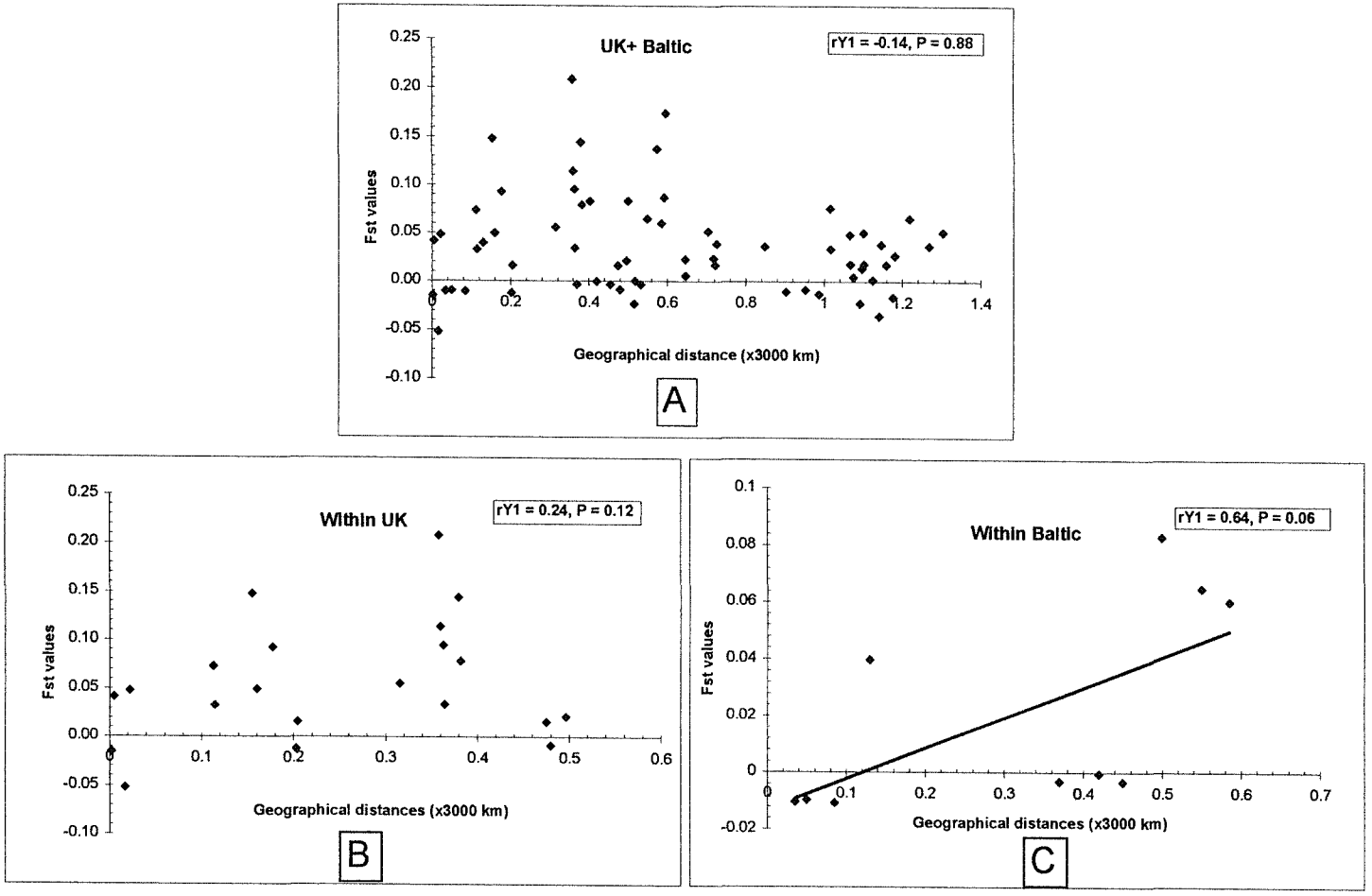


Figure 4.6 Correlation between Fst values and geographical distances both broad-scale (A) and local-scale (B and C)

analysis showed a high genetic similarity among populations from the Baltic, the west coast of Sweden, the British Isles and North America. The morphometric analysis showed more heterogeneity than the isozyme studies and revealed a separation between the populations from the Baltic, the British Isles, and North America. The morphometric variation, however, could be affected by environmental factors such as predators causing shell thickening and opercular reduction (Appleton and Palmer, 1988) and temperature affecting the characteristics of opercular valves (Barnes and Healy, 1965, 1971).

Extensive larval dispersal could cause the genetic similarity between the British and Baltic barnacle populations found in this study. By using the duration of the planktonic larval phase, the ability of larvae to disperse in *E. modestus*, which requires 10-15 days from hatching to settling, was about 20-30 km per year (Crisp, 1958). For *S. balanoides*, which exists as planktonic nauplii for 21-35 days and up to 4 weeks as pelagic cyprids, the larvae can disperse about 70-100 km per year (Flowerdew, 1983). Using the data above, Furman (1989) suggested that the larvae of *B. improvisus* require 14-27 days from hatching to settling. Therefore, the dispersal ability of the larvae could be approximately 30-70 km per year. Therefore, theoretically, the planktonic larvae of *B. improvisus* have the potential to disperse between the Baltic and Britain, but it is unlikely given their behaviour which seems to retain them at the head of estuaries (Bousfield, 1955; de Wolf, 1973; Hedgecock, 1982; Flavell, 1996). However, the genetic similarity between the British and Baltic populations might not be caused by high larval dispersal ability alone. It is possible that the Baltic and British populations were derived from the same ancestral gene pool and that their separation has happened not long enough ago to detect the differences, as was suggested in the report on Antipodean and European populations of *E. modestus* (Flowerdew, 1984). *B. improvisus* was first recorded in the Baltic in 1844 (Gislén, 1950), but the first record of

B. improvisus in Britain was not known. However, a monograph by Darwin (1854) suggested that the species might be present in 19th century. Therefore, we assumed that the separation occurred about 150-200 years ago. It would be difficult to detect the genetic structure of this barnacle species with high dispersal ability larvae between these two regions. However, it is also important to consider that the first records of the species do not necessarily mean that the first colonisation of the species in the Baltic occurred at that time. The species might have lived there long before its discovery.

4.4.2 Local-scale structure

In the previous study in enzyme variation of *B. improvisus* around the British Isles (Furman *et al.*, 1989), 11 enzymatic loci were used. Five of them were considered to be monomorphic (Idh, Mdh, Apk, 6Gpd, and Hk). Differences in allele frequency distribution were found in only 3 polymorphic loci (Pgm, Gpt, and Mpi). The other 3 polymorphic loci (Apk2, Pgi, and Aco) showed no differences in allele frequency distribution. In my study, significant differences among the British populations were found. The analysis of the structure between the south (River Severn, Itchen, Hamble and Thames) and the north-west coast (River Dee, Mersey, and Ribble) of the British Isles populations also showed significant differences among populations within groups but no significant differences among groups (see Table 4.4). This suggested that there was no clear genetic structure within the British populations as also reported in the previous study (Furman, *et al.*, 1989). The level of gene flow between neighbouring estuaries should also be very low since differences were also found among populations within regional groups (south and north-west coasts). With such a long larval life, there is the potential for high larval dispersal ability, and the larvae should be able to easily disperse between neighbouring estuaries within the British Isles provided the estuaries are not too far apart. The genetic differentiation among the populations, found in this study, suggests that there should be biological and/or physical barriers to the dispersal

ability of the larvae. Since populations *of B. improvisus* around the British Isles are generally found in rivers or estuaries, salinity may then be a barrier preventing the migration between rivers or estuaries. However, the study of salinity tolerance on eggs, larvae, and adults of *B. improvisus* (Furman *et al.*, 1989) suggested that salinity could not be a barrier of the migration of the larvae since they can survive in a wide range of salinity. The behaviour of the planktonic larvae and the direction of water currents and tidal currents (both seaward and landward) could be key factors explaining of larval retention in estuaries as suggested by Bousfield (1955).

Furthermore, the populations of *B. improvisus* around the British Isles are ephemeral populations (Furman, 1989a). For this reason, it would be very difficult to detect their population genetic structure. These unstable barnacle populations and their histories are also not well documented. The differences found amongst British estuary populations could therefore be the effect of the differences in founders when the colonisation occurred. Then, larval retention in estuaries (Bousfield, 1955; de Wolf, 1973; Hedgecock, 1982; Flavell, 1996) may be the key factor in keeping the genetic identity in each estuary. It is therefore likely that ships fouled by adult barnacles which are inadvertently transported between the Baltic and British region could play an important role in the colonisation *of B. improvisus* populations around the British Isles, as adults release viable larvae into estuaries. This could be supported by the fact that the *B. improvisus* populations around British Isles are found mostly in the estuaries, which are located near harbours or docks. Furman *et al.*, (1989) also suggested that the abundant populations were found in larger estuaries with heavier traffic, where large commercial ships are present.

In the Baltic, there was no significant difference among populations within the groups and among the groups (between the Danish Belts and Gulf of Finland). This suggests

that there is a high level of gene flow between the populations within the Danish Belt and Gulf of Finland areas in contrast to the results in the British populations. Similar results were also found in previous studies (Furman, *et al.*, 1989; Furman, 1989b).

Another possibility to explain the overall lack of Baltic genetic differentiation is that the colonisation of the populations in the Baltic has not happened long enough ago to form sub-populations within the region. However, there were some differences among the Baltic populations. This was due to the variance in the population from the Swedish west coast which showed strong differentiation from other populations in the Baltic (see table. 4.2c). Moreover, there was no genetic differentiation among the Baltic populations when the Swedish west coast population was excluded from the analysis (see table 4.6). The genetic differentiation found between the Swedish west coast population and the others in the Baltic could be the result of a number of factors. Firstly, the differentiation may have been caused by selection pressure because of the dramatic fluctuation in salinity occurring in the Danish Belts region (mouth of the Baltic sea). Such a selection has been reported in *Mytilus edulis* (Theisen 1978; Varvio *et al.* 1988), and *Gammarus zaddachi* (Bulnheim and Scholl, 1982). Furman *et al.* (1989) however showed that eggs, larvae, and adults of *B. improvisus* could survive in wide ranges of saline conditions. It is therefore unlikely that this genetic differentiation is the result of selection pressure, since selection is also not likely to have any effect on mtDNA used in this study compared with the allozymes used in the study described above (Furman *et al.*, 1989; Furman, 1990). However, selection may have resulted in isolation and subsequent divergence of mtDNA. Secondly, the differentiation may have been caused by a problem with the sampling, since the samples obtained from the Swedish west coast were obtained from laboratory maintained, as described in section 4.2.2. Therefore, samples should have consisted of only one generation and the survival of the samples may have been different from natural sites, where there is more

competition for food and space. This could have led to strong genetic differentiation in this population in comparison to other Baltic populations.

My study was unable to determine population genetic structuring between the Baltic and British populations. This is likely to be due to the lack of difference between the Baltic populations and the problems with the temporal population structuring in the British Isles populations. A more extensive population sampling strategy may reveal such population structuring, although this is likely to be difficult due to the few numbers of individuals found in many British sites. However, from the results so far obtained, it would be reasonable to note that the level of gene flow among populations within the Baltic Sea is higher than within the British Isles.

4.4.3 Dispersal patterns

The distribution of *B. improvisus* populations around the British Isles is considered to be patchy and isolated (Furman *et al.*, 1989) and the populations mostly occur at the upper reaches of the British estuaries. In contrast, distribution is continuous within the Baltic Sea, although the species is most abundant in harbours (Furman, 1989b). The dispersal patterns of the larvae within these regions is therefore an interesting aspect of this study. As described above, gene flow among populations within the Baltic Sea appears to be higher than between British estuaries. This suggests that big estuaries (such as the Baltic Sea), allow larvae to disperse throughout the area and maintain genetic homogeneity within the estuary. In contrast, in the populations around the British Isles which are isolated, the larval migration from one estuary to the other may take place at a very low level. Therefore a massive number of larvae have to be produced to provide a tiny number of larvae that migrate, adapt, and become new recruits to populations in the new environment. If migration between nearby estuaries does occur, why is *B. improvisus* not found along the coast between the estuaries

where populations are present? Even though the answer to this question is still unknown, it may be because the competition for food and space with other barnacles and marine organisms in the open sea is too great, and there may be a preference for low salinity water conditions in this barnacle species. Larval retention in estuaries may also have an effect on the isolation of their populations.

Dispersal of larvae between the Baltic and British populations are likely to be the result of adult migration by ships (Southward and Newman, 1977), since the broad-scale distribution of this barnacle species around the world is patchy (Newman and Ross, 1976). The lack of genetic differentiation between European and American populations reported by Furman (1990), also supports the hypothesis above.

Chapter Five

The occurrence and evolution of a unique intergenic spacer in the mtDNA of *Balanus improvisus* (Darwin) and *Balanus eburneus* (Gould)

5.1 Introduction

The animal mitochondrial genome generally consists of 37 genes encoding for 13 proteins, 2 rRNA, and 22 tRNA genes. There is only one non-coding region, also known as the control region, which contains the initiation site for mtDNA replication and RNA transcription (Brown, 1983, 1985). The gene content within animal mtDNA is highly conserved, but an extra $tRNA^{met}$ gene has been reported in the blue mussel (*Mytilus edulis*) (Hoffmann *et al.*, 1992). Gene organisation within animal mtDNA was once thought to be conserved, but the recent availability of complete mtDNA genome sequences of both vertebrates and invertebrates suggests that gene rearrangements have occurred more often than previously thought. It has also been found that the rearrangements have mostly involved the movement of tRNA genes (Hoffmann, *et al.*, 1992; Kumasawa *et al.* 1996). The sizes of animal mtDNA are generally in the range of 1.6 to 1.9kb (Brown, 1985). However, there are now a large number of reports showing that a large size variation in animal mtDNA can occur. There are two main causes of the variation. Firstly, the variation of tandem repeated sequences mostly occurring in the control region has been reported in scallops (Gjetvaj *et al.*, 1992), fish (Minnow, Broughton and Dowling, 1994; Sturgeon, Miracle and Campton, 1995), mammals

(Hoelzel *et al.*, 1993 and 1994), and birds (Berg *et al.*, 1995). Secondly, the direct tandem duplication of coding and non-coding sequences has been found in lizards (Stanton *et al.*, 1994; Zevering, *et al.*, 1991; Moritz and Brown, 1987), snakes (Kumazawa *et al.*, 1996), and honeybee (*Apis mellifera*) (Cornuet *et al.*, 1991). However, the origin of the extra sequences found in the salamander, family (Ambystomatidae), are still unclear (McKnight and Shaffer 1997).

As mentioned in a previous chapter (Chapter 3 section 3.3.4.2), a unique DNA sequence was found in the mtDNA of *B. improvisus*. It is of great interest to try to understand the origin and evolution of this barnacle and of the sequence. Based on "Revision of the Balanomorpha barnacles; including a catalog of the species" (Newman and Ross, 1976), "True barnacles" belong to the order Thoracica. It consists of four suborders, namely Lepodomorpha (the stalked barnacles), Brachylepadomorpha (mostly extinct), Verrucomorpha (asymmetrical sessile acorn barnacles), and Balanomorpha (sessile acorn barnacles). The Balanomorpha is composed of three superfamilies namely, Chthamaloidea, Balanomorphoidea, and Balanoidea (see also fig 5.1). There are two hypotheses for the origin of the Balanomorpha. Firstly, a monophyletic hypothesis, that the Chthamalidae were derived from a scalpelliform barnacle and gave rise to Balanomorphoidae and Balanoidae. The Chthamaloidae are usually regarded as more primitive than the Balanoidae. The fossil record showed that *Catophragmus* (a chthamalid) appeared in the late Cretaceous, and the Balanoidae appeared later in the early Eocene. Secondly, in a polyphyletic hypothesis, the three superfamilies in the Balanomorpha have an independent evolutionary lineage and are directly derivable from the Lepadomorpha.

Phylogenetic work to date has concentrated on higher order relationships within the Cirripedia (Billoud *et al.*, 2000; Glenner, *et al.*, 1995; Spear *et al.*, 1994). At a fine scale

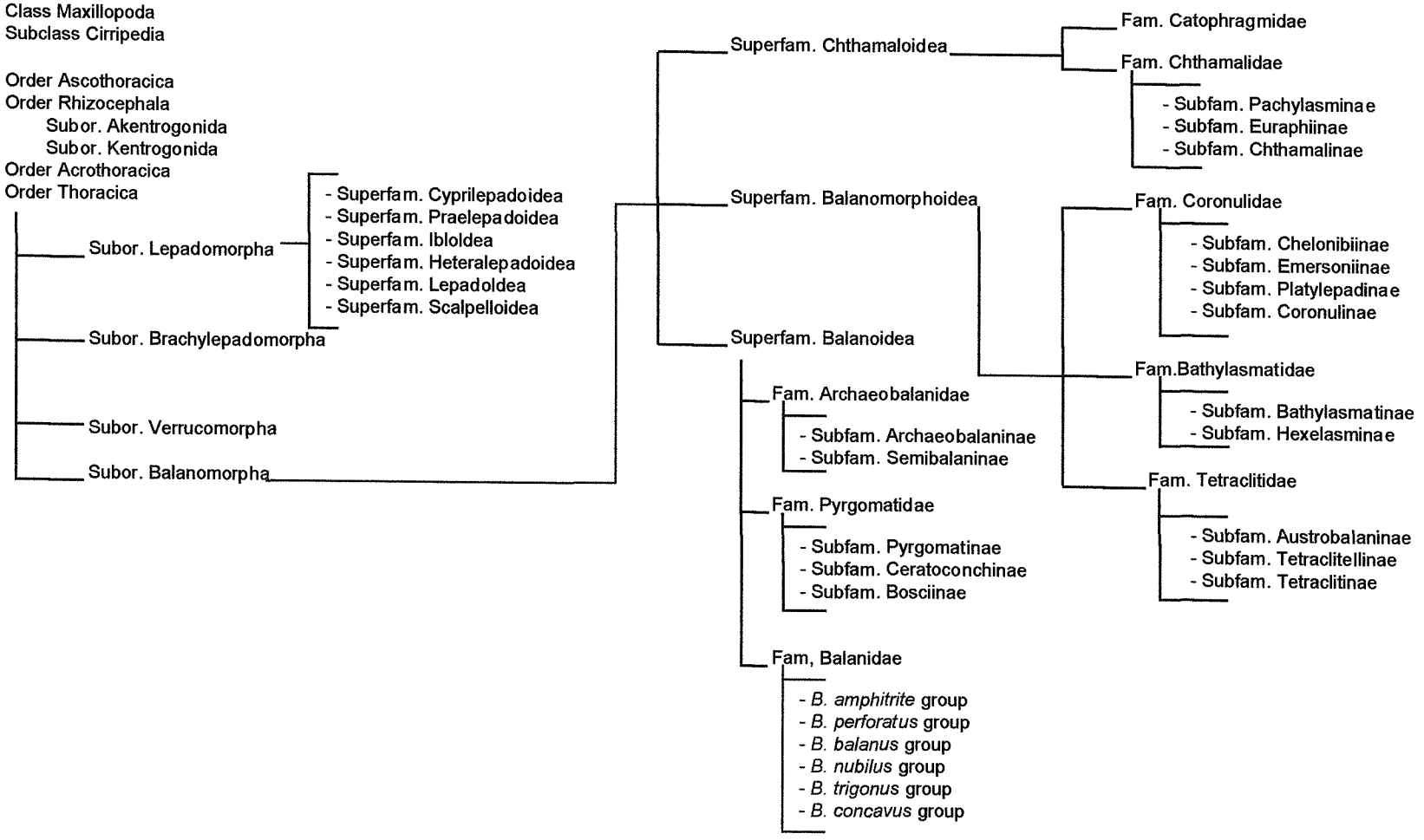


Figure 5.1 The classification of balanomorpha based on Newman and Ross (1976) and Newman (1987)

(i.e. within family or subfamily), relationships are still unclear. Since *B. improvisus* was considered to be in the *B. amphitrite* group on morphological grounds (Henry and Mclaughlin, 1975), it is important to understand the phylogeny of *B. amphitrite* group and its close relatives. This could provide information concerning the evolutionary lineage of a unique spacer found in the mtDNA of *B. improvisus* as described above.

In this chapter, a further investigation of the unique spacer found in *B. improvisus* was carried out, with particular reference to its distribution, origin and evolution. The distribution of the spacer located between COI and $tRNA^{leu}$ genes in other thoracica and general features of the spacer were investigated. The evolutionary lineage and origin of this spacer were also studied using available molecular information and phylogenetic analyses.

