GENETIC BIODIVERSITY OF THE EUROPEAN BARNACLE *CHTHAMALUS MONTAGUI*

by

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Abstract

Biodiversity ultimately is genetic diversity. Genetic diversity within species is eroded before negative trends in biodiversity become evident as loss of species or habitats. Hence, monitoring biodiversity at the genetic level may indicate what will happen at higher levels of organisation if the trend is allowed to continue.

There is a pervasive belief that marine ecosystems are less vulnerable to biodiversity loss than terrestrial ones, due to marine species' high dispersal ability and connectivity, large geographic ranges, low genetic differentiation among populations and high genetic variation within populations. Many studies offer compelling evidence that it is not so: loss of genetic variation due to natural and anthropogenic factors has been detected even in marine species with potentially high dispersal.

In this context the genetic pattern of the European barnacle *Chthamalus montagui*, a species with high dispersal capability, was investigated from three different perspectives using polymorphic microsatellite loci as molecular markers.

The effect of structures created to protect coastal areas in the Adriatic Sea, was investigated to test the hypothesis that artificial substrates can act as "corridors" facilitating gene flow among previously isolated populations.

The genetic pattern of central populations was compared to that of peripheral/marginal populations over the range of *C. montagui* in the UK, to test the hypothesis that marginal and peripheral populations tend to be less genetically variable than central ones.

For both studies results were consistent with the formulated hypotheses at the 3 analysed loci.

Finally, a broader survey of the NE Atlantic and Mediterranean range of this barnacle was carried out to assess spatial scales of genetic variation. A clear differentiation between Atlantic and Mediterranean samples was detected; however, the major source of genetic variation was within sites at a very small spatial scale.

The information gained generates insights for marine genetic management and conservation planning.

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Author's declaration

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Chapter 1

General Introduction

1.1 The context of the project

My PhD research programme was part of a larger three year project titled "EUMAR - European Marine Genetic Biodiversity" financed by the EU, within the fifth Framework Programme, running from January 2002 until June 2005.

The overall objective of EUMAR was to find means to progress from general ideas about biodiversity, via a firm knowledge base and through the results of the project, to guidelines for genetic biodiversity management in the coastal zone. This aim was achieved by combining genetic and demographic modelling and empirical data to estimate short- and long-term effects of different threats to genetic diversity.

A broad range of model species (littorinids, dogwhelks, limpets, polychaetes, barnacles etc.), all from coastal habitats but with different life histories and demographic characters, were investigated by seven European partner laboratories. The first part of the project referred to natural levels of spatial and temporal genetic variation, to identify the scales on which human activities may act. The second one assessed anthropogenic impacts, such as the introduction of artificial habitats, habitat fragmentation and artificial selection. My project focussed on barnacles and investigated the genetic patterns in relation to spatial scales, peripheral/marginal populations and artificial substrates.

1.2 Genetic biodiversity

Biodiversity is a word with multiple meanings depending on the biological scale to which it is applied (Thorne-Miller and Catena, 1991; Norse, 1993; Heywood and Watson, 1995; Ormond *et al.*, 1997).

In the text of the Convention on Biological Diversity held in 1992 in Rio de Janeiro, "Biological Diversity" is defined as "the variability among living organisms from all sources including, inter alia, terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part; this includes diversity within species, between species and of ecosystems" [Article 2] (ISCBD, 1994).

Given these various scales of biodiversity, the biological diversity of an area is conveniently described at three levels:

1. Infra-specific or genetic diversity is the variation among individuals within a population and among populations of a plant or animal species. The genetic makeup of a species is variable between populations of a species within its geographic range. Loss of a population results in a loss of genetic diversity for that species and a reduction of total biological diversity for the region (Féral, 2002).

2. Species diversity is the total number and abundance of plant and animal species in an area. The number of species currently described on Earth is between 1.4 and 1.7 million (Stork, 1988; Wilson, 1992), but the Global Diversity Assessment suggests a conservative estimate of 1.75 million (Heywood and Watson, 1995; Duffy and Lloyd, 2007). More species have been described on land than in the sea (Gray, 1997), but some authors suggest that in the deep sea there are from 10 million (Grassle and Maciolek, 1992) to 500,000 (May 1992; Briggs, 1994) undescribed species.

3. The third level concerns the variety of natural communities or ecosystems within an area. These communities may be representative of or even endemic to the area. It is within these ecosystems that all life dwells (Féral, 2002).

In the Biodiversity Convention an ecosystem is defined as "a dynamic complex of plant, animal and micro-organism communities and their non-living environment interacting as functional unit". The boundaries of such systems are loosely defined and are especially difficult to demarcate in the sea since the fluxes of energy and material within and exported from a system are rarely known. The most frequently used quantitative measure of biodiversity for a given marine area is habitat diversity rather than ecosystem diversity (Gray, 1997). In ecological terms, physical areas and biotic components that they contain are termed habitats. Habitats have clear boundaries and they are easier to envision (e.g. a coral reef, an estuary). Three levels of habitat diversity can be distinguished: alpha (within-habitat), beta (between-habitat) and gamma (landscape) diversity. The last one, defined as a mosaic of habitats over larger scales, often hundreds of kilometres, is important in relation to biodiversity conservation (Gray, 1997).

It is clearly important, therefore, to specify what scale (hence, what type of diversity) is being studied. However, biodiversity is dynamic in its nature and covers a complex set of relationships within and between these different levels of organisation. Species and their populations are in continuous evolutionary change (Féral, 2002).