5.2 Materials and methods

5.2.1 Samples.

Thirteen barnacle species were used in this study. They were collected from various places (see table 5.1). All barnacle samples were preserved in absolute ethanol, stored at –20°C, and used as soon as possible.

5.2.2 DNA extraction

DNA was extracted using the modified CTAB protocol (see section 3.2.1) for all barnacle species, except for *B. amphitrite* for which the protocol described below was used, since degraded DNA was obtained when CTAB or Phenol:Chloroform protocol was used.

Table 5.1 List of taxa used in this study with their classification (based on Newman and Ross, 1976), and the location where they were collected

Taxon	Classification based on (Newman and Ross, 1976)	Collection information
Pollicipes pollicipes	Order Thoracica, sub order Lepadomorpha Superfam. Scalpelloidea	Sines, South west Portugal By Dr. Teresa Cruz
Verruca stroemia	Order Thoracica, sub order Verrucomorpha	South west England By Prof. S.J. Hawkins
Euraphia depressa	Order Thoracica, sub order Balanomorpha Superfam. Chthamaloidea, Fam. Chthamalidae	Pontetto, Genoa, Italy By Dr. Federica Pannacuilli
Chthamalus stellatus	Order Thoracica, Sub order Balanomorpha Superfam. Chthamaloidea, Fam. Chthamalidae	Cork, Ireland By Dr. A.M. Power
Chthamalus montagui	Order Thoracica, sub order Balanomorpha Superfam. Chthamaloidea, Fam. Chthamalidae	Cork Ireland By Dr. A.M. Power
Tetraclita squamosa squamosa	Order Thoracica, sub order Balanomorpha Superfam. Balanomorphoidea, Fam. Tetraclitidae	Butterfly beach, Hongkong By Dr. G.A. Williams
Elminius modestus	Order Thoracica, sub order Balanomorpha Superfam. Balanoidea, Fam. Archaeobalanidae	Riever Itchen, Southampton, UK By Sanit Piyapattanakorn
Semibalanus balanoides	Order Thoracica, sub order Balanomorpha Superfam. Balanoidea, Fam. Archaeobalanidae	South west England By Prof. S.J. Hawkins
Balanus perforatus	Order Thoracica, sub order Balanomorpha Superfam. Balanoidea, Fam. Balanidae	South west England By Prof. S.J. Hawkins
B. crenatus	Order Thoracica, sub order Balanomorpha Superfam. Balanoidea, Fam. Balanidae	Albert Dock, Liverpool, UK. By Prof. S.J. Hawkins
B. amphitrite	Order Thoracica, sub order Balanomorpha Superfam. Balanoidea, Fam. Balanidae	Genoa Habour, Genoa, Italy By Dr. Federica Pannacuilli
B. improvisus	Order Thoracica, sub order Balanomorpha Superfam. Balanoidea, Fam. Balanidae	River Itchen, Southampton, UK, By Sanit Piyapattanakorn
B. eburneus	Order Thoracica, sub order Balanomorpha Superfam. Balanoidea, Fam. Balanidae	Genoa Habour, Genoa, Italy By Dr. Federica Pannacuilli

The thorax of *B. amphitrite* was homogenised with 250 μl lysis buffer (6M of Guanidine Thiocyanate (Promega) and 0.1M of Sodium Acetate (Sigma)) and incubated at room temperature for 30 minutes. The solution was then centrifuged at 6500rpm and the supernatant removed. The DNA was precipitated with 2 volumes of absolute ethanol, kept at –20°C for 1 hour, and centrifuged at 14000rpm for 15 minutes to collect the DNA pellet. The pellet was resuspensed with 200μl of TE and followed by standard Phenol:Chloroform extraction (Sambrook *et al.*, 1989)

5.2.3 The presence of the spacer

This intergenic spacer was found during the development of genetic markers for *B. improvisus* (see section 3.3.4.2). To investigate the presence of this spacer in other barnacle species, a set of primers amplifying this spacer was developed. The mtDNA sequence of COI-COIII of *B. improvisus* was obtained (see fig. 3.3), and highly conserved regions were identified by aligning *B. improvisus* DNA sequences with other DNA sequences of crustaceans and insects. A *Balanus improvisus* primer (BI-COIF2) was designed to allow more specific PCR amplification for barnacles. The COII-croz primer developed for the honeybee *Apis mellifera* (Crozier *et al.* 1989) was retained as the reversed primer since the mismatch within the sequences did not affect its specificity for *B. improvisus*. This set of primers produced specific PCR products (COI-COII) from various species of barnacles. A 10 μl PCR reaction routinely used consisted of 5-10ng of extract DNA, 1X PCR buffer (Advanced Biotechnologies), 2.5 mM $MgCl_2$, 0.2 mM of dNTPs, 10 pmol of each primer [BI-COIF2 (5' ATA CCW CGW CGW TAT TCR GAN 3') and COII-Croz (5' CCA CAA ATT TCT GAA CAT TGA CC 3')], and 0.5 units of *Taq* polymerase (Advanced Biotechnologies). The reaction was carried out as follows: 95°C for 5 minutes followed by 35 cycles of 94°C for 30 seconds, 53°C for 45 seconds and 72°C for 1 minute with a final extension at 72°C for 10 minutes using PE 480 Thermocycler (Perkin Elmer).

5.2.4 Obtaining the control region sequence of *B. improvisus*

A set of degenerate primers (12sRNAF (5' GTH TAA CCG CKA YKG CTG GC 3') and tRNAmetR (5' TGG GGY ATG AAC CCR BTA GC 3')) (see fig. 5.2a) was designed to amplify the control region of *B. improvisus*, but they failed to amplify a specific PCR product. Therefore, the primer 16SBR (5' CCG GTC TGA ACT CAG ATC ACG T 3'), a universal primer used in the amplification of 16sRNA gene (Palumbi 1996a) and Spac2R (5' CCA CAA GAC CTT AGT AAA GGT 3') designed based on *B. improvisus* DNA sequence was employed to amplify a 6kb PCR product including the control region (see fig. 5.2b). The expanded long template PCR system (Boehringer Mannheim) was employed to amplify the 6kb PCR product. A 50μl PCR reaction routinely employed consists of 30-50ng of extracted DNA, 1X PCR buffer III, 0.35mM dNTPs, 10pmol of each primer, 0.75 unit of expand long *Taq* polymerase. The PCR reaction was carried out as follows: 94°C for 2 minutes followed by 30 cycles of 92°C for 10 seconds, 52°C for 30 seconds and 68°C for 6 minutes. After 10 cycles the extension time was increased by 20 seconds in each cycle with a final extension at 68°C for 15 minutes.

5.2.5 Sequencing of PCR products

PCR products were run on 1% agarose gel. The products were then excised from the gel and recovered using Qiaquick Gel extraction (Qiagen). Sequencing of COI-COII genes and control region was carried out using Big Dye cycle sequencing kit (Perkin Elmer) following the manufacturer's instructions (for PCR product). BICOIF2 was used to obtain the sequences of COI, $tRNA^{leu}$, and COII sequences for those barnacle species that had no spacer. Since the spacer was found in *B. improvisus* and *B. eburneus*, to obtain the complete sequences of the spacer including $tRNA^{leu}$ and partial sequence of COII. A primer, BICOIF3, (CCA CAA TTT ACT ATC CGA TA) was specifically designed for use on these species. For the control region sequence of *B.*

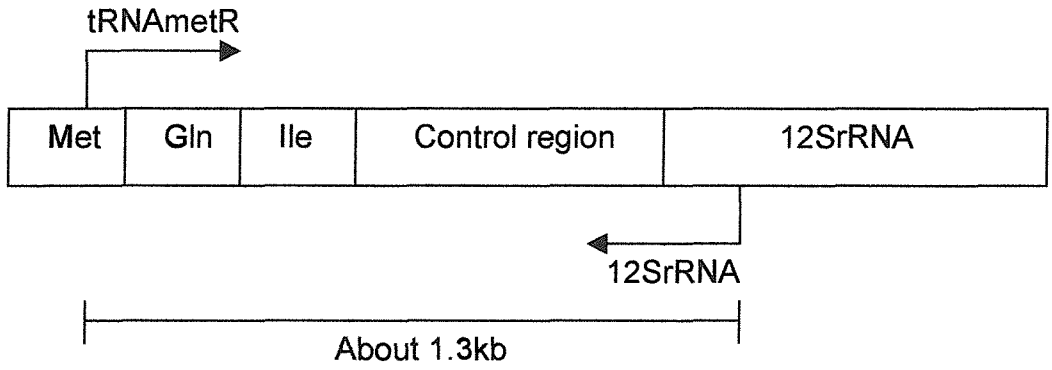


Figure 5.2a Linear diagram showing the regions spanned by the primers, tRNAmetR and 12SrRNAF, approximate size of the PCR product, and the gene organisation based on the mtDNA sequence of *D. yakuba* (Wolstenholem, 1985) [Met ($tRNA^{met}$), Gln ($tRNA^{gln}$), and Ile, ($tRNA^{ile}$)]

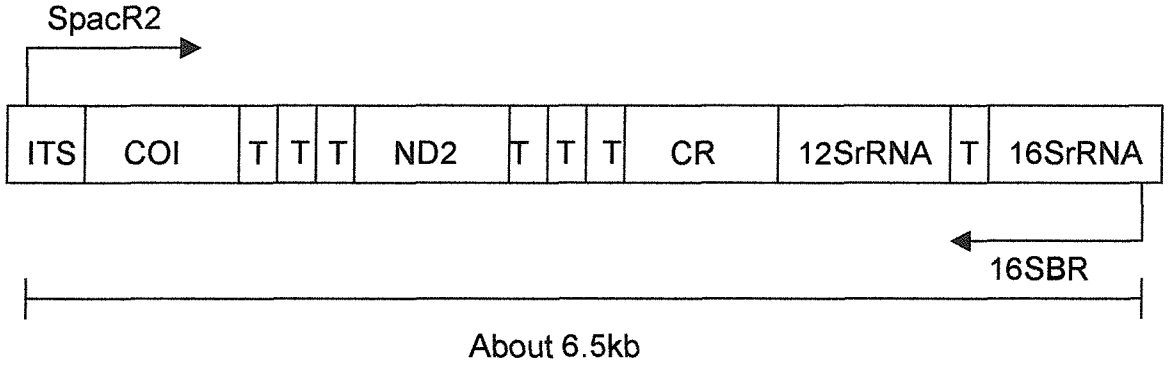


Figure 5.2b Linear diagram showing the regions spanned by the primers, SpacR2 and 16SBR, approximate size of the PCR product, and the gene organisation based on the mtDNA sequence of *D. yakuba* (Wolstenholem, 1985) and *B. improvisus* COI-COIII sequence (see chapter3). [ITS (intergenic spacer), COI (cytochrome oxidase subunit I), ND2 (dehydrogenase subunit II), CR (control region), T (tRNA genes)]

improvisus, the degenerated primers (12sRNAF & tRNAmetR) were used as primers in the sequencing reaction to obtain the control region sequence of *B. improvisus* using the 6kb PCR amplification as the DNA template for sequencing reactions.

5.2.6 Sequence analysis

The gene identification was carried out by comparing the sequences obtained from this study with known sequences of closely-related species using a DNA alignment program, Clustral X (Thompson, 1997) and Blast search. The DNA sequences were translated to amino acid using Maclade version 3.08a computer program (Maddison and Maddison, 1992). The amino acid codes of invertebrate mitochondrial DNA (*Drosophila*) were used for this translation. tRNA genes were identified by their secondary structure and their anticodon. The secondary structure of single stranded DNA was analysed by the computer software called "*RNAstructure*" (Mathews *et al.* 1999), which can be downloaded on (http://128.151.176.70/RNAstructure.html).

5.2.7 Phylogeny analysis

To understand the evolutionary lineage of the unique intergenic spacer found in *B. improvisus* and *B. eburneus*, the phylogenetic study of *B. amphitrite* group is needed. There has been no report about the phylogeny of this group of barnacles so far. Therefore, the phylogenetic analysis in this study was concentrated on the *B. amphitrite* group. Therefore, *B. improvisus*, *B. amphitrite*, and *B. eburneus* (*B. amphitrite* group), *B. crenatus* (*B. balanus* group), *B. perforatus* (*B. perforatus* group), *S. balanoides* (Archeobalanidae) *C. montagui and C. stellatus* (Chthamalidae), *V. stroemia* (Verrucomorpha), and *P. pollicipes* (Lepadomorpha) were included in this analysis. The partial sequence of COI and COII genes and complete sequence of $tRNA^{leu}$ were used. The analysis was carried out using PAUP* beta version 4.0b4a computer software (Swofford, 1998). Distance, maximum parsimony, and maximum

likelihood analyses were all employed and *P. pollicipes* was treated as an outgroup. A total of 473 characters were used, and assumed to be unordered characters. Gaps (-) were treated as missing data. The distance matrix was estimated using the TrN substitution model (Tamura and Nei, 1993), and variation among sites was set to be equal. The neighbour-jointing method (Saitou and Nei, 1987) was used to produce phylogenetic trees. The maximum parsimony analysis was carried out using branch and bound search to obtain the most parsimonious phylogenetic tree. The initial upper bound was obtained by stepwise addition. The character was weighted using the successive weighting method (Farris, 1969) with rescale consistency index (Farris, 1989). The maximum likelihood analysis was carried out using heuristic search. The model of substitution used was general time reversal (Yang, 1993; 1994). All 6 substitution types and base frequencies were estimated by maximum likelihood. Site specific variation was estimated using character-partition "*CodonPosition*" and applied for the variation among sites of the sequences. Bootstrap analysis was performed in all analyses (1000 replicates with distance and maximum parsimony analysis and 100 replicates with maximum likelihood analysis).

5.3 Results

5.3.1 PCR amplification of COI-COII

The primers (BICOIF2 and COII-croz) produced a specific PCR amplification (13 species tested so far). The sizes of the PCR products were approximately 0.9-1.2kb in length. The 0.9kb PCR product was produced from 11 species of barnacles, except for *B. improvisus* and *B. eburneus*, which produced 1.2kb PCR products. After 21 individuals of *B. eburneus* were screened, one sample produced the PCR product, which is about 1.1kb.

5.3.2 The intergenic spacer

The partial DNA sequences of COI-COII genes of various barnacle species were obtained. The larger PCR products in *B. improvisus* and *B. eburneus* were the result of a unique intergenic spacer, which is located between COI and $tRNA^{leu}$ genes as found in the previous study (see section 3.3.4.2). This unique sequence was identified as an intergenic spacer because this DNA fragment is located just after the end of COI gene and the beginning of $tRNA^{leu}$ gene. This spacer has not been found in the other 11 barnacle species screened so far. The first 50bp at 5' end of the spacers were highly conserved, 88% similarity between *B. improvisus* and *B. eburneus*, but the sequences after that were highly variable (see fig. 5.3).

The large-scale length variation, found in *B. eburneus*, is the result of the deletion of 170bp occurring in the middle part of the spacer in some individuals (see fig. 5.3). This phenomenon did not occur in *B. improvisus*. The variations within the spacer of *B. improvisus* were mostly point mutations or base substitutions and the variation in TA repeat unit near 5' end of the spacer (see fig. 5.4). There is also no large-scale length variation within the spacer in *B. improvisus* (result from RFLP analysis chapter 4). The secondary structures of the spacers showed stable stem-and-loop structures (see fig. 5.5, 5.6, 5.7). The large deletion within the spacer of *B. eburneus* was the absence of the long stem-and-loop structure in the middle section of the structure (see fig. 5.5 and fig. 5.6). However, the mechanism causing this large variation is still unclear. The comparison between the secondary structure of the spacers obtained for *B. improvisus* and *B. eburneus* showed short conserved sequences at the basis of the long stem-and-loop structure in the middle section of the structure in both species (see fig. 5.3, 5.6 & 5.7). These conserved sequences were not found in the short form of the spacer found in *B. eburneus*.

```
                 COI ──►
B.E.1            TTTAGAATGAGTTCATACTACTCCTCCTCATTATCATAGTTACGATGAACTTCCACAATT
B.E.2            TTTAGAATGAGTTCATACTACTCCTCCTCATTATCATAGTTACGATGAACTTCCACAATT
B.E.7            TTTAGAATGAGTTCATACTACTCCTCCTCATTATCATAGTTACGATGAACTTCCACAATT
B.I.15           TTTAGAATGAGTTCATACTACTCCTCCTCACTATCATAGTTATGATGAACTTCCACAATT
                 ****************************** *********** ****************
                        ///Spacer
B.E.1            TACTATCCGATAATTCTAAAAAATAAATTTATTTTAGATTTTAATCTTACTAAAAGTTA
B.E.2            TACTATCCGATAATTCTAAAAAATAAATTTATTTTAGATTTTAATCTTACTAAAAGTTA
B.E.7            TACTATCCGATAATTCTAAAAAATAAATTTATTTTAGATTTTAATCTTACTAAAAGTTA
B.I.15           TACTATTCGATAATTCTATAAAATAAATATAGT--AGATTTTTAATCTACCTAAAGTTTA
                 ****** *********** ********* ** *  *************  *****  ***

B.E.1            AGCTTTTTAGAATTCTCAATCAAAGTCTAAAAAAATTTCCGCGGTGAATTTGCAT-AT--
B.E.2            AGCTTTTTAGAATTCTCAATCAAAGTCTAAGAAAATTTCCACGGTGAATTTGCAT-ATGT
B.E.7            AGCTTTTTAGAATTCTCAATCAAAGTCTAAAAAAATTTCCGCGGTGAATTTGCAT-ATGT
B.I.15           A--TACTTAAGAATTTTAACC----TTTTTAAGGTTACGATAGGTGAATCTGTAAGACCT
                 *  *  ***  * * * ** *    * *   *   *      ******* ** *  *

B.E.1            ------------------------------------------------------------
B.E.2            AGGGATACCCCACTGAGGTTCCCTACTTCACATGCTTAAATACCGGCAAGCAGACCGATC
B.E.7            AGGGATACCCCACTAAGGTTCCCTACTTCACATGCTTAAATACCGGCAAGCAGACCGATC
B.I.15           CGAACAACCTTCCTAGTGGTATTCCTTTGTTACACTTATATCTCCGATGACCAGCCGGCC

B.E.1            ------------------------------------------------------------
B.E.2            GGTCTGGGAAAATTTATTTTTGGCTTGTTGGTGTTTAACCGTGTGAAGAAGGGAGCCTCA
B.E.7            GGTCTGGGAAAATTTATTTTTGGCTTGTTGGTGTTTAACCGTGCGAAGAAGGGAGCCTTA
B.I.15           GGCTTTAGTATAATGGTCAAAGGAGCTATAGT-TGTAAC-----AAAGGGATACTGCTAA

B.E.1            ------------------------------------------------CAAAAATTGATT
B.E.2            GTGGGGTATCCCTACATATACAAGTTCACCTACAATGAGTTTGTGTATCAAAAATTGATT
B.E.7            GTGGGGTATCCCTACATATACAAGTTCACCTACAATGAGTTTGTGTATCAAAAATTGATT
B.I.15           GAAGGTTGTACGAGGTTTTATAAGTTCACCCAGATTGTTACAATAT-TTATATATTTATT
                                                              * * *** ***
                                                          tRNA^leu ──►
B.E.1            AGATTGGGAC----AGTTTTCGGTTATTTAAACTGTTTCTG------TAAAATCTGAAAT
B.E.2            AAATTGGGAC----AGTTTTCGGTTATTATAACTGTTTCTG------TAAAATCTGAAAT
B.E.7            AGATTGGGAC----AGTTTTCGGTTATTATAACTGTTTCTG------TAAAATCTGAAAT
B.I.15           ATATATCTATGTTTAACCTACAAGAAATA-AACCTTTACTAAGGTCTTGTGGTCTGAAAT
                 * **    *     *   * *    * *  ***  ** **       *    ********

B.E.1            GGCAGATTAGTGCTGTAGATTTAAGATCTACCCAAGAAAGTTAAAATCCTTCTTTCAGAA
B.E.2            GGCAGATTAGTGCTGTAGATTTAAGATCTACCCAAGAAAGTTAAAACCCTTCTTTCAGAA
B.E.7            GGCAGATTAGTGCTGTAGATTTAAGATCTACCCAAGAAAGTTAAAACCCTTCTTTCAGAA
B.I.15           GGCAGATTAGTGCTGTAGATTTAAGATCTACCCAAAAAGGTTTAAGTCCTTTTTTCAGAA
                 ********************************** ** *** **  **** ********
                    COII──►
B.E.1            TTTAATGTCAACATGATCACAATTAAGATTTCAAGATAGAGCTTCACCTTTAATAGAAGA
B.E.2            TTTAATGTCAACATGATCACAATTAAGATTTCAAGATAGAGCTTCACCTTTAATAGAAGA
B.E.7            TTTAATGTCAACATGATCACAATTAAGATTTCAAGATAGAGCTTCACCTTTAATAGAAGA
B.I.15           TTTAATGTCAACATGATCACAATTAAGTTTTCAAGATAGAGCTTCCCCATTAATAGAAGA
                 ************************** ***************** ** ***********
```

Figure 5.3 The alignment and identification of the spacer in *B. improvisus* (B.I.) and *B. ebumeus* (B.E.). Black bold letters indicate the spacer sequences. The conserved block sequences are shaded in black. (*) indicates identical sequences and (-) indicates gaps in sequence.

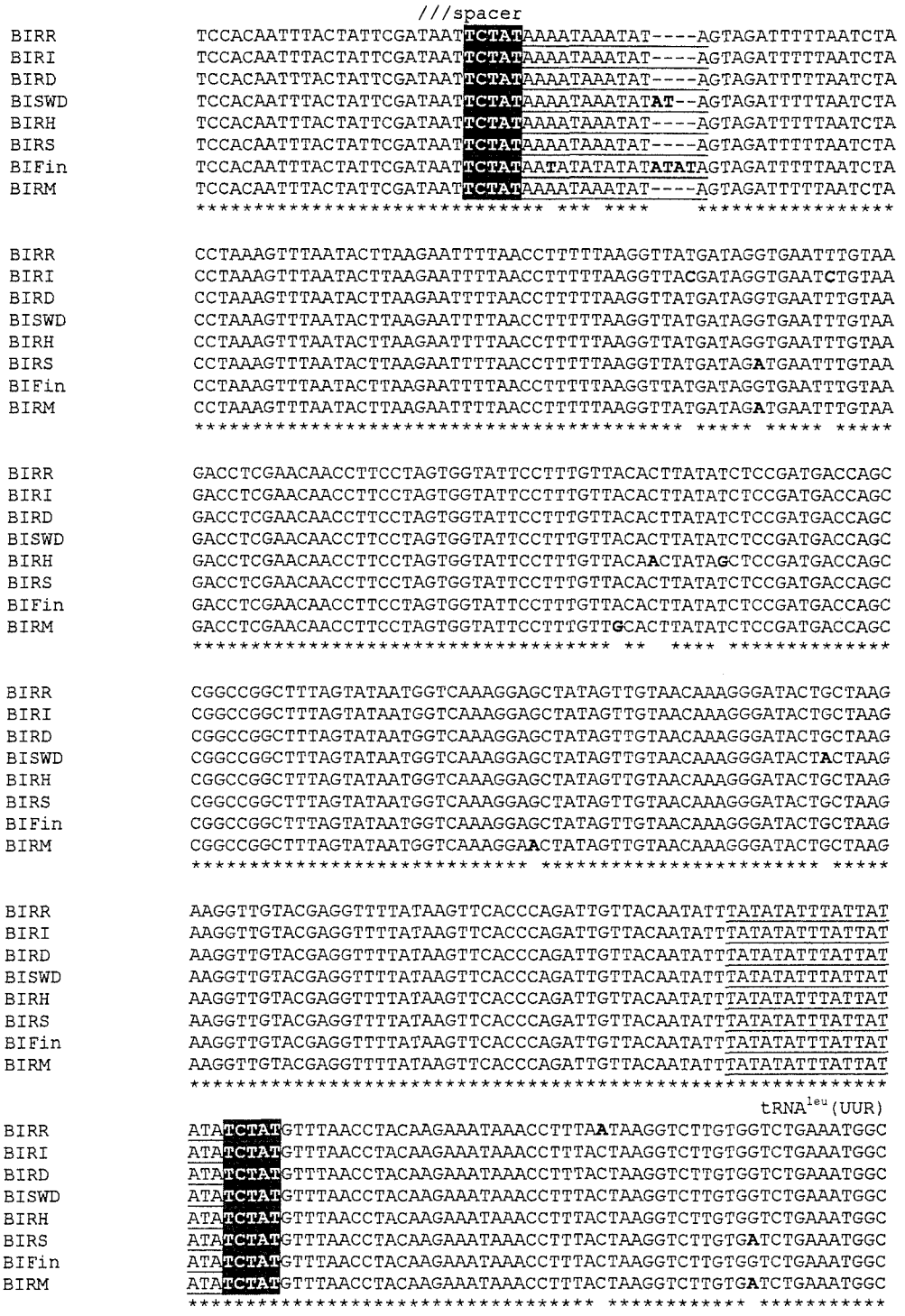


Figure 5.4 Sequence alignment of the *B. improvisus* spacer from eight samples obtained from various geographical populations [River Ribble (BIRR), River Itchen (BIRI), River Dee (BIRD), River Hamble (BIHR), River Severn (BIRS), River Mersey (BIRM), West Coast of Sweden (BISWD) and Gulf of Finland (BIFin)]. The putative direct repeat sequence was shaded with black. Variation between sequences are indicated by bold letters.

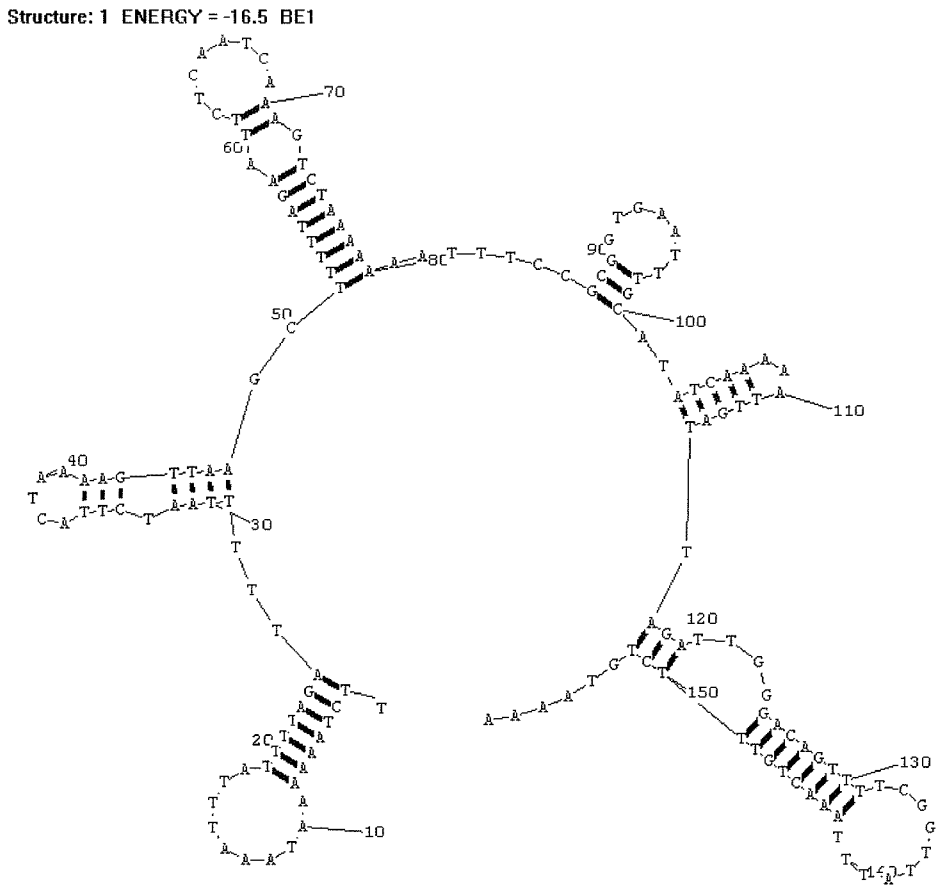


Figure 5.5 The putative secondary structure of the single-stranded DNA sequence of the short form of the spacer obtained from *B. eburneus*

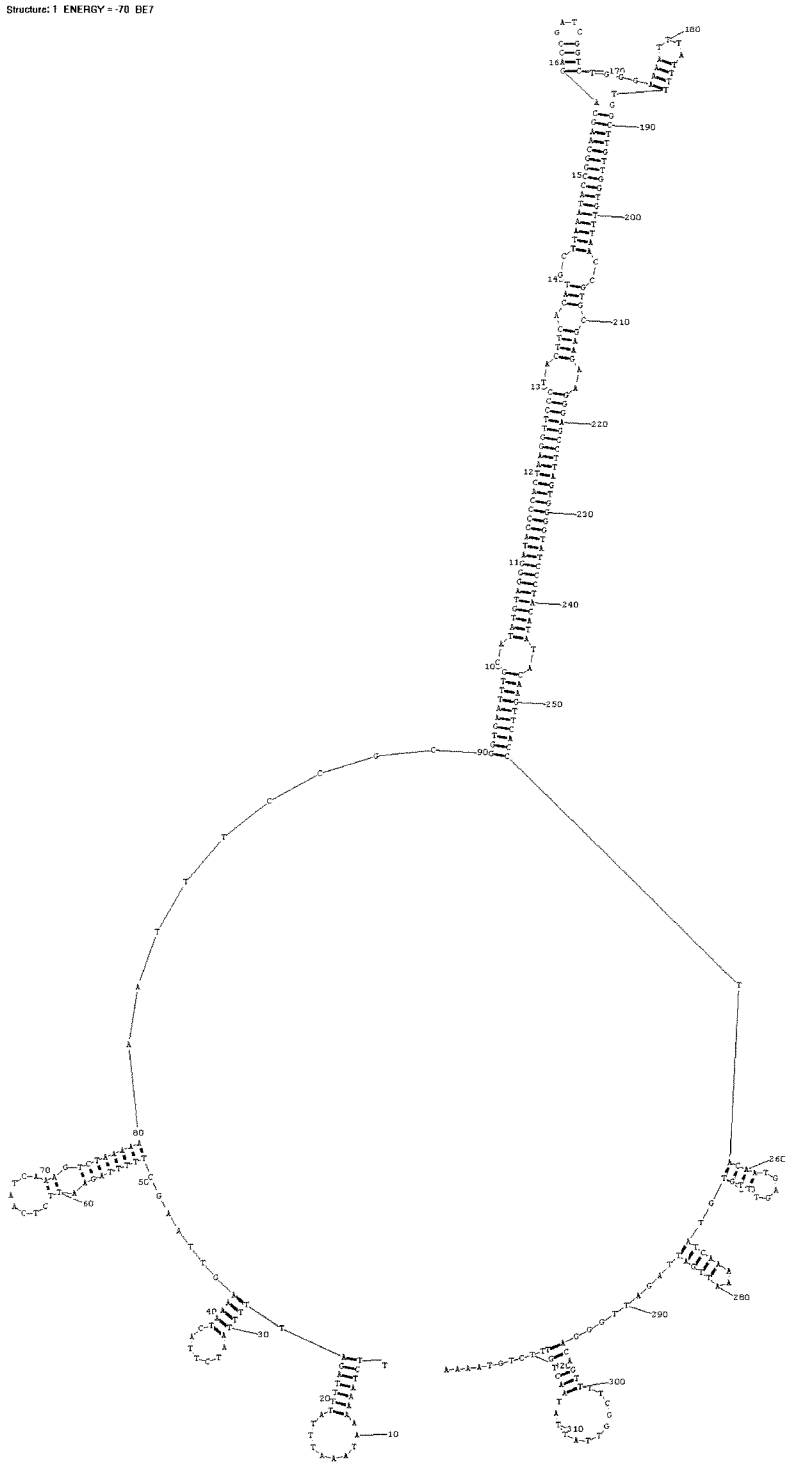


Figure 5.6 The putative secondary structure of the single-stranded DNA sequence of the long form of the spacer obtained from *B. eburneus*

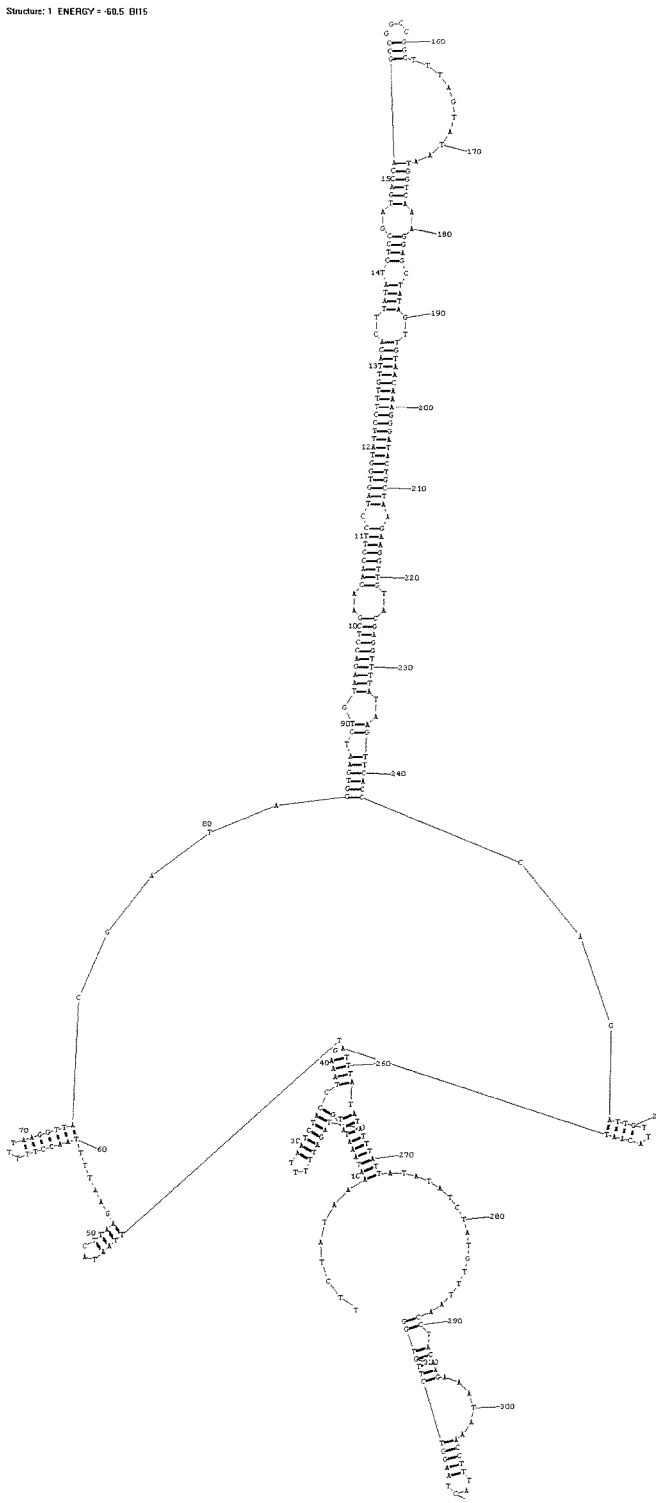


Figure 5.7 The putative secondary structure of single-stranded DNA sequence of the spacer obtained from *B. improvisus*

5.3.3 Control region amplification and sequence in *B. improvisus*

A specific 6 kb PCR product was successfully amplified from *B. improvisus* using a set of primers (Spac2R and 16SBR described in section 5.2.4) (see fig. 5.2b). The PCR product was then used as a DNA template to obtain the control region sequence of *B. improvisus*. The primers (12sRNAF and tRNAmetR) were successfully used to obtained the control region sequence. By using the mitochondrial gene organisation of *Drosophila yakuba* (Wolstenholem, 1985), the control region should be located between $tRNA^{Ile}$ and the small rRNA genes (12s rRNA). Therefore, The identification of the control region was carried out by the use of the conserved region of $tRNA^{Ile}$ to identify its 3' end (see fig. 5.8). This should indicate that the sequence was the control region *of B. improvisus*. The 5' end of the control region was difficult to identify since it is close to the12s rRNA gene, and the sequence obtained was not in the conserved region of 12s rRNA gene. This makes it very difficult to identify the 5'end of the control region. However, the putative 5'end of the control region was identified using the average position of the end of its sequence similarity with several arthropod mitochondrial 12s rRNA genes (see fig. 5.8). The control region consisted of 187A, 38C, 41G, 131T (80.1% A+T and 19.9% C+G). The length of the control region of *B. improvisus* is about 397bp, which is fairly short compared with 690bp in *Daphnia pulex* (Crease, 1999), and 1780bp in *Artemia franciscana* (Genbank accession no. X69067).

5.3.4 Phylogenetic analysis

5.3.4.1 Sequence alignment of COI-COII genes

The alignment of the sequences showed that the stop codon of the COI gene *of B. amphitrite* has one nucleotide missing (TA instead of TAA) and there is no spacer between COI and $tRNA^{leu}$(UUR) for *B. amphitrite*. However, the stop signal of COI gene in *B. amphitrite* could be caused by the polyadenylation process. The *V. stroemia* sequence also had short spacers between COI gene and $tRNA^{leu}$(UUR) (17bp) and

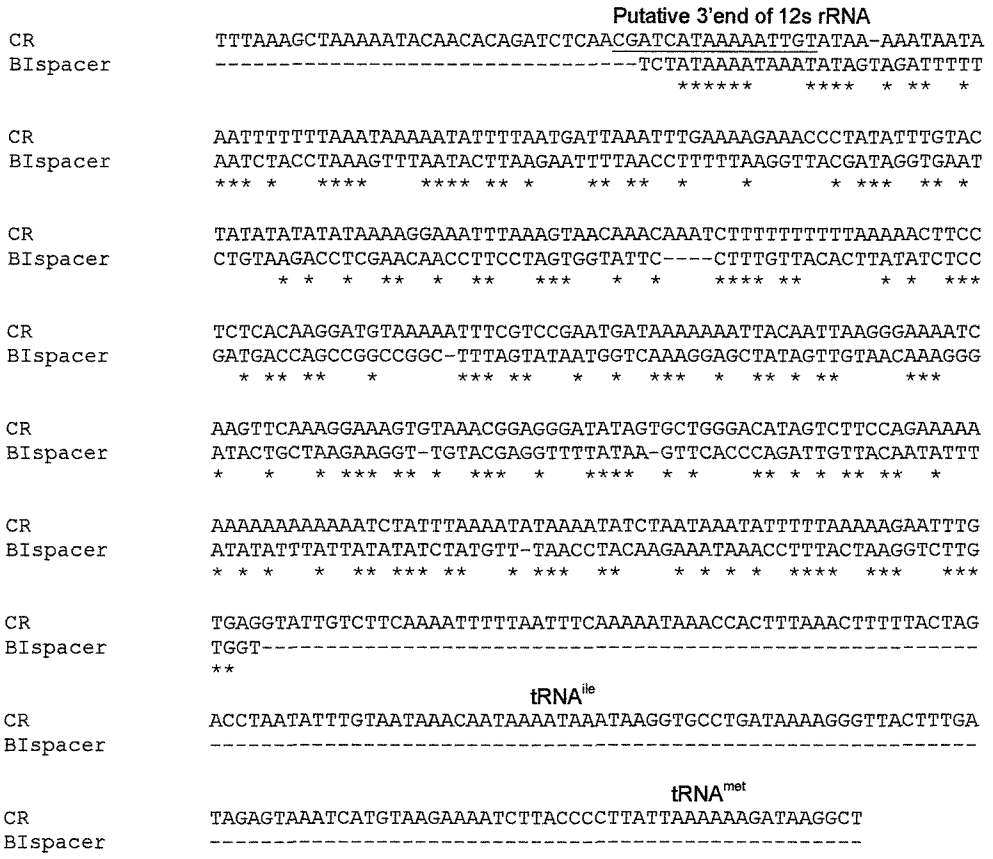


Figure 5.8 Sequence alignment between the spacer and control region sequence of *B. improvisus* (* indicates the similarity between the two sequences). The underlined letters indicate the putative area of the 3'end of 12S rRNA gene.

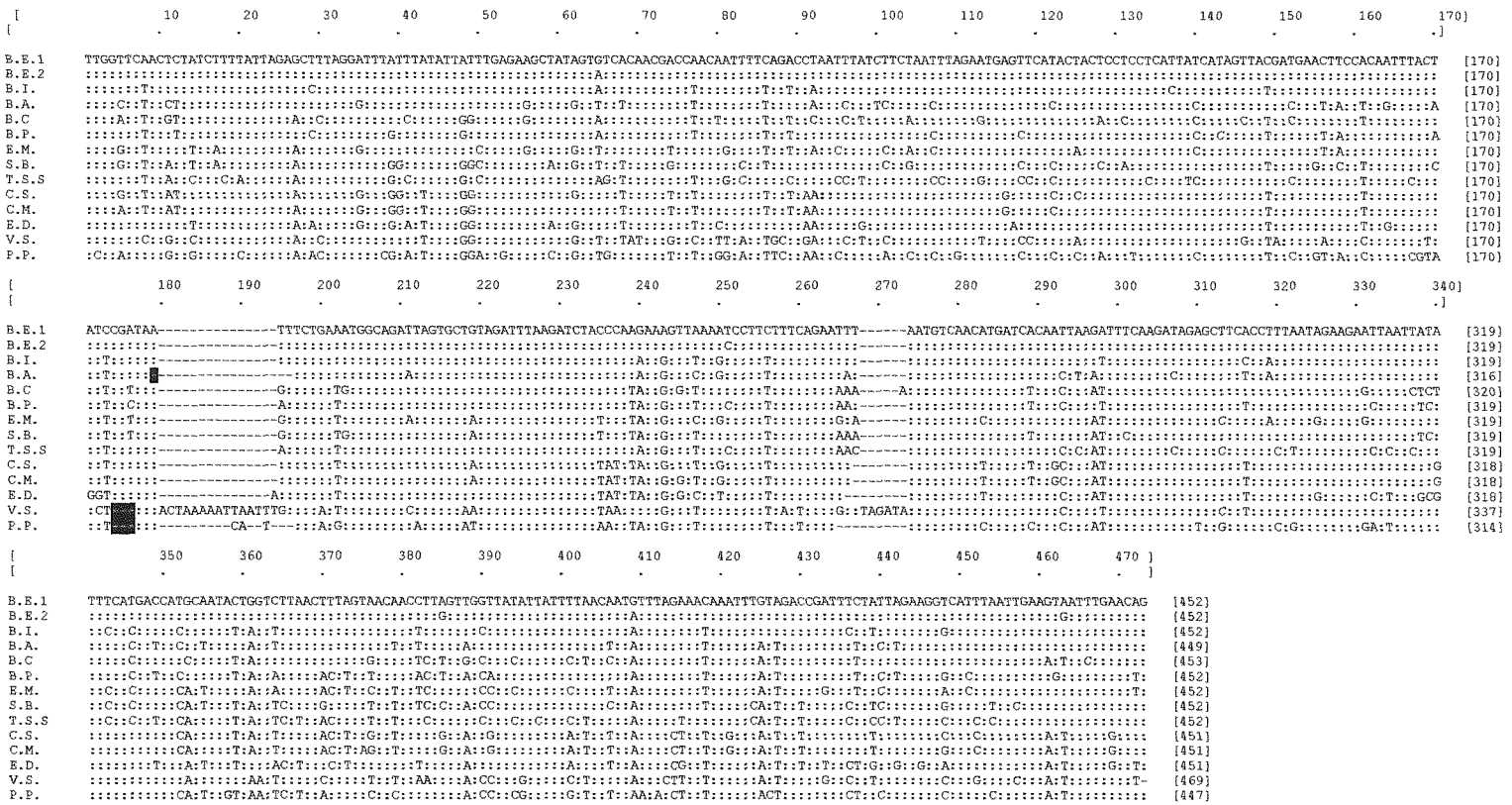


Figure 5.9 Sequence alignment of the COI-COII sequence from thirteen barnacle species showing one nucleotide missing at the stop condon of the COI gene in *B. amphirite* (B.A.), and one amino acid triplet codon missing before the stop codon of the COI gene in *V. stromia* (V.S.) and *P. pollicipes* (P.P.), shaded in black. (:) indicates characters that match those in the sequence above. (-) indicate gaps in sequences. (B.E. = *B. eburneus*, B.I. = *B. improvisus*, B.A. = *B. amphitrite*, B.C. = *B. crenatus*, B.P. = *B. perforatus*, E.M. = *E. modestus*, S.B. = *S. balanoides*, T.S.S. = *T. squamosa squamosa*, C.S. = *C. stellatus*, C.M. = *C. montagui*, E.D. = *E. depressa*, V.S. = *V. stroemia*, and P.P = *P. pollicipes*)

```
B.E.1     GSTLSFISALGFIYIIWEAMVSQRPTIFSPNLSSNLEWVHTTPPHYHSYDELPQFTIR*MSTWSQLSFQDSASPLMEELI    [80]
B.E.2     :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::    [80]
B.I.      :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::    [80]
B.A.      :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::    [80]
B.C       ::::::::T::::::G:::::::::::::::::::::::::::::::::::::::::::::::::::N:::::::::::::    [80]
B.P.      ::::::::::::V::V:::::::::::::::::::::::A:::::::::::::::::::::::::::::::::::::::::    [80]
E.M.      ::::::::T::::::::::::::::::V:::::::::::::::::::::::::::::::::::::::N:::::::::::::    [80]
S.B.      ::::::::T:::G::G:::::::::::::::::::::::A:::::::::::::::::::::::::::N:::::::::::::    [80]
T.S.S     :::::L::T:::V::V:::::A::::V::::::::::A::::::F::::::::::::::::::::N:::::::::::::    [80]
C.S.      ::::::::T:::GF:G::::::::::::::Q:::::::::::::::::::::::::::::::::::::N:::::::::::::    [80]
C.M.      ::::::::T:::GF:G::::::::::::::Q:::::::::::::::::::::::::::::::::::::N:::::::::::::    [80]
E.D.      ::::::::T:::VF:G::::::::::::::Q::::::::I:::::::::::::::::G::::::::N:::::::::::::    [80]
V.S.      ::::::::T::::F:G::::::M:::L:AR::::::D:A::::::::::N:I:::IT-::::::::::::::::::::::    [79]
P.P.      ::::::::T:::VF:G:::::A::::V::Q::::::::A::::S::::::::::V:-::::::::N::::::::::::I:    [79]

B.E.1     MFHDHAMLVLTLVTTLVGYIILTMFSNKFVDRFLLEGHLIEVIWT    [125]
B.E.2     :::::::::::::::::::::::::::::::::::::::::::::    [125]
B.I.      ::::::::::::::::::A:::::::::::::::::::::::::::    [125]
B.A.      ::::::::::::::::::::::::::::::I::::::::::::::    [125]
B.C       S::::P::::::::::::A::::::::::::I::::::::::::I:::    [125]
B.P.      S::::::::::::::::A::::::::::::I::::::::::::G:::    [125]
E.M.      :::::T::::::::::::A::::::::::::I::::::::::::::    [125]
S.B.      S::::T::::::::::::A::::::::::::I::::::::F::::::    [125]
T.S.S     :::::T::::::::::::A::::::::::::I::::::::::::::    [125]
C.S.      :::::T::::::::::::::::I:::T:::I::::::::::::I:::    [125]
C.M.      :::::T::::::E::::::::I:::T:::I::::::::::::I:::    [125]
E.D.      A::::T::::::::::::::::M:::T:::I::::::::::::I:::    [125]
V.S.      :::::T::I::::::M:A:V:::::L:::I::::::::V::I:::    [124]
P.P.      :::::T::I::::::::A:V:V::MT:::T::::::::F::I:::    [124]
```

Figure 5.10 The amino acid sequences of the COI-COII showing the lack of one amino acid Arginine (R) in *V. stroemia* and *P. pollicipes* shaded in black

also between $tRNA^{leu}$(UUR) and COII genes (10bp). In addition, other barnacle species also have short spacers between those genes but they are less than 8bp in length (See fig. 5.9). *V. stroemia* and *P. pollicipes* have one amino acid missing at the end of COI gene (Arginine (R)) (see fig. 5.10). This could be a distinct character for Lepadomorpha and Verrucomorph. However, only one example was screened for each group.

5.3.4.2 Phylogeny of *B. amphitrite* group

The best trees obtained from all three analyses showed the same structure. However, the 50% consensus tree obtained from maximum likelihood analysis was slightly different form distance and maximum parsimony analysis (see fig. 5.11, 5.12, 5.13). The analysis showed monophyly between the Chthamaloidea and Balanoidea with a strong bootstrap value (>94%). This result supported the hypothesis of the Balanomorpha being monophyletic. Although the analyses were preliminary studies for the phylogeny of *B. amphitrite* group, with only three representatives of the group, *B. improvisus* and *B. eburneus* appear to be descendents of *B. amphitrite*. The results also showed that this particular group of barnacles was well separated from the others and it seems to be at the summit of the Balanidea phylogeny. However, clearer phylogeny of *B. amphitrite* group still has to be carried out with more taxa from the group including samples from other groups such as *B. nubilus*, *B. trigonus* and *B. concavus* group.

5.4 Discussion

5.4.1 The distribution and evolutionary lineage of the spacer

The Cirripedia consist of four orders the Ascothoracica, Rhizocephala, Acrothoracia, and Thoracica (Newman and Ross, 1976). In this study on the distribution of the

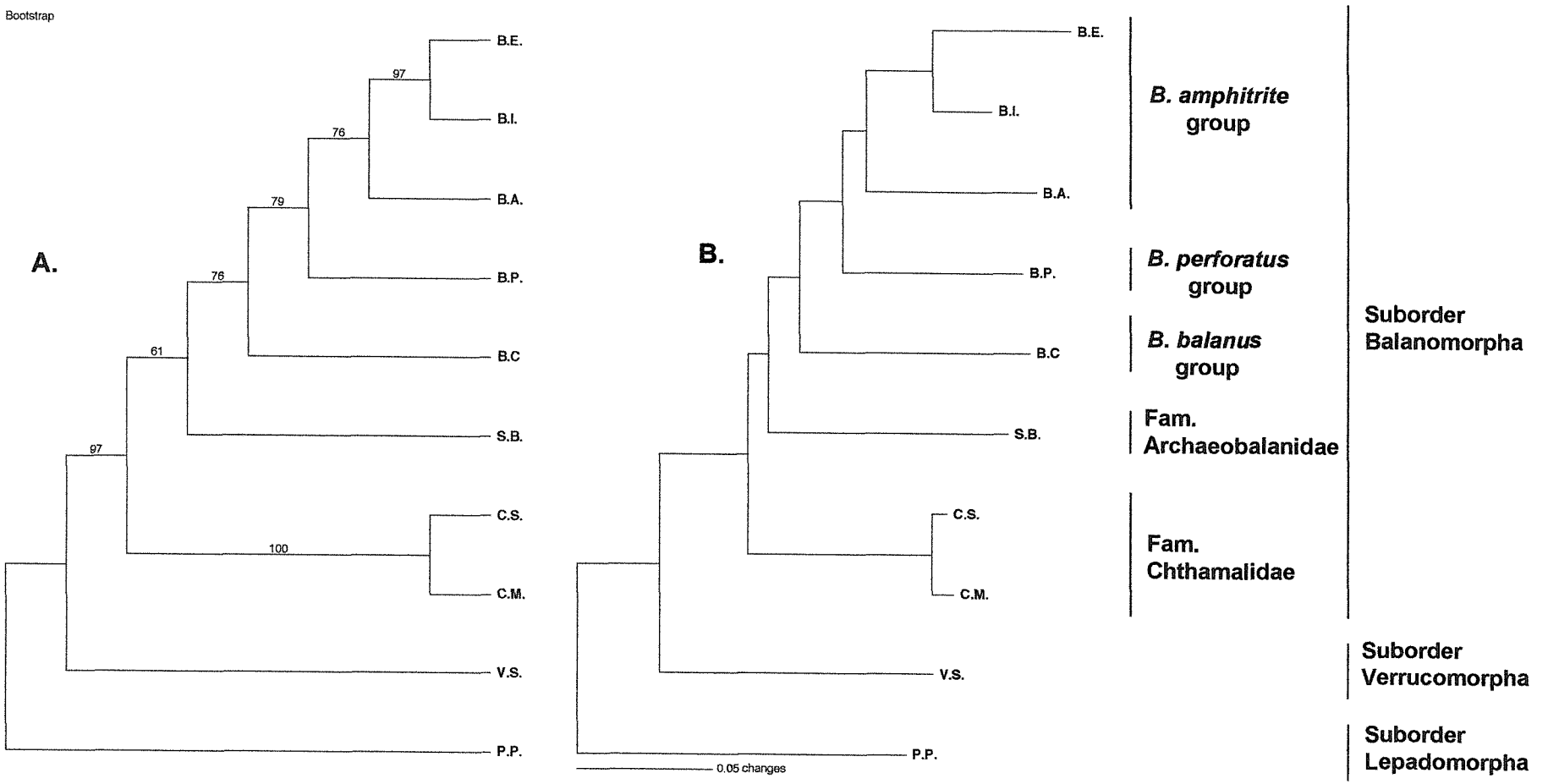


Figure 5.11 Neighbour-joining phylogenetic trees obtained from distance analysis using Tamura- Nei (TrN) substitution model (Tamura and Nei, 1993). **A.)** The 50% majority-rule consensus phylogenetic tree with bootstrap value (1000 repilcates resampling), **B.)** The best phylogeny tree with minimum evolution score = 0.8119. (B.E. = *B. eburneus*, B.I. = *B. improvisus*, B.A. = *B. amphitrite*, B.P. = *B. perforatus*, B.C. = *B. crenatus*, S.B. = *S. balanoides*, C.S. = *C. stellatus*, C.M. = *C. montagui*, V.S. = *V. stroemia*, P.P. = *P. pollicipes*)

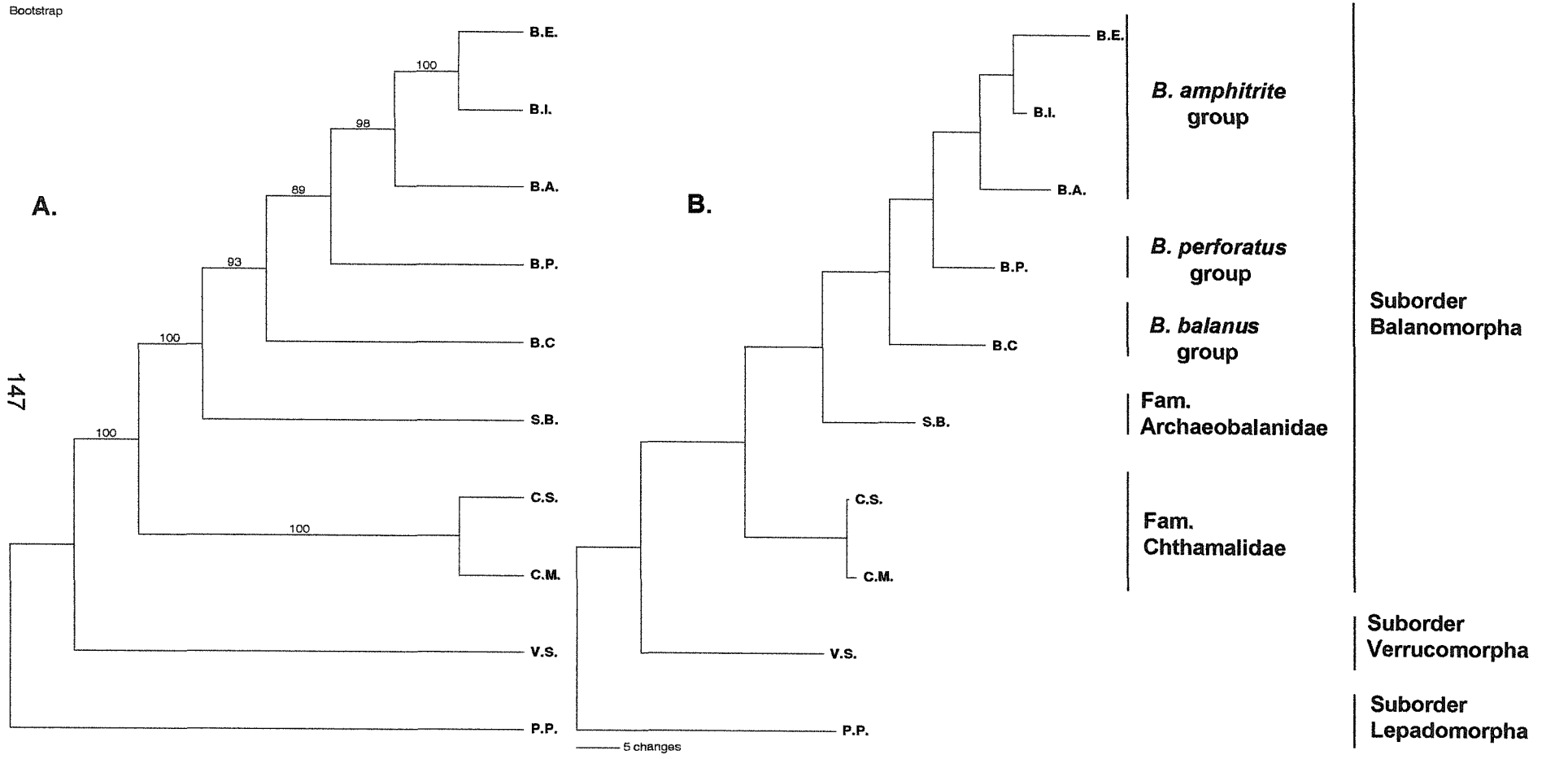


Figure 5.12 Phylogenetic trees generated by maximum parsimony analysis with 117 parsimony-informative character. *P. pollicipes* (P.P.) was used as an outgroup. Tthe Branch-and-bound algorithm was employed to obtain the most parsimonious tree (upper bound was estimated by stepwise addition). **A.)** the 50% consesus tree with bootstrap value (1000 replicate resampling), **B.)** The most parsimonious tree (tree length = 164, consistency index = 0.9, retention index = 0.8248, rescaled consistency index = 0.7435). (B.E. = *B. eburneus*, B.I. = *B. improvisus*, B.A. = *B. amphitrite*, B.P. = *B. perforatus*, B.C. = *B. crenatus*, S.B. = *S. balanoides*, C.S. = *C. stellatus*, C.M. = *C. montagui*, V.S. = *V. stroemia*, P.P. = *P. pollicipes*)

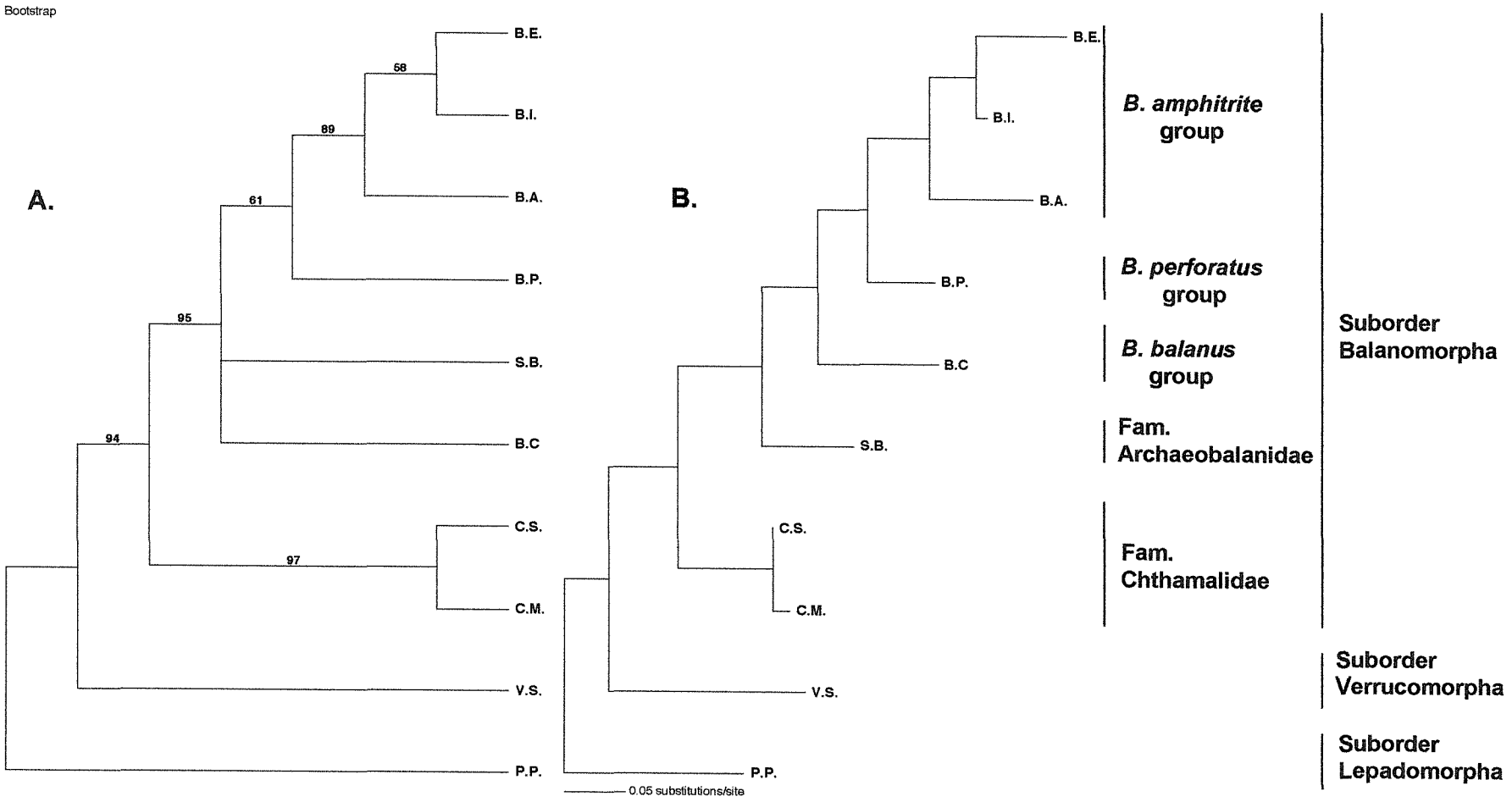


Figure 5.13 Phylogenetic trees generated by maximum likelihood analysis. *P. pollicipes* (P.P.) was used as an outgroup. An heuristic search was employed to obtain the best tree. Nucleotide frequency and substitution rate matrix were estimated via maximum likelihood. General time reversal was used as the substitution model with site-specific rates estimated using character-partition "CodonPosition". **A.)** the 50% consensus tree with bootstrap value (100 replicate resampling), **B.)** The best maximum likehood tree obtained (-Ln likelihood= 2127, (πa= 0.346, πc= 0.147, πg=0.125, πt=0.382), (a=0.354, b=3.603, c=1.46, d=1.717, e=6.245, f=1), (Non-coding=0.564, codon position1=0.466, codon position2=0.163, codon position3 =2.699). (B.E. = *B. eburneus*, B.I. = *B. improvisus*, B.A. = *B. amphitrite*, B.P. = *B. perforatus*, B.C.= *B. crenatus*, S.B. = *S. balanoides*, C.S. = *C. stellatus*, C.M. = *C. montagui*, V.S. = *V. stroemia*, P.P. = *P. pollicipes*)

spacer, the focus was on the Thoracica which contains four suborders; Lepadomorpha (stalk barnacles), Brachylepadomorpha (mostly extinct), Verrucomorpha (asymmetrical sessile barnacles), Balanomorpha (sessile acorn barnacles). After the screening of the distribution of the spacer in various species belonging to order Thoracica (except for suborder Brachylepadomorpha), the spacer was found in only two species of acorn barnacles, family Balanidae (*B. improvisus* and *B. eburneus*). Unsurprisingly, these two barnacles are in the same group (*B. amphitrite* group) (Newman and Ross, 1976, Henry and Mclaughlin, 1975), and they have been reported to be morphologically close (Bassindale, 1964). However, the spacer was not found in *B. amphitrite*, which makes it clear that this unique sequence is not a distinct character for all barnacles in *B. amphitrite* group. Therefore, this insertion was not passed on during the evolutionary lineage of all Balanomorpha since it does not occur in other Balanomorpha, Verrucomorpha or Lepadomorpha. This suggests that the spacer appeared after the separation of *B. improvisus* and *B. eburneus* from the others.

Therefore, a potential hypothesis for this event is that it might be a chance event occurring on a specific lineage of *B. improvisus* and *B. eburneus* and might have occurred recently in terms of evolutionary time. The Balanidea are most likely to have appeared in the early Eocene 38-54 mya (Newman 1976, and Glenner *et al.*, 1995). Therefore the insertion should have occurred after that. The phylogenetic analysis in this study also supports this hypothesis. It also suggested that the spacer has a specific evolutionary lineage and is restricted to *B. improvisus* and *B. eburneus.* The insertion event should have occurred after their separation from *B. amphitrite.* However, it is still unclear whether only one insertion event occurred just once after the separation of *B. improvisus* and *B. eburneus* from *B. amphitrite*, or if two insertion events independently occurred after the separation of *B. improvisus* and *B. eburneus* (see fig. 5.14). However, the conserved sequences found at 5' end of the spacer and

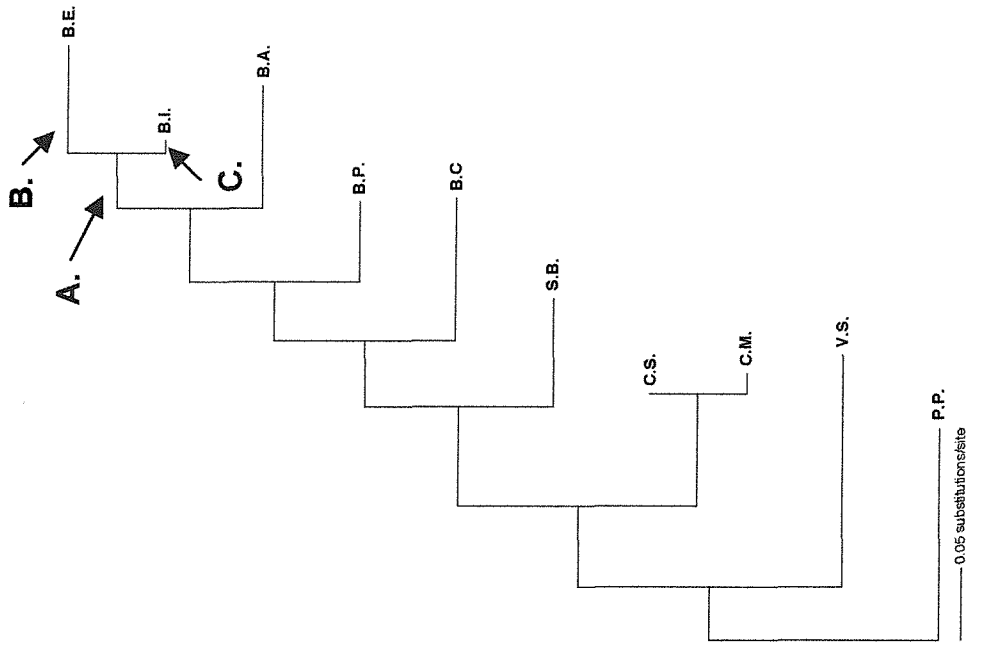


Figure 5.14 Two possible insertion events of the spacer [single event (**A.**) and two independent events (**B.** and **C.**)] (B.E. = *B. eburneus*, B.I. = *B. improvisus*, B.A. = *B. amphitrite*, B.P. = *B. perforatus*, B.C.= *B. crenatus*, S.B. = *S. balanoides*, C.S. = *C. stellatus*, C.M. = *C. montagui*, V.S. = *V. stroemia*, P.P. = *P. pollicipes*)

the short conserved sequence (see fig. 5.3) found in both barnacle species support the view that only one insertion event occurred and the spacers might be derived from the same origin. This finding disagrees with the evolutionary lineage of the spacer found in the salamander family Ambystomatidae, which may have been produced by several insertions occurring independently on the same "hot spot" of mutation (McKnight and Shaffer, 1997). However, more representatives from the *B. amphitrite* group are needed to obtain a clearer phylogeny of the groups and perhaps, this should provide a clearer understanding of the insertion event.

5.4.2 The persistent occurrence of the spacer

The duplication in lizards (genus *Cnemidophorus*) was found only in some individuals in the population (Stanton *et al.*, 1994). This suggests that the duplication occurred recently, infrequently and restricted to particular individuals in the population. Conversely, the spacers found in *B improvisus* and *B. eburneus* were present in all samples screened in this study. This showed the persistent occurrence or the stability of the spacer as described in snakes (Kumazawa, *et al.*, 1996) and salamanders (McKnight and Shaffer, 1997). Therefore, there are two possible hypotheses for this occurrence. Firstly, there could have been a population bottleneck in the past. The Balanidea appeared in early Eocene 38-54 mya (Newman and Ross, 1976, Glenner *et al.*, 1995). The middle Eocene to late Oligocene, about 30 –35 mya, was a critical period of organisms living on earth since there were major changes in global climate and ocean circulation (Berggren and Prothero, 1992). Therefore, it is possible that this might have major effects on the survival of these two barnacles and such a population bottleneck could play an important role in the persistent occurrence of this sequence. Secondly, the period of 30-35 million years is long enough to allow the spacer to spread through populations.

5.4.3 Does the spacer have a function?

The spacer appears to have no function of coding for any proteins because there are many stop codon sites on all 6 ORFs (opening reading frames) and also the insertion and deletion that occurred within the spacer also supports this idea. However, the spacer probably is of no harm or any disadvantage to the genome. Therefore, it would be said that there is no selection to eliminate this spacer from the genome. This does not agree with the idea that the evolution of animal mtDNA tends to reduce in size since the smaller the size, the more efficient in replication (less time) (Rand and Harrison, 1989). Alternatively, when the nonfunctional DNA segment has been removed the gene order should be more stable and it could be difficult to rearrange genes without any effect on the function of genes in the genome (Wallace, 1982). Therefore, It is possible that the spacer could have positive advantages to the genome. One possibility is that the duplication, followed by deletion could allow the gene rearrangement to occur. Also, Wong and Clayton (1985, 1986) reported that stem-and-loop structure plays a role as the signal for the enzyme that initiates the synthesis of mtDNA L-strand during the replication. The secondary structures of the single strand of the spacer showed such structures (see fig 5.5, 5.6, 5.7). It might be possible that this spacer served as an alternative initial site of replication in the genome as suggested for *A. mellifera* (Cornuet *et al.*, 1991).

5.4.4 The origin of the spacer

There are three possible processes that might explain the origin of this spacer: 1) the transposition of the tRNA genes, 2) the duplication of coding and non-coding genes within mitochondrial genome, and 3) the insertion of sequences from other genomes (nuclear DNA or tranposons.)

The transposition of tRNA genes is the first aspect that could be the explanation for the origin of this spacer because gene rearrangements in animal mitochodrial DNA mostly involve the movement of tRNA genes (Kumazawa *et al.*, 1996; Wilding *et al.*, 1999). However, the secondary structure of this spacer did not show any structure of tRNA genes, and there was no tRNA sequence matched with the spacer sequence after Blast search. By aligning the spacer sequence with known tRNA genes of other arthropods, there were many overlapping areas when different tRNA gene sequences were used in the analysis. Furthermore, the rearrangement of tRNA genes should not occur between highly closely-related species belonging in the same group (*B. amphitrite*). Therefore, it is unlikely that this spacer is a cluster of tRNA genes.

The chance duplication of non-coding or coding regions is probably the most likely explanation for this event since there are many reports about this phenomenon in animal mitochondrial DNA (see section 5.1). If it was the result of the duplication of coding genes, there should remain some similarity of the spacer sequence to the relevant coding gene in mitochondrial genome after Blast search because most of the coding genes in animal mitochodrial DNA are fairly conserved. But Blast search results showed no sequences having strong similarity with this spacer. Consequently, the duplication of coding genes is probably not the answer. However, the spacer could be duplicated from the variable region in the animal mitochodrial genome. Therefore, when Blast search was performed, there is no similarity between the spacer and any sequences in the database. Animal mitochondrial DNA has only one non-coding region called the control region or A-T rich region. The duplication of this sequence has been reported in snakes (Kumazawa *et al.*, 1996) and fish (minnow (Broughton and Dowling, 1994)). Therefore, the control region of *B. improvisus* was obtained as described in section 5.2.4. By comparing the spacer with the control region sequence, they showed about 50% similarity (see fig. 5.8). From this result, it might be that the spacer is the

duplication of the control region, but it happened a sufficiently long time ago to allow such variation between these two sequences. However, most of the literature that discusses about the duplication described above showed that both original and duplicated sequence had very strong similarity (Kumazawa *et al.*, 1996; Stanton *et al.*, 1994; Broughton and Dowling, 1994; Cornuet *et al.*, 1991). Therefore, It is difficult to conclude that this spacer is a duplication of the control region. The mitochondrial genome of *B. improvisus* should provide a clear explanation whether the spacer has been duplication from other genes within its own mitochondrial genome.

The last hypothesis is that the spacer originates from the duplication of sequences from other genomes. There is no report about this phenomenon in any animal mitochondrial genome. However, it has been mentioned by McKnight and Shaffer (1997) in their finding of the intergenic spacer between Cyt b and $tRNA^{pro}$ genes in the salamander, family Ambystomatidae. They have found only the direct repeat sequence at the 5'end of the additional sequence, but there is no direct repeat at the 3'end nor an inverted repeat sequence close to it. The general character of transposon or transponsable element is the direct repeat sequence at the 3' and 5'end followed by the inverted repeat sequence (see Lewin 2000). Therefore, it is unlikely that additional sequence is the transposable element. The intergenic spacers found in *B. improvisus* appear to have direct repeat sequence at the 5'end of the sequence (5'TCTAT3') and near the 3'end (about 41bp inside from the 3'end of the sequence). There is an inverted repeat sequence close to those direct repeat sequences. However, inverted repeat sequences were TA repeat motif and they were not completely complementary to each other. Furthermore, there is no direct repeat (5'TCTAT3') near the 3'end of *B. eburneus* spacer sequence as found in *B. improvisus* (see fig. 5.3&5.4). Therefore, It is difficult to believe that these intergenic spacers have originated from transposons (mobile genetic elements).

By using the evidence so far obtained, especially, the similarity at the 5' end of the sequence between *B. improvisus* and *B. eburneus* and the similarity in secondary structure and their evolution lineage revealed by phylogenetic analysis, it seems likely that these spacers have the same origin. However, the origin of these unique sequences is still unclear, but extremely interesting.

Chapter Six

A preliminary investigation on the mating strategy of *Balanus improvisus* using microsatellite analysis

6.1 Introduction

Barnacles are hermaphrodites. Cross-fertilisation is commonly found in these sessile organisms (for more details see section 2.1.3), but self-fertilisation behaviour has also been reported in a few barnacle species, namely *B. perforatus*, *C. stellatus*, *V. stroemia* and *B. improvisus* (Crisp, 1954; Barnes and Crisp, 1956; Furman and Yule, 1990). In contrast, some barnacles are obligate cross-fertilisers (e.g. *S. balanoides*, *B. crenatus*, and *E. modestus*) (Barnes and Crisp, 1956).

In *B. improvisus*, a study on self-fertilisation was carried out in an aquarium (Furman and Yule, 1990). It showed that isolated individuals of this barnacle species carried fertilised eggs and a brood. This suggests that they are capable of self-fertilisation. In this study, further investigation into the mating strategies of *B. improvisus* was carried out. A field study was set up and molecular techniques were applied to reveal the mating strategies of this barnacle species. This study was only a preliminary investigation, since only one highly polymorphic microsatellite marker (BI35) was available (see table 3.3). The two main questions to be addressed in this study are: 1) does self-fertilisation occur in isolated individuals? and 2) do multiple fertilisation events occur in grouped individuals?

6.2 Materials and methods

6.2.1 Site of study

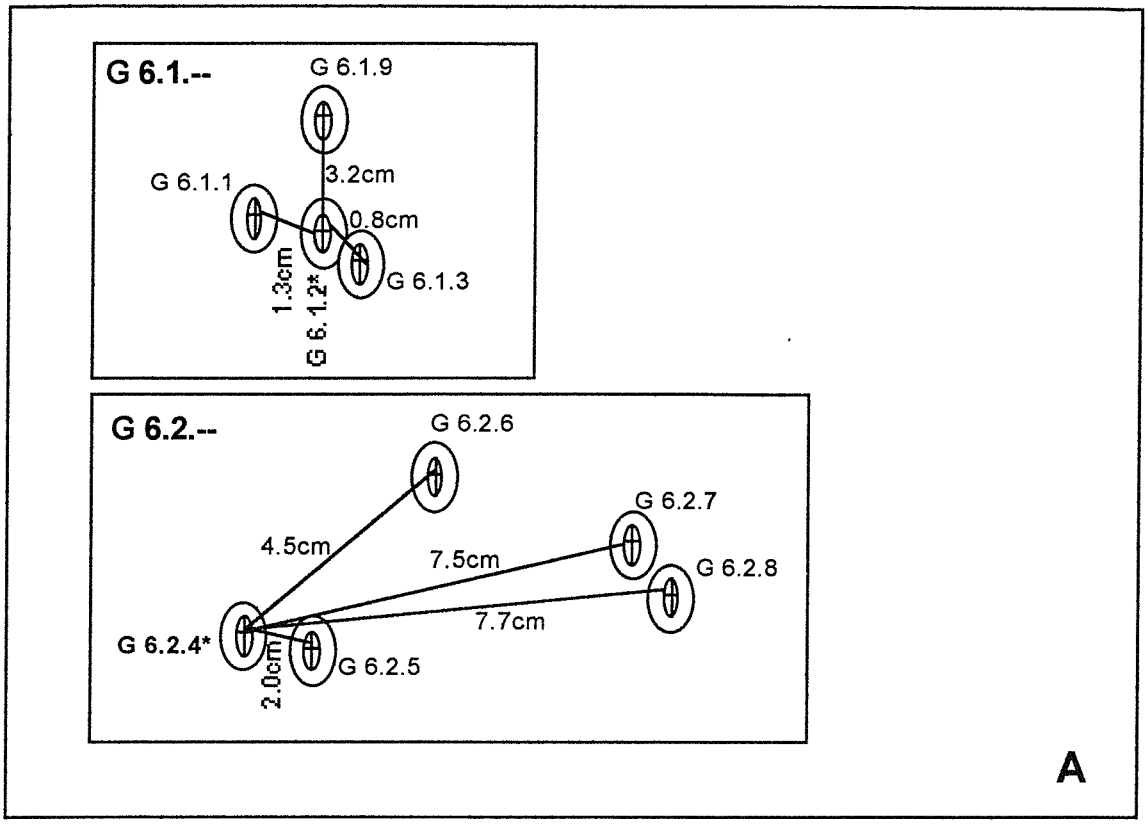
This study was carried out at Cobden Bridge, River Itchen, Southampton (see section 2.3.1 and fig. 2.5)

6.2.2 Self-fertilisation experiment (a field study)

6.2.2.1 Experimental set up

This experiment was set up on the exterior of the concrete wall of Cobden Bridge at more or less the same height. A total of sixteen quadrats (~29X21cm (A4 size)) were set up. Eight were used for the investigation of self-fertilisation in isolated individuals, and eight were used to analyse cross- and multiple-fertilisation events in grouped individuals (see fig. 6.1)

In the self-fertilisation experiment, *B. improvisus* individuals falling within the quadrats were kept isolated by eliminating their neighbours. The closest distance between isolated individuals was 3.5cm. This was to ensure that cross-fertilisation could not take place between these individuals, where the 3.5cm was set using data from nearest distance, and average length of penis measurements for *B. improvisus* (see section 2.3.2.2 (male activity)). Since the breeding season (female activity) of *B. improvisus* was shown to be suspended between November and January (see section 2.3.2.2 (female activity)), the experiment was set up in December 1998. This was to confirm that broods obtained in this study were from the same reproductive year.



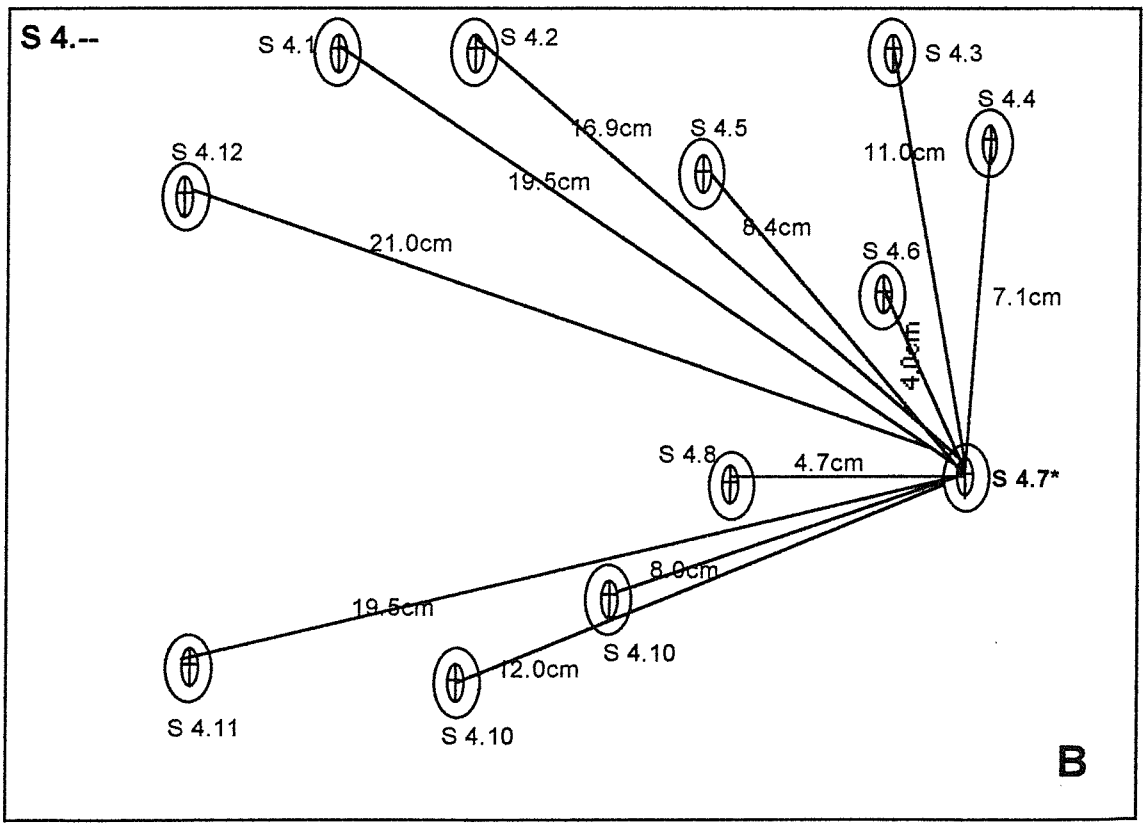


Figure 6.1 Maps of the clusters of barnacles (A) grouped individuals (G 6.1.-- and G 6.2.--) (B) isolated individuals (S 4.--). (*) indicates individuals containing broods. Distances between individuals containing broods and their neighbours are also displayed.

For the recognition of quadrat areas and *B. improvisus* samples in each quadrat, epoxy putty (Milliput®, The Milliput Company, Dolgellau, Gwynedd, UK.) was used to mark at the corner position of the quadrats on the concrete wall. The positions of the putty, and of *B. improvisus* individuals lying within the quadrat were then marked onto acetate sheets (A4 size). These sheets were then used to recognise the quadrats and *B. improvisus* samples.

6.2.2.2 Sample collection

B. improvisus samples were collected during the peak of the species' breeding season between June and July (see section 2.3.2.2). Samples were collected on four separate occasions: early June, middle of June, early July, and middle of July. Samples in two quadrates of each experiment (self- and cross-fertilisation) were collected each time. The percentage of individuals which died, were reproductively inactive, and which contained an egg-mass and brood, were recorded. All the samples were then labelled, placed in absolute ethanol, and stored at –20°C as quick as possible for DNA analysis.

The chi-square test was employed to test the hypothesis: reproductive activity in grouped barnacles is different from in isolated barnacles.

6.2.3 Parental study (a molecular analysis)

6.2.3.1 DNA extraction for adult barnacles

The DNA extraction protocol used in this study for *B. improvisus* adults was as described in section 3.2.1.

6.2.3.2 DNA extraction for larval barnacles

The DNA of *B. improvisus* larvae was obtained using *micro*LYSIS™ solution (Microzone limited). 7μl of *micro*LYSIS™ solution was added into a 0.5ml microcentrifuge tube. A *Balanus improvisus* larva was then placed into the tube. Next, a drop of mineral oil (~10-15μl) was added, and the solution was then briefly centrifuged. The lysis reaction was then carried out using a PCR machine with the conditions set as follows: 65°C for 5 minutes, 96°C for 2 minutes, 65°C for 4 minutes, 96°C for 1 minute, 65°C for 1 minute, and 96°C for 30 seconds. The solution was then ready for immediate use or stored at 4°C for subsequent analysis.

6.2.3.3 Molecular analysis

Only the BI35 microsatellite locus (see table 3.3) was used in this investigation. The PCR conditions used at this locus was described in section 3.2.7.2 and in table 3.3, but for the larvae, 1μl of DNA obtained in section 6.2.3.2 was used in PCR reactions. The PCR products were then analysed on an automated sequencer as described in section 3.2.7.3.

6.3 Results

6.3.1 Self-fertilisation in *B. improvisus* (a field study)

After screening 98 isolated individuals and 114 grouped individuals of *B. improvisus* (212 in total), it was seen that some of the isolated individuals contained broods in their mantle cavity. This suggests that self-fertilisation occurs in *B. improvisus* or the broods are the reproductive output obtained by the fertilisation between eggs and the sperms transferred by the water column. The data also showed that there were more dead organisms found within isolated individual samples than within grouped

individual samples (see fig 6.2). A highly significant difference in the number of deaths of *B. improvisus* living in the isolated and grouped conditions was also found using chi square test ($\chi^2_{(1)}$ = 16.96, P<0.01), (see also table 6.1a). From the table, the individual components of χ^2 were examined. The largest values found were: 7.75 (dead individuals in the isolated condition) and 6.65 (dead individuals in the grouped condition). Looking at these values more closely, there were more observed dead individuals in the isolated condition than expected. In contrast there were less observed dead individuals in the grouped condition than expected. This indicates that death is positively associated with the isolated condition, but it is negatively associated with the grouped condition. Therefore, this observation suggests that isolated individuals might be more susceptible to physical stresses (current, desiccation, and silting) and biological interaction (predation).

A significant difference in the reproductive activity between *B. improvisus* individuals living in the isolated and grouped conditions was also found (($\chi^2_{(2)}$ = 6.60, P<0.05) (see table 6.1b). From the table, the largest values of individual components of χ^2 were 2.83 (individuals containing an egg-mass in the isolated condition) and 1.89 (individuals containing an egg-mass in the grouped condition). This indicates that there are more individuals found carrying an egg-mass within the isolated condition than within the grouped condition. This is likely to result from the isolated individuals having to carry their eggs longer than the grouped individuals, since self-fertilisation is required for isolated individuals to produce their broods and this is likely to occur later, after cross-fertilisation was not possible (Furman and Yule, 1990).

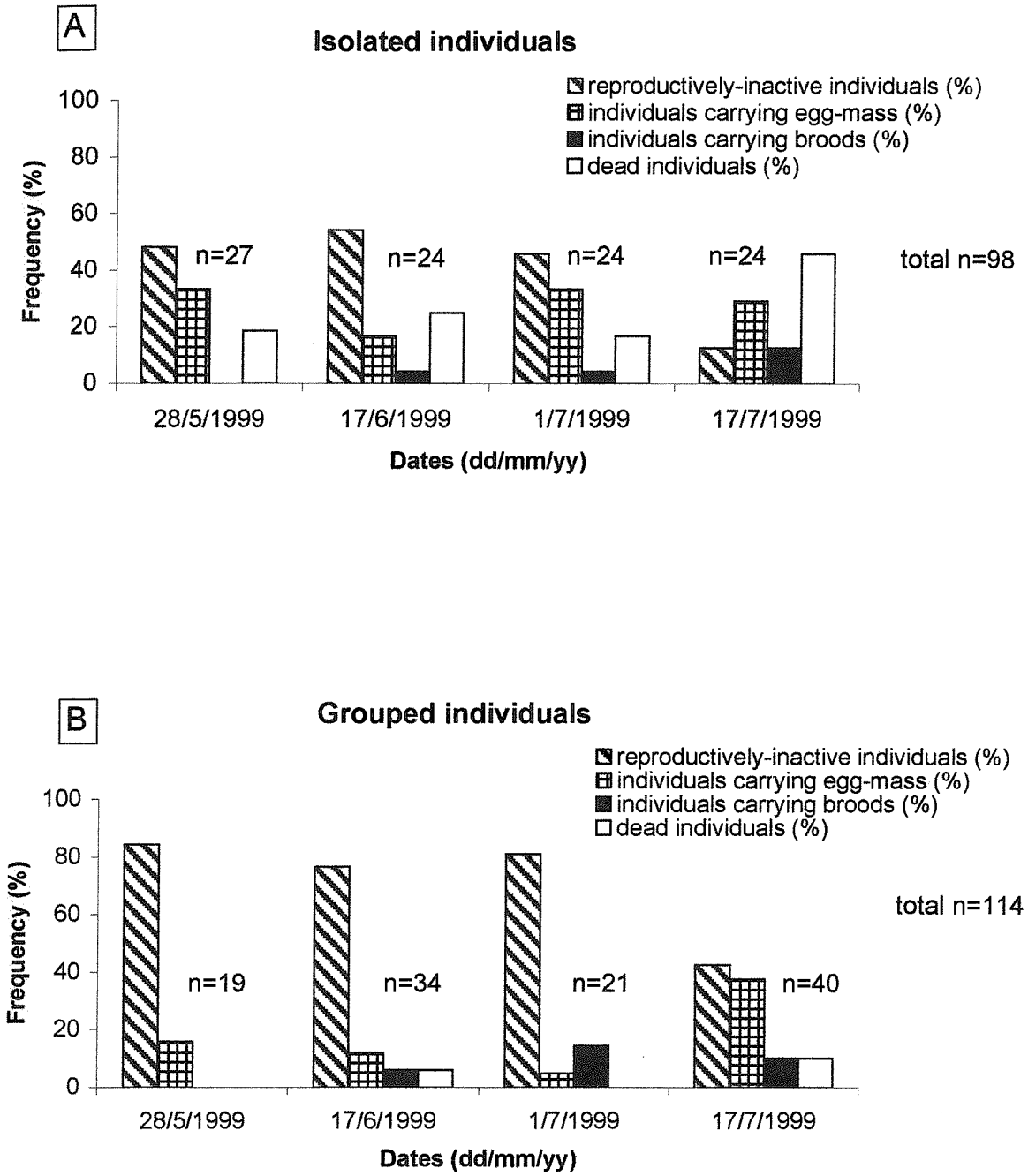


Figure 6.2 Frequency histogram of the reproductive activities of *B. improvisus* isolated (A) and communal (B) individuals at the Cobden Bridge site, River Itchen, Southampton.

Table 6.1 Calculation of expected frequencies, Individual components [$(O\text{-}E)^2/E$)] of χ^2, and χ^2 [$\chi^2 = \sum (O\text{-}E)^2/E$, O = observed frequencies and *E* = expected frequencies]. (A) Chi square test showing the differences in the frequency of surviving barnacles living in the isolated and grouped conditions. (B) Chi square test showing the differences in reproductive activity between barnacles living in the isolated and grouped conditions.

A. ($\chi^2_{(1)}$=16.96, P<0.01)

Conditions	Survivors		Total (n)	χ^2
Isolated	Living individuals	Dead individuals		**16.96**
O (n)	**72.00**	**26.00**	**98.00**	
E (n)	83.21	14.79		
$(O\text{-}E)^2/E$	1.38	7.75		
Grouped	Living individuals	Dead individuals		
O (n)	**108.00**	**6.00**	**114.00**	
E (n)	96.79	17.21		
$(O\text{-}E)^2/E$	1.18	6.65		
Total (n)	**180**	**32.00**	**212.00**	

B. ($\chi^2_{(2)}$=6.60, P<0.05)

Conditions	Reproductive activities			Total (n)	χ^2
Isolated	Inactive individuals	Individuals containing egg-mass	Individuals containing broods		**6.60**
O (n)	**39.00**	**28.00**	**5.00**	**72.00**	
E (n)	46.00	20.4	5.60		
$(O\text{-}E)^2/E$	1.07	2.83	0.06		
Grouped	Inactive individuals	Individuals containing egg-mass	Individuals containing broods		
O (n)	**76.00**	**23.00**	**9.00**	**108.00**	
E (n)	69.00	30.60	8.40		
$(O\text{-}E)^2/E$	0.71	1.89	0.04		
Total (n)	**115.00**	**51.00**	**14.00**	**180.00**	

6.3.2 Molecular analysis

6.3.2.1 Grouped individuals

Three clusters of grouped individuals were analysed: cluster A, B, and C (see also table 6.2a, b, and c). In cluster A (G 6.1.--), the analysis revealed that the larvae had the same genotype as their mother (188,188). However, the closest neighbour (putative father) genotype was also (188,188) (see table 6.2a.). Therefore, cross-fertilisation might have occurred within this cluster, but the genetic marker analysis could not detect this since both mother and putative father shared the same genotype (188,188).

In cluster B (G 6.2.--), the analysis unveiled the larval genotypes: (192,192), (192, 232), and (232,232). These corresponded to the heterozygous genotype of their mother (192,232). This indicated self-fertilisation of the mother (G 6.2.4) whose nearest neighbour had a different genotype, G 6.2.5 (genotype (174,174)) 2.00 cm away (see table 6.2b).

In cluster C (G 8.1.--), the data showed that all larvae were heterozygous, having genotype (168,172) which corresponded to their mother G 8.1.9 having genotype (168,168), and their putative father G 8.1.8 (being 0.6cm away from the mother, G 8.1.9), having genotype (172,172) (see table 6.2c)

6.3.2.2 isolated individuals

Three clusters of isolated *B. improvisus* individuals were analysed: cluster D, E, and F (see also table 6.3d, e, and f). The microsatellite analyses of clusters E (S 4.--) and F (S 7.--) (see table 6.3e&f) indicated self-fertilisation behaviour in these isolated individuals as all larvae inherited their maternal genotypes (202,202) for cluster E and (172,172) for cluster F (see table 6.3e&f).

Table 6.2 Assessing the nature of fertilisation in grouped *B. improvisus* individuals: the left hand columns show the microsatellite genotypes of the adult barnacles and the larvae. The right hand columns show the distances between brood-containing adult (*) and its neighbours, and also the frequency (n) of larval genotypes. A-C indicate separated clusters of *B. improvisus*

A

Cluster A (G 6.1.--)	
Adult samples/genotypes	**Distances from brooding individual (cm)**
G 6.1.1/(172,172)	1.30
*G 6.1.2/(188,188)	0.00
G 6.1.3/(188,188)	0.80
G 6.1.9/(192,192)	3.20
Larval genotypes	**No.of larvae (n)**
(188,188)	28
Total	28

B

Cluster B (G 6.2.--)	
Adult samples/genotypes	**Distances from brooding individual (cm)**
*G 6.2.4/(192, 232)	0.00
G 6.2.5/(174,174)	2.00
G 6.2.6/(164,164)	4.50
G 6.2.7/ (190,194)	7.50
G 6.2.8 /(186,200)	7.70
Larval genotypes	**No. of larvae (n)**
(192,192)	22
(232,232)	13
(192,232)	12
Total	47

C

Cluster C (G 8.1.--)	
Adult samples/genotypes	**Distances from brooding individual (cm)**
G 8.1.6/(174,214)	3.30
G 8.1.7/(186,186)	3.50
G 8.1.8/(172,172)	0.60
*G 8.1.9/(168,168)	0.00
G 8.1.10/(170,170)	1.40
G 8.1.11/(192/192)	3.90
Larval genotypes	**No. of larvae (n)**
(168,172)	29
Total	29

Table 6.3 Assessing the nature of self-fertilisation in isolated *B. improvisus* individuals: the left hand columns show the microsatellite genotypes of the adult barnacles and the larvae. The right hand columns show the distances between brood-containing adult (*) and its neighbours, and also the frequency (n) of larval genotypes.

D

Cluster D (S 6.--)	
Adult samples/genotypes	**Distances from brooding individual (cm)**
S 6.8/(188,188)	9.40
S 6.9/(174,202)	5.50
S 6.10 / (Dead)	11.20
S 6.11/(164,164)	6.00
*S 6.12/(176,176)	0.00
Larval genotypes	**No. of larvae (n)**
(176,176)	33
(218,218)	9
(176,218)	18
Total	60

E

Cluster E (S 4.--)	
Adult samples/genotypes	**Distances from brooding individual (cm)**
S 4.4/(174,174)	7.10
S 4.5 / (Dead)	8.40
S 4.6/(192,192)	4.00
*S 4.7/(202/202)	0.00
S 4.8/(204,204)	4.70
S 4.9/(202,202)	8.00
Larval genotypes	**No. of larvae (n)**
(202,202)	25
Total	25

F

Cluster F (S 7.--)	
Adult samples/genotypes	**Distances from brooding individual (cm)**
S 7.7 / (Dead)	4.20
*S 7.8/(172,172)	0.00
S 7.9 / (Dead)	3.80
S 7.10/(208,208)	5.20
Larval genotypes	**No. of larvae (n)**
(172,172)	28
Total	28

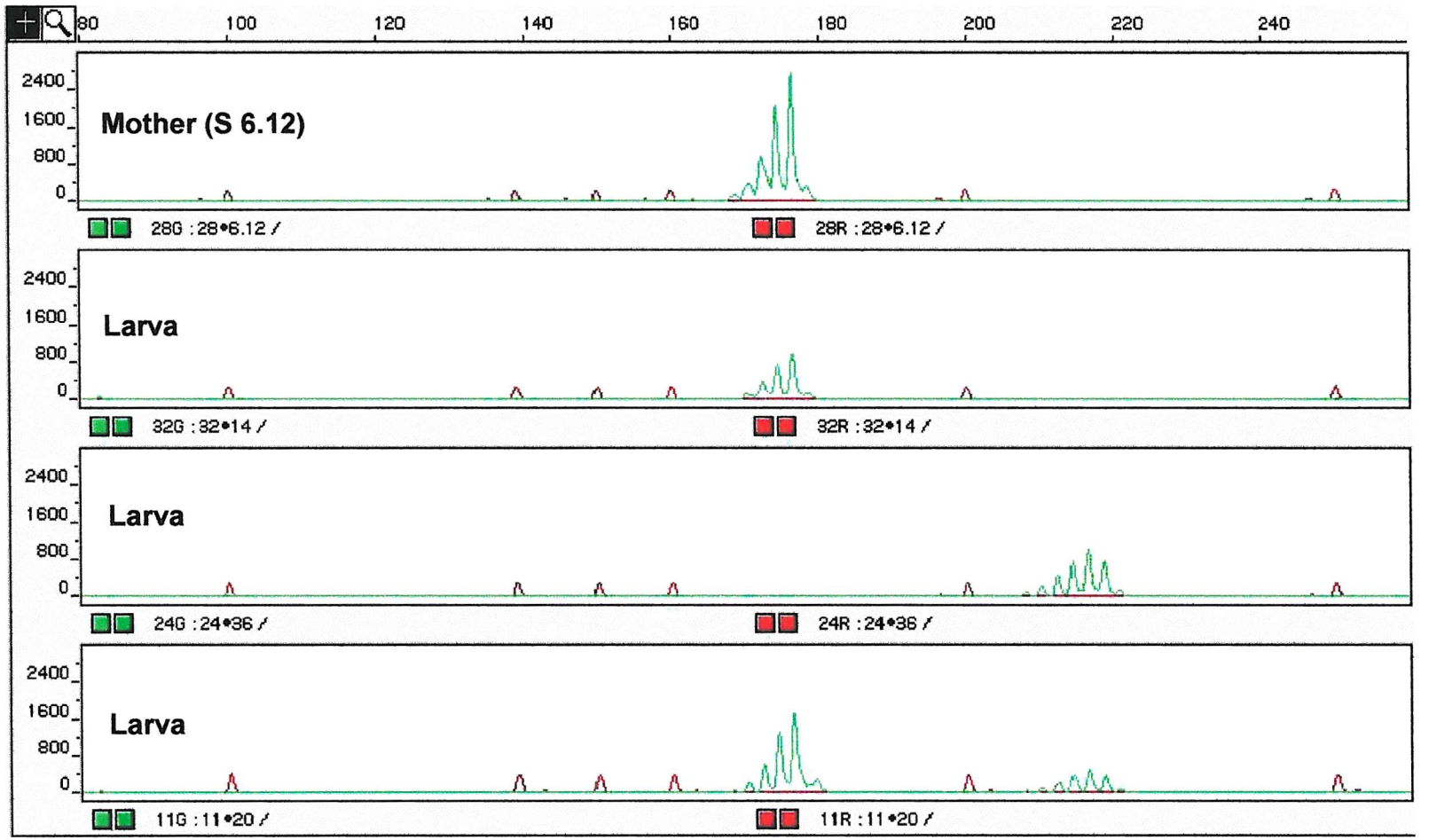


Figure 6.3 Chromatogram of the parental analysis of the sample, S 6.12, using the microsatellite marker (BI35) (in green). Red peaks indicate standard DNA size markers (Tammar 350, Perkin Elmer)

An ambiguous result was found in the analysis of cluster D. There were a total of three genotypes found in the larvae of this cluster ((176,176), (176,218), and (218,218) see table 6.3d and fig. 6.3). The larvae having genotype (176,176) were likely derived from self-fertilisation events. The larvae having genotype (176,218) seem to the offspring of cross-fertilisation events, but there is no neighbouring individual containing the 218 allele. There are two possible explanations for this situation. Firstly, foreign sperms could be transferred in the water column, or secondly the organisms could store the foreign sperms somewhere within their bodies. The larvae having genotype (218,218) have no linkage to the mother (genotype (176,176)) (see table 6.3d). This was an unexpected phenomenon. It could be the result of larval adoption behaviour as suggested in the crustacean *Leptomysis spp.* (Wittmann, 1978). If this behaviour is present in *B. improvisus*, a specific type of larvae seems to be selected for adoption, because only one particular genotype (218,218) of adopted larvae was observed in this study.

It is possible that the above results are a consequence of the problems in molecular markers such as DNA contamination or null alleles. In this study, the main source of DNA contamination is likely to be from the mother because the larvae were still in their mother's mantle cavity when the dissection was performed. Therefore, the larvae having genotype (176,218) might be the adopted larvae having genotype (218,218), but the allele (176) was obtained from the maternal DNA contamination. This supports the idea of adoption occurring in *B. improvisus*.

For the null allele situation, allele (218) could be a null allele, which was amplified for the larvae, but not for the mother. If this were the case, the genotype of the mother would be heterozygous (176,218), and the expected larval genotypes could be a mixture of (176,176), (176,218), and (218,218) genotypes as were obtained in the

data. However, it is hard to accept that this situation occurred only in the mother because in theory, the null allele should be found in the larvae as well.

6.4 Discussion

This project is an investigation at the molecular level into the mating strategies of *B. improvisus*. The work represents a preliminary study, since only one locus of microsatellite marker was available for use. The study confirmed that isolated individuals of *B. improvisus* do have self-fertilisation behaviour (see table 6.2b, and 6.3e&f) as reported by Furman and Yule (1990), and that this behaviour could be found in individuals lying as close as 2.0cm apart (see table 6.2b). Self-fertilisation in *B. improvisus* might play an important role in the colonisation and maintenance of its unstable, small, and patchily distributed populations (Furman *et al.*, 1989; Furman and Yule, 1990), since the organism can reproduce without the requirement of a neighbouring barnacle to cross-fertilise. Therefore, only a small number of *B. improvisus* individuals are needed for the colonisation (Crisp and Southward, 1953). Although self-fertilisation was observed in *B. improvisus*, Furman and Yule (1990) suggested that it only occurred when cross-fertilisation could not take place. The idea that self-fertilisation employed by this barnacle species was also shown by the consistent lack of heterozygosity seen in small *B. improvisus* populations, where most of the individuals in the populations were isolated from each other (Furman *et al.*, 1989).

An ambiguous result (as described in section 6.3.2.2) emerged in this study, which could not be simply accounted for. It suggested that *B. improvisus* might be capable of adopting the larvae from other individuals of the same species. Although, this behaviour was found in another crustacean species, *Leptomysis spp.* (Mysidacea) (Wittmann, 1978), no strong evidence was found in this study to support this

hypothesis (see section 6.3.2.2). Assuming, however, that adoption does occurred, and that larvae are randomly adopted from the water column, in all probability, several different genotypes of adopted larvae should be found in the mantle cavity of the same mother. However, in this study, only one foreign allele (218) was found in the larvae of individual, S 6.12, (see table 6.3d). If adoption occurs, it implies that only a specific genotype of larvae is selected for which is unlikely.

It is extremely difficult to believe that such a complicated process of adoption as just described does occur in *B. improvisus*. It is possible instead that this ambiguous result could be caused by problems associated with the genetic marker itself as described in section 6.3.2.2, since only one set of samples (isolated individual cluster D (S 6.--)) provided such a result. However, if this behaviour does occur, an interesting aspect is how the adult barnacles take up the planktonic larvae into their mantle cavity (e.g. by chance adoption of the larvae in the water column during feeding or the selected adoption of the offspring of their neighbour).

In conclusion, this investigation confirmed that self-fertilisation occurs in *B. improvisus*, and can take place between individuals separated by only 2cm. In contrast cross-fertilisation was shown to occur between individuals separated by less than 1cm apart in distance. There was no evidence of multiple-fertilisation occurring under this study. In the future, more clusters (sets of samples), and reliable genetic markers are needed to provide a clearer understanding of the mating strategies in *B. improvisus*, including adoption (if indeed it does occurs).

Chapter Seven

General discussion

This project can be divided into two main sections. The first section involves a field study on the basic ecology and biology of *B. improvisus* (Chapter 2). The second section (incorporating Chapters 3-6) is molecular ecological and evolutionary studies on *B. improvisus*. In Chapter 3, molecular markers for *B. improvisus* were developed. In Chapter 4, a mtDNA PCR-RFLP technique was used to investigate the population genetic structure of this barnacle species. In Chapter 5, the unique spacer, found in the in mitochondrial genome of *B. improvisus* during the development of the molecular markers, was further investigated in term of its evolution and origin. In Chapter 6, the mating strategies employed by *B. improvisus* were studied using microsatellite analysis. In this final Chapter, the results obtained from the previous chapters, the limitations of the study, and future work are discussed.

7.1 Distribution, dynamics and genetic differentiation of the populations of *B. improvisus*

In the British Isles, the distribution of *B. improvisus* is restricted to the estuaries where the species is found mostly in the upper reaches of the estuaries. Their populations are isolated, since they are not found along the coastline in between the estuaries, where they inhabit (Furman *et al.*, 1989). Comparing the data from the survey carried out in this study with data obtained from Furman (1990) (see table 2.2), these isolated populations are ephemeral (see also section 2.4.1), and the abundant populations are

more stable than the smaller populations (Furman, 1990). This suggests that the reproductive output within the estuaries, which is directly related to the abundance of the populations and local larval supply, could play a major role in maintaining the populations of this barnacle within the estuaries around the British Isles. This is also supported by firstly, the reports on *B. improvisus* larval retention in estuaries (Bousfield, 1955; Flavell, 1996) and secondly, the significant genetic differentiation seen among the populations from different estuaries around the British Isles found in this study (Chapter 4). This shows that the level of gene flow (or the level of larval migration) between the British populations is low. Since the populations of this barnacle are isolated and unstable, the genetic differentiation found between the populations could be caused by the genetic differences in the founders during colonisation and also by the multiple invasion of new recruits released by the mature adults travelling by ships.

In contrast, in large brackish water bodies such as the Baltic (essentially a big estuary), where the populations of this barnacle are more continuously distributed (Furman, 1990), no significant genetic differentiation among the Baltic populations were found, when the population from Swedish West Coast was excluded (for details see section 4.4.2). The genetic similarity among these populations could be the result of larval migration within the estuary. Therefore, recruitment in these populations derives from the reproductive output of other populations within the estuary including its own. It is thus fair to note that gene flow between the populations within the Baltic (big estuaries) is higher than between the populations around the British Isles, and no population subdivision were seen among the Baltic populations analysed in this study. However, a comparison of the level of gene flow between the Baltic and between the British populations might not be reasonable, since the Baltic could be considered as one big estuary, which contains many populations. Therefore, the Baltic populations analysed

in this study were effectively obtained from only a simple connected large system. In contrast, the British populations were from highly fragmented populations.

Consequently, the gene flow between Baltic populations should be considered as the gene flow within one estuary, and it is likely that larval retention also occurs in the Baltic. Nevertheless, the lack of genetic differentiation between the populations in British Isles and the Baltic suggests that the level of gene flow between them is high. Assuming larvae cannot themselves escape from the Baltic, then one potential source of gene flow between the Baltic and the British populations is the migration of mature adults by ships. This migration could also play an important role in the success of the global distribution of *B. improvisus* (Henry and Maclaughlin, 1975; Newman and Ross, 1976) (see section 2.12).

7.2 The evolution of the unique intergenic spacer

The discovery of the unique intergenic spacer between the COI and $tRNA^{leu}$ genes in *B. improvisus* and *B. eburneus* is an intriguing result. There has been no previous report on a spacer being found in any barnacle mitochondrial genomes. By screening the species distribution of the spacer (from Lepadomorpha to Balanomorpha), the results indicated that the spacer was not passed on the evolutionary lineage of barnacles since the spacers identified are restricted to only certain barnacle species (e.g. *B. improvisus* and *B. eburneus* so far). Therefore, the insertion of the spacer into mtDNA genomes of these two barnacle species appears to have arisen by a chance event of sequence duplication. However, the mechanism of the duplication and the origin of the duplicated sequence are still unknown.

Phylogenetic analysis in this study showed *B. improvisus* and *B. eburneus* are evolutionarily close and that the *B. amphitrite* group to which both these species belong

is the most advanced of the Balanidea analysed so far. However, this analysis could not provide enough evidence to resolve whether only a single or two independent duplications of the spacers occurred during the evolution of the two barnacle species. An intensive phylogenetic analysis of the Balanidea is required to overcome that question. By comparing the general features of the spacers in *B. improvisus* and *B. eburneus*, (e.g. sequence similarity and secondary structure, see section 5.3.2), it could be seen that, in all probability, they are derived from the same origin. Therefore, it is likely that a single duplication event is responsible for their emergence.

7.3 Self-fertilisation in *B. improvisus*

Self-fertilisation behaviour in *B. improvisus* was reported first by Furman *et al.*, 1990. A further study examining self-fertilisation in this species is presented in this study and involved the use of molecular analysis (microsatellites) (Chapter 6). The results of this study confirmed the ability of selfing in isolated individuals of *B. improvisus* and supported the observation that it can only occur when cross-fertilisation cannot take place (Furman and Yule, 1990). The result of the nearest distance analysis also showed that *B. improvisus* prefers to settle close to its conspecific species. This also indicates that cross-fertilisation is more preferential than self-fertilisation. However, this study also showed that there are more isolated individuals of *B. improvisus* compared with those of *E. modestus* (see section 2.3.2.1), but the reasons for this isolation are presently unclear (see section 2.4.2 for more details).

In small populations where the barnacles settle far away from their conspecific species (e.g. Conwy, suggested by Furman *et al.*, 1989), selfing could cause population inbreeding (lack of heterozygosity). On the other hand, this ability could facilitate the colonisation of *B. improvisus* populations, since they are free from the obligation to

settle close to their conspecific species for cross-fertilisation and small founder populations can maintain themselves.

7.4 Limitations of this study

At Cobden Bridge, River Itchen, Southampton, *B. improvisus* was mostly found settling on small stones. This introduces certain limitations on the experimental set up to investigate the background biology and ecology of this barnacle species, which are not applicable with other barnacle species that tend to be found on rocky shores. One clear example is that quadrats cannot be set up, and it is also impossible to collect the data from the same set of stones every month since the water current moves the stones around all over the field under study. For this reason, the data obtained for this study were the average of the data collected from 20 different stones. Using this number of stones should overcome the artificial variation caused by the use of a different set of stones for data collection at each sampling time. However, the setting up of quadrats and collection of data from fixed quadrats are preferable where practical.

It is also necessary to point out the need for a long-term ecological study into *B. improvisus*. However, the populations of this barnacle are generally small and unstable. Therefore, it could be very difficult to carry out a long-term investigation, especially for a study, which involves killing the organisms. In this project, the work was carried out in over a single year. The reason for this are not only because of the above reason, but are also due to the intensive time requirement for population genetic studies, specifically for the development of methodology. This intensive time requirement and associated labour was necessary in this work because there was no genetic tools and information available for *B. improvisus*, when this project started. The ultimate aim of the study however, is not only the development of genetic tools, but also the use of

those tools to answer particular ecological questions. The latter is a fairly time consuming process, including screening of large numbers of samples.

As noted above, the populations of *B. improvisus* are ephemeral, and they can be recolonised by founders derived from different sources (populations). This could be a major factor reponsible for the lack of genetic structure among *B. improvisus* populations found in this study. If this is the case, it will be extremely difficult to understand the genetic structure of such unstable populations. Another limitation that must be noted here is that, inbreeding caused by self-fertilisation (Furman *et al.*, 1989) cannot be studied using a mtDNA marker, since it has no recombinant property (Wilson *et al.*, 1985).

In the study of the evolutionary lineage of the unique spacers found in *B. improvisus* and *B. eburneus*, the main limitation was that there was no previous knowledge, and therefore information on this spacer for any other barnacle species. Therefore, it was difficult to design the experiments to study the evolutionary lineage and origin of these spacers. It also required a lot of time and effort to complete those experiments. The study on the evolutionary lineage of the spacers was carried out indirectly using phylogenetic analysis and it provided reasonable results (see section 5.3.4.2 & 5.4.1). The analysis on the origin of the spacers proved more difficult. It would have been fairly straightforward, if there were DNA sequences in the database (Genbank). Unfortunately, there was nothing. However, from the results obtained so far, three hypotheses have been presented to try to explain the origin of the spacers as discussed in section 5.4.4.

Finally, it is clear that at present the main problem limiting detailed understanding of the mating strategies in *B. improvisus* is the limit in the number of highly polymorphic

microsatellite markers used for the identification of individuals. Therefore, to achieve the original aims of this study, few more markers will be required. In terms of technique, despite it involving complicated molecular techniques (see section 3.2.6), it is fairly straightforward to obtain new microsatellite sequences. The most difficult task is optimisation of the PCR conditions to produce a specific PCR product.

7.5 Future work

As far as the ecological study on *B. improvisus* is concered, it would be very interesting to carry out a long-term study of the dynamics of large and small populations, especially those found at the upper reaches of estuaries. This should reveal the relationship between the abundance of larval supply and the abundance of adults within the estuaries. The dispersal patterns of the larvae within the estuaries are also worth investigating, since this should show whether the majority of the larvae retain in the estuaries or not.

Various genetic markers and techniques have been developed for *B. improvisus* as well as for other barnacle species in this project, but more microsatellite markers need to be developed for future investigations into population structure and mating strategy. The sequence of the microsatellite isolated in this study are shown in the appendix 1 including the sequences of PCR primers that have already tested (see the results in table 3.2). It might be worth designing new PCR primers and testing with those new primers. If new microsatellite markers need to be isolated, then repeat units other than (CA)n would be best such as (TA)n or (GA)n, since most of the (CA)n repeat units have already been isolated in this study.

With the work on the unique spacers, further investigation of their species distribution must be carried out. The problem with such species distribution studies is how to select

the species or group of organisms to screen, since these organisms are very diverse (Cirrepedia) (see Darwin, 1854, Newman and Ross, 1976). The results obtained in this study showed that the spacers were found in barnacle species belonging to the *B. amphitrite* group (Henry and Mclaughlin, 1975, Newman and Ross, 1976), namely, *B. improvisus* and *B. eburneus*, which are evolutionarily close. The other interesting aspect is that both of these species live in brackish water. Therefore, the screening should primarily concentrate on this group of barnacle species living in brackish water (e.g. *B. pallidus*, Southward, per. com.). It might also be worth to have a further study on phylogeny of this group of barnacles.

A further evolutionary study of interest on spacers could be the analysis of their geographical distribution (around the world). This is important to examine in the light of the experimental data which showed the consistent occurrence of the spacers among *B. improvisus* samples from the Baltic and the British Isles, and in contrast, the ephemeral occurrence found in lizards (genus *Cnemidophorus*) (Stanton *et al.*, 1994). The interesting question is how have the spacers spread throughout the populations? Two hypotheses have been proposed. Firstly, the duplication of the spacers could have occurred long enough ago to allow them to spread throughout the populations. Secondly, a population bottleneck could have occurred in the past where most of the survivors contained the spacers in their mtDNA genomes (see section 5.4.2 for discussion). From screening the geographical distribution of the spacers, there are two possible outcomes. Firstly, the spacers could occur in all samples (populations). This could indicate that all the populations of *B. improvisus* around the world are from the same origin, but it could not provide an answer for the hypotheses above. Secondly, the spacers could occur in only some of the samples (populations). This could indicate that a population bottleneck could occur in the past.

7.6 Final remarks

As discussed, *B. improvisus* has proven to be an interesting barnacle species because of its distinct patchy distribution, population dynamics and evolution. Even though this project is the first ecological and evolutionary study on this barnacle species at the DNA level, it has provided some invaluable information, such as the observation of larval retention in estuaries, insights into the phylogeny of *B. amphitrite* group and their relatives, and the identification of a novel intergenic spacer in *B. improvisus* and *B. eburneus*. Furthermore, the genetic markers and the techniques developed in this study are generally applicable, not only to *B. improvisus* but also to other barnacle species and may become useful tools for future researchers studying these sessile organisms.

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Appendices

Appendix 1. DNA sequences contain microsatellite repeats (bold letters). The underlined characters indicate PCR primer sequences.

BI 45

CCTGATGGCGTGACATCCATAAACCTTTTCTGTGCTATAATCAGAACATCGTTTAGTCA
CGCACGTCGCAGAATACCTGGCTGCCTTCAATTCGTGTGTTGTAACTGGGATTTAGGTC
AGTTGGGTCTGAAAACGGCCATTCTAGGCCAAGTGGGAAACCGGCGTGAATAGTTAGGG
AGCACTGTCACCATTTGGTTCGGTGTGATTTGAGCACCGGTCCTGGGACGATTGGGCTA
TTGGGCTGTCTCAGTCCAACACTTGCAC**GTGTGTGTGTGTGTGTGTGTGTGTGT**AGT

BI 57, BI 14.2, BI 15.2 & BI 74

CCCAGTAGAGCGTCCCACAGAGGAGGGACGTCCGTGTCCCGGCTCTACAGGATACTGTC
CACTCTACTACCACAGTACAGTTGTACTGTACATGACTATATTATACAGACTCATGTCA
CAGCGCTAGTACTCCGTCAGTCAGGACACTGTCTCACTGGATGTGTGACGGTGTCACGT
TGGGGAGGGTGTCATGTATTA**CACACACACACACACACACACACA**TATGATGAGATGAT
C

BI 33

GGGCTTAATAATTTATCCAGAATTGAAGAATGAAAAGAATGCTTTAGCATGCTTTTCAG
CATGAAATTCCCATTGGCGTGTCGCTAAAAAAGTTGGGCTTGGCCTGTTT**GTGTGTGTG**
TGTGTGTGTGTGTGTTCAAACACTTTGGCAGCTGGAGCCGAAACGGGTGGTCCGATC

BI 47, BI 2.1, & BI 79

CGCGTCCTCGCAAGTCACGGTAGGCCGAGTGTGTAATCCCATTTTGTCCTCGGTAGTCA
CAAGTGGAAAGTG**CACACACACACACACACACACACA**TCGAGAGGGAACCGGTAACAGC
TTGAGCCAGGTCTGTCCCGTGCGTTGTGAGTACGCCGCCGCCTGGTTCGGCTAGGAACA
GATGTAGTCATGTCACACATTTGAAGCAAAAGTTGGTCAGCAGGACAATATCTCGGCCG
AGAGCATCTGTGATC

BI88, BI 7, & BI 2.2

GACCGTCAGCTTGCCAGATGCCCTAGAGTAAGTTGCATCCGTTTAACTTGTATTATATG
ACAAAGGCAACGTCAACAATGCACAGCTTGGTTTGGTGCCCCTGCATAAGCGAGCAAAA
GTTATTTGCATTTCAAAATAATTTCATCAAACATGTGTTGTCATGCGTGATGTCATGGC
CCATGCGACGTCATCACTGAGTA**GTGTGTGTGTGTGTGTGTGCGTGTGTGTCTGTGT**GG
TTACAGTGCTCGGCAGCTGATC

BI 35

TTATTCAGACGGAGGCTGAGCGCCGAAGGCGCGAAGCCGGAGTCTAACAATCGATGCAA
ATCCGAGTAAGGTCA**TGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTG**
TGTGTGTGTGTCCTCCCATCCTCCTCTGTCAGCAAATTGCTTAAGAAGCCCCAAGAAGT

TCTAGATAAGTGTCAGTGGAGGTACATTCGAAGGTTTGTCGCTTCGTTTTACCACCAGG
TCACCAAG

BI 39

CAAATTTGGCATGTGGCTAGACACTGCATAGGTAGGTGCGTTAAAAGGGCCAGGGAGGA
TGTAACCACACCCGTGGATAGGTGGCGCGGCACGTGTCACGTGCCACCCGAGGTGCCAC
ACGAGGTAATACACAGTGTCGATCAACAATGTAATTTTCTATGGCACGTATACATACGA
AAGCGCGTGCACGATTTATAAAAGGTTAATAACCTGAACAATGTCATTTGTGTGCTCCA
GTTGAAATTATATCAAGAAGACCCCATGAACAAACGGCGTGTGTCCGTATCTTAGGGAG
TGGCGCAT**GTGTGTTGTGTGCGTGTGTGCGTGTGTGCGTGTGTGCGTGCGTGCGTGCGT**
GTGTGTGTGTGCTATATCCTTATCATATGACTTTGCCTCTTATTGGGCGTGCGTGCGTG
CCTCCTTCATATGATAGAGCACGTGGGCATATAATATTACGTAGCACGTATATTTGAAT
AAATAAA

BI 41 & BI 4.2

GTATGAGCATCGTATGAACATGATAGTATGAACATGGTATGGACAGAATATGAGCAATG
TATGAACCATGTATGAACAATGCGCTTAAAA**CACACACACACACACACACACACACACA**
CACACACACACACACACACACACACACACACACACACACACACACACACACACACACACCTAT
ACGTGTACTGCGCTGCGAGGCGTACTGGTTCATTCGTGCCTTTTTCTTTCACGCATATT
CTTATATTCAGGTTATTTTTTAACGGTTTGATTGGTTTGTTAACACTTTATTTGTTTGC
TTAGTTATCATCTATCTTAGTTCTGACACCGCAATGTCAAGTGGTACAGCGCAAGATC

BI 61 & BI 11.1

TTTTGCGGTCAAGCCAGTTCCATATTCTGAGATATAATCGCCGCTACACACATACATTC
AGACAGACACATACATTTACGCACATATGCACACACATGCACACACATACGCATTGACA
CACGCAGGAAGA**CCCCCCCCCCACACACACACACACGACACGCATACACACACACACACA**
CACACACAAACACACACACACACACGCACACACGCACACACGCACACACACGCACACAC
GCACACACACACGCTCTCTCTTACCTACAACATACTTACCTCAGTTCACTTACTCACTG
ACAAATACGCTTGCTGACTTTGTTTTTCTCTCGTTACGTGTATGCGC ATTTTT

BI 85, BI76, & BI77

GGTTGTCATTGGGGTTAGGACACCGGTCCGGCCTAGCCAGGCGGGCTTTAGGCACTTTT
TATTCGTTTTAACCGTGTATTAACTTGTGAAGATGTACTGTATGCGTGTGAAAACACTG
AAAAGTGTACGTGAAA**CACACACACATACACACACACACACACACACACACACACGCAC**
GCACGCACACCTCTTCTTATTTCATTAGGTCATGTTATGTTAAGTTTGTTATGTTAAAA
AATTGCAAGGACCTGTGGTATGCGTTGTGAGCCTGTGGCCCCGGCCTGACCGTTACGCG
TGCGTGTGTGGCTTAGGAACAGGCCTGCAG

BI 4

GCTGCCGGCATCTAGTCCAGAATTGAAGGATGAAAAGTATGCGTTAGCATGCTTTTCAG
CATGAAATTCGCATTGGCGTGTCGGTCAAAAAGTTGGGTCTCGGCTGTTT**GTCAGTCAG**
TCAGTGTGTGGGTGTGTGTGTGTGTGTGTGTGTCCAAGGACTTTTGCTTCTGGAGCCAA
AACGGTCGGATCTGAGCCGAGCGAAAGCACGTGCAATCTTCACTGGCTAATAATGGTCC

BI 54

CCATTGCGGAGTAATGGCAAGTGGGACTTTATCAACACTGTGTGTCACCTCATGTGGCA
CCTCGTGTAGCACGTGCCACGTGACGTGCCACCCAGCCATGGGTGTGGTCACATCCTCC
TGGACCTCTTTAAGCCACCTACCTGGTCGGCGTCTAACGACGTGCCAAATTTGAGTACG
AT**CACACACACACACACACACACA**CGTNCTACACGCACTACCACATGCCGTCCGCCTACCT
GGCCATCACCACGCTCTGCATCACCTACCAGCTCATGAAGCTCGCCAGCAGGTCAGG

BI 3341

CACTTCTCAGAGGTGCAACAACCTGTCAACCTGTCGCATACTGTCATTCGATCACGGAA
ATCTGTCGCACAGCCTAACATCACTCAAAAT**GCGCGCGCGTGTGTGTGTGTGTACACAG**
TGCACTGCGTGTGTGTGTGTGTACACTGTGCACTGCGTGTGTGTACAGAAACTGTGCAC
TGTTCACTTGTGTAGTTTGTGTCGTTTTTCCTTCTCATGCGCATGTTTGAAGACGACAA
GAACAAACTGGCTAATGCAAGCCTCGTAAAAACAAACTTTTTCCAAAAGCACTCCCCTA
TTACATTAGCGAGATATTGCAATGG

BI 46.2

GATCATCGACACAAGTGGCCAGCAGT**GAGAGCGCGCGCGCGCACACACACACACACACA**
CACACCTTTTCTGTCCCGGTCGATCCGCTGATGTGCAGGCAGCCAGCGTGACGCGATGC
CCACGGCTTGCAGGGGCACGTCCGGCTGACCTGAATACGCTGCCAGCAGCTCGGTCCGC
GCCAGTGGTCATCTGTGTGTATCGGCCGTCTTGCTTCTCCT

BI 22.2

GGATGTCTACATTTGCTCCTTGGCTGTGTTATTCAGACGGAGGCTGAGCGCTGAAGGCG
CGAAGCCGGAGTCAAACAATCGATGCAAATCCGAGTTGGGTCA**TGTGTGTGTGTGTGAG**
TGTGTGTGTGTGTGTATGGTATTTGAGATTATCTCGTGAATCACAGCACTTAGCGAAAC
AAATGAGGTACCATAGTGTAGGTCTCAGTCGCGCGCATTGCTAAGGCACCATTAAAACT
TTCCTGGTGTANAGGTGTGCACGTGCC

BI 107.2

GATCCGTCGCTCCTGTGCTGAGCTATAACCGAAAACCCAAAAGCAGACATA**CAAACAAA**
CAAACAAACAAACACACAAATTACAGATAGCGGACCTCACCAGAATGAAACCTTCGGTA
ACCCTCGTTACCATTCGTCTTAGCCTACGGTTTTGGACTAGATACGGACCCACGCGTTA
TGTGTGTGTCCGGGGCCAAAACCGTAGCCTTTCAACCTAATTCTTCGGCTTGTAATGCA
TCAAATTAGTTTGTGCACGTGCGCACGTGCAGTTTCACATCGGGCACCCTCACTGACCT
TTGGAAGCCACCTTACTAAGTGGTCTTCAT

BI 26.2

GGTCCAGGTGCAGCGAGTCGGCCCGGTACTCCACCTGCGG**CACACACACACACAGACAC**
ACACACAGGACAGGTCAGGTCCAGGTGCAGCGAGTCGGCCCGGTACTCCACCTGCGG**CA**
CACACACACACACACACACACAGGACAGGTCAGGTCCAGGTGCATCGAGTCGGCACGGT
ACTCCACCTGCAG**CACACACACACACACAGACACACACAGACACACACACA**GTACAGAT
C

BI 14.3

GATCATGACACGGGCGCACAGCGTCCGGTCGCCGGGACGCCG**CACACACACACACACA**G
CTCATTCACGTCACACACTAAGTAGTGCAGCGGCGCGTACCTCCAATGTCACTGGTGAC
GCCGCACATACAGTTCATGGACGCACGGACACGCGGGCGGCGCATACACACCTGAAATA
TCAGCGCGCGCATTCGTGCGGGCTGACGCCGCCCACAGCTCATTGACGCCACACAGGTC
ACACCTTCAATGTCACTAGCGACGGTGATCGCAGAATTT

BI 58, BI 59, & BI 31

TCGAGCGAGAGCACGTGCAATCTTCACTGGCTAATCATGATCCAAAATTTGGCAGATTC
GCACGGCACGTGCACACCTCTACACGAGGAAAGTTTTAATGGTGCCTAAGCAATGCGCG
CGACTGAGACCTACACTATGGTACCTCATTTGTTTCGCTAAGTGCTGTGGTTCACGAGA
TAATCTCAAATACCATA**CACACCCACACACACACACCCACACACACACA**TACATGACCT
AACTCGGATTTGCATCGATTGTTTGACTCCGGCTTCGCGCCTTCGGCGCTCAGCCTCCG

TCTGAATAAAAAACAAAATTCCGCCACAATGGAAATGCCAAAAGGCGAAGCATCATCAG
TCTTGGTGAGATC

BI 35.3

CCAGTGTGTCTGCCAAACGAATGTAGTGC**GTGTGTATGTGTGTGACTTGTGTGTGTGGT**
GGCGTGCGTTTGTTACTTTTTTTTTTAACTTCACCCCCAACGGGTCATAGACCCAATTAC
AGGAGGTGCTCCTTCTCCTCCTACATGGTTTGTGTTTACGAGGTCTTACCAAAGGACCT
CACCAGATCGGGCGAGACACCAACCATGGGCGACCCCGGTGA

BI 44.1

GATCTAAGGGTCCCCCGAATGGCCGTTGTGTATCCATGCAGCGCGGCGCCCGGCGCGGT
GGCCGCCCGGTATCAATACGGAGGAGTGTTTCAGGCCGGCGGGCAGTGCAGTGAT**GGGG**
CGGGGCGGGGCGGGGCGGGGATAACTGTGTGATAATGTGCGAATGTTACCAGGGGTGGC
ACCTGACCCCTGGTGTCACAATGGTGTCATGACGAAGACCGACTCTGATCGCGGTGGCG
CCACCCGCCCCGATCGCAGAATTCGCACGAGTACTACGCAGAATAATCCC

Appendix 2. The gain (1) and loss (0) matrix of restriction sites

	Restriction endonucleases					
Hapoltypes	*TaqI*	*EcoRI*	*DdeI*	*NdeII*	*HinfI*	*VspI*
H1	0111000111111100	1101	0101100001111111010	11101001	011111010	00111000111100
□H2	0111010111111100	0001	0101100001111111010	11101001	011111011	00111000111100
□H3	0111000111111100	1001	0101100001111111010	11101001	011111011	00111000111100
□H4	0111010111111100	1101	0101100001111111010	11101001	011111010	00111000111100
□H5	0111010111111100	0001	0101000001111111010	11101001	011111011	00111000111100
□H6	0111000111111100	1101	0001100001111111010	11101001	011111010	00111000111100
□H7	0111000111111100	1001	0101100001111111010	11101001	011111010	00111000111100
□H8	0111000111111100	0101	0101100001111111010	11101001	011111010	10111000111100
□H9	0111000111111100	1101	0101100001111111011	11101001	011111010	00111000111100
□H10	0111000111111100	1101	0101100001111111000	11101001	011111010	00111000111100
□H11	0111010111111100	0001	0101100001111111010	11101001	011110011	00111000111100
□H12	0111010111111100	0101	0101100001111111010	11101001	011111011	00111000111100
□H13	0111010111111101	0001	0101100001111111010	11101001	011111011	00111000111100
□H14	0111000111111100	1101	0101000001111111010	11101001	011111010	00111000111100
□H15	0111000111111100	1001	0101100011111111010	01101001	011111010	00111000111100
□H16	0111000111111100	1111	0101100001111111010	11101001	011111010	00111000111100
□H17	0110000111111100	1001	0101100011111111010	01101001	011111010	00111000111101
□H18	0111000111111100	1001	0101100011111111010	01101001	011111011	00111000111100
□H19	0111000111111100	1001	0101100011111111010	01101001	011111011	00111001111101
□H20	0111000111111100	1101	0111100001111111010	11101001	011111010	00111000111100
□H21	0111000111111100	1001	0101100011111111010	01101001	011111011	00111000111101
□H22	0111000111111101	1001	0101100011111111010	01101001	011111011	00111000111100
□H23	0111000111111100	1001	0101100011111111000	01101001	011111011	00111000111100
□H24	0111010111111100	0001	0101100011111111000	11101001	011111011	00111000111101
□H25	0111000111111100	0101	0101100001111111010	11101001	011111010	00111000111100
□H26	0111000111111100	1001	0101100011111111010	01101001	011111010	00111000111101
□H27	0111000111111100	1001	0101100001111111000	11101001	011111010	00111000111110
□H28	0111010111111100	0001	0101100001111111010	11101001	011111011	00111000100100
□H29	1111000111111100	1001	0101100011111111010	01101001	011111011	00111000111100
□H30	0111000111111100	1001	1101100001111111010	11101001	011111011	00111000101100
□H31	0111000111111100	1001	0101100001110111010	11101001	011111011	00111000111100
□H32	0111000111111100	1001	0101100011111111010	01101001	111111010	00111000111101
□H33	0111000111111110	1101	0101100001111111010	11101001	011111110	00111000111100
□H34	0111000111111100	1001	0101100001111111010	01101001	011111011	00111000111100
□H35	0111000111111100	1101	0101100001111111010	11101001	011111010	00111000111110
□H36	0111000111111100	1101	0101101001111111010	11101001	011111010	00111000111100
□H37	0111000111111100	1101	0101110001111111010	11101001	011111010	00111000111100
□H38	0111000111111100	1101	0101100000111111010	11101001	011111010	00111000111100
□H39	0111010111111100	1001	0101100001111111010	11111001	011111011	00111000111100
□H40	0111000111111100	1001	0101100011111111010	11101001	011111010	00111000111100
□H41	0111000111111100	1001	1101100001111111010	11101001	011111011	00111000111100
□H42	0111000111111100	1001	0101100001111111110	11101001	011111010	00111000101100
□H43	0111000111111100	1101	0101100001111111010	11101001	011111011	00111000111100
□H44	0111000111111100	1001	0101100011111111010	11101001	011111011	00111000111100
□H45	0111000111111100	1101	0101100001111111010	01101001	011111010	00111000111101
□H46	0111000111111100	0001	0101100001111111010	11101001	011111011	00111000111100
□H47	0111010111111100	1001	0101100011111111010	01101001	011111010	00111000111100
□H48	0111000111111100	1101	0101100001111111010	01101001	011111011	00111000111100
□H49	0111000111111100	1101	1101100001111111010	11101001	011111011	00111000111101
□H50	0111000111111100	1101	0101100001111111010	11101001	011111010	00111000111101
□H51	0111000111111100	1101	0101100001111111010	11101001	011111010	00111000111000
□H52	0111001111111100	1001	0101100011111111010	01101001	011111011	00111000111100
□H53	0111000111111100	1001	0101101001111111010	11101001	011111011	00111000111100
□H54	0111000111111100	1101	1101100001111111010	11101001	011111010	00111000111100
□H55	0111000111111100	1001	0101100001111111010	11101001	011111111	00111000111100
□H56	0110000111111100	1001	0101100011111111010	01101001	011111011	00111000111100
□H57	0111000111111100	1001	0101100010111111010	01101001	011111011	00111000111101
□H58	0111000111111100	1001	0101100001111110010	11101001	011111011	00111000111100
□H59	0111000111111100	1000	1101100001111111010	11101001	011111011	00111000111100
□H60	0111000111111100	1000	0101100011111111010	01101001	011111011	00111000111100
□H61	0111000111111100	1101	0101100001111111010	11101001	011111010	00111000101100
□H62	0111000111111100	1101	0101100001111111010	11101000	011111010	00111000111100
□H63	0111000111111101	1101	0101100001111111010	11101001	011111010	00111000111100
□H64	0111000111111100	1101	0101100001111111010	11101001	011111110	00111000111100
□H65	0111000111111100	1101	0101100001111111010	11101001	011111010	00111010111100
□H66	0111000111111100	1101	0001100001111111010	11101001	011111010	00111010111100
□H67	0111000111111100	1101	0101100001111110010	11101001	011111010	00111000111100

	Restriction endonucleases					
Hapoltypes	*TaqI*	*EcoRI*	*DdeI*	*NdeII*	*HinfI*	*VspI*
□H68	0111010111111100	0001	0001100001111111010	11101001	011111011	00111000111100
□H69	0111000111111100	1001	1101100001111101010	11101001	011111011	00111000101100
□H70	0011000111110100	1101	0101100001111111010	11101011	011011010	00111000111100
□H71	0111000111111100	1101	0101000001111111010	11101001	011111010	00111000111101
□H72	0111000111111100	1001	0101100011111111010	01101101	011111011	00111000111101
□H73	0110000111111100	1001	0111100011111111010	01101001	011111011	00111000111100
□H74	0111000111111100	1101	0101100001111111010	01101001	011111010	00111000111100
□H75	0111000111111100	1101	0101100101111111010	11101001	011111010	00111000111100
□H76	0111000111111100	1001	0100100011111111010	01101001	011111011	00111000111101
□H77	0111010111111100	1001	0101100001111111010	11101001	011111011	00111000111101
□H78	0111010111111100	1001	0101100001111111010	11101001	011111011	00111000111100
□H79	0101000111111100	1001	0101100011111111010	01101001	011111011	00111000111101
□H80	0111010111111100	0001	0101100001111111010	11101001	011011011	00111000111100
□H81	0111000111111100	1001	0101100011111111110	01101001	011111011	00111000111100
□H82	0111000111111100	1101	0101100001111111010	10101001	011111110	00111000111100
□H83	0011000111111100	1001	0101100001111111010	11101001	011111011	00111000111100
□H84	0111000111111100	1101	0101100001011111010	11101001	011111010	00111000111100
□H85	0111010111111100	0001	0101100001011111010	11101001	011011011	00111000111100
□H86	0111010111111100	0001	0101100001111111010	11101001	011111010	00111000111100
□H87	0111010111111100	0101	0101100001111111010	11101001	011110011	00111000111100
□H88	0111000111111100	1001	0101100011011111010	01101001	011111011	00111000111101
□H89	0011000111110100	1001	0101000011111111010	01101011	011011011	00111000111100
□H90	0111000111111100	1001	0101100011111111010	01101001	011111010	00111000101100
□H91	0110000111111100	1101	0101100001111111010	11101001	011111010	00111000111100
□H92	0011000111110100	1101	0101100001111111010	11101001	011111010	00111000111100
□H93	0111000111111101	1001	0101100001111111010	11101001	011111011	01111000111100
□H94	0111010111111100	0001	0101100001111101010	11101001	011111011	00111000111100
□H95	0111000111111100	1001	0101100001111110010	11101001	011111010	00111000111100
□H96	0111000111111100	1001	0101100011111111010	01101001	011111011	00111000101100
□H97	0111000111111100	1001	0100100011111111010	01101001	011111011	00111000111100
□H98	0111010111111100	0001	0101100001111111010	11101001	011111011	01111000111100
□H99	0111010111111101	1001	0101100011111111010	01101001	011111011	00111000111100
□H100	0111000111111100	0001	0101100001111111010	11101001	011111010	00111000111100
□H101	0111000111111100	1001	0101100011111111010	01101001	011111011	00111100111100
□H102	0111000111111100	1001	1101100001111111010	01101001	011111011	00111000111101
□H103	0111000111111100	1101	0101100001111111010	11101001	011111010	00111000100100
□H104	0111000111111100	1101	0101100001111111010	11101001	011011010	00111000111100

Appendix 3. Publications

Iyengar A., Piyapattanakorn, S., Heipel, D.A., Stone, D.M., Howell, B.R., Child, A.R., Maclean, N. (1999). A suite of highly polymorphic microsatellite markers in turbot *(Scophthalmus maximus* L.) with potential for use across several flatfish species. *Molecular Ecology* 9, 368-371.

Power, A.M., Piyapattanakorn, S., O'Riordan, R.M., Iyengar, A., Myers, A.A., Hawkins, S.J., Delany, J., McGrath, D., Maclean, N. (1999). Verification of cyprid size as a tool in the identification of two European species of *Chthamalus* barnacles using mtDNA-RFLP analysis. *Marine Ecological Progress Series* 191: 251-256

A. Iyengar, S. Piyapattanakorn, D.M. Stone, D.A.Heipel, B.R. Howell, S.M. Baynes, N. Maclean (2000). Identification of microsatellite repeats in turbot (*Scophthalmus maximus* L.) and dover sole (*Solea solea*) using a RAPD-based technique: characterization of microsatellite markers in dover sole. *Marine Biotechnology* 2 , 49-56.

Harper G.L., Piyapattanakorn, S., Goulson, D., Maclean, N. (2000). Isolation of microsatellite markers from the Adonis blue butterfly (*Lysandra bellargus*). *Molecular Ecology* 11: 1948-1949.