Genetic diversity is at the lowest hierarchy in this biodiversity sequence, which enhances — not diminishes — its importance (Templeton *et al.*, 2001). Genetic differences among individuals within a species provide the foundation for diversity among species and ultimately the foundation for the diversity among ecosystems. Genetic diversity determines the ecological and evolutionary potential of species (Féral, 2002); it is the raw material of evolutionary change, including adaptation and speciation (Templeton *et al.*, 2001). Without genetic diversity, a population cannot evolve, and it cannot adapt to environmental change. It is the clay for evolutionary adaptation and ultimately speciation, and its role is fundamental in the ability of a species to persist when challenged by various environmental pressures (e.g. disease outbreak, food shortage, climate change) (Allendorf and Luikart, 2007).

The ultimate view of biodiversity is that it *is* genetic diversity (Avise and Hamrick, 1996). Even if this seems an extreme view, the fact that biodiversity changes at the genetic level often precede changes at species and ecosystem levels cannot be ignored. That is, before the negative trends in biodiversity are observed as loss of species or habitats, the genetic diversity within species will be eroded. Thus, assessing biodiversity at the genetic level may be an indicator of what will happen at higher levels of organization in a particular area.

Genetic diversity is created by the process of **mutation**, which is responsible for allelic diversity (alternative forms of genes at the same locus - alleles). The allelic diversity within a reproducing population is translated into genotypic diversity through the mechanisms of gamete formation and

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union (system of mating): during gamete formation, alleles at different loci are put together into various combinations by the processes of recombination and assortment, which greatly augments the potential for genotypic diversity (Templeton *et al.*, 2001).

Mutation and recombination are the processes by which new alleles are created and they should be equally transmitted from one generation to another, but allelic frequencies can change in populations, such that some variants may increase in frequency at the expense of others. In fact, their evolutionary fates are governed by three other forces: natural selection, migration and genetic drift (Hartl and Clark, 1997; Weir, 1990; Thorpe and Smartt, 1995; Féral, 2002).

Natural selection operates via differential survival and reproductive success of individuals: organisms having advantageous variations are more likely to survive and reproduce than organisms lacking them. Those individuals with well-adapted phenotypes will make a great contribution on to the next generation. Consequently, adaptive variants will become more prevalent through the generations, while harmful or less useful ones will be eliminated. This means that some alleles will increase in frequency, while others decrease and some may be lost. This process plays a leading role in evolution (Ayala, 1982).

Genetic drift is the accumulation of random events that change the makeup of a gene pool slightly, but often compound over time. The process alters the gene frequencies of a population by chance events that determine which allele will be carried forward while others disappear (Féral, 2002).

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The importance of genetic drift as a source of genetic differentiation is inversely related to population size. When the reproducing population is large, the allele frequency of each successive population is expected to vary little from the frequency of its parent population unless there are selective pressures acting on those alleles. On the other hand, when the effective breeding population is small, random processes can cause a disproportionately greater deviation from the expected result. Therefore, small populations are more subject to genetic drift than large ones (Cavalli-Sforza and Edwards, 1967).

Migration or gene flow occurs when individuals move from one population to another and interbreed with the latter. Gene flow does not change allele frequencies for the whole species, but may change them locally when the allele frequencies in the migrants are different from those in resident individuals (Ayala, 1982).

Genetic drift, which causes the local breeding population to lose allelic diversity, decreases genetic variation within population but increases genetic differentiation among populations, whereas gene flow, which brings new allelic diversity into the local population, increases variation within, but reduces differentiation among local populations (Templeton et al., 2001).

The balance between drift, selection and gene flow and its impact on genetic variation in the local population's gene pool is important for three reasons: (a) the possibility that genetic uniformity makes populations more likely to experience high infection rates and rapid spread of pathogens; (b) the possibility that loss of local genetic diversity will increase inbreeding,

reducing the population's ability to respond to environmental change through the process of adaptation, with a progressive reduction of population size, increasing the risk of a bottleneck, in which a significant percentage of a population is killed or otherwise prevented from reproducing; and (c) the possibility that local adaptations will be unable to spread throughout the species from their local population of origin, leading to speciation (Templeton *et al.*, 2001).

Therefore, loss of genetic variation within and among populations may reduce the overall evolutionary potential of species; thus, the first step in biodiversity conservation is to acquire knowledge of genetic diversity and of the dynamic mechanisms through which is regulated (Cognetti and Maltagliati, 2004).

1.2.1 Marine genetic biodiversity

In the marine domain there are more animal phyla than on land: 35 phyla occur in the sea but only 11 on land. Phyletic diversity is highest on the seabed; of 35 marine phyla only 11 are represented in the pelagic realm. Although the pelagic realm has an enormous volume compared with the benthic realm, most of marine species diversity is benthic rather than pelagic. This is probably a consequence of the fact that the marine fauna originated in benthic sediments (Gray, 1997).

Moreover, in general, marine species have higher genetic diversity than freshwater and terrestrial species (Gray, 1997). In a comparative study Ward *et al.* (1994) showed that average heterozygosity was similar in

marine and freshwater subpopulations, but was considerably less in freshwater species than in marine species counterparts.

Spatial scales in the marine environment can vary by more than ten orders of magnitude (Butman and Carlton, 1995), and genetic diversity occurs over different spatial scales, at distances ranging from a few millimetres to several thousand kilometres. The scale at which physical distance between organisms determines the level of genetic relationships among them varies among species in relation to their respective life cycles and dispersal capabilities (Procaccini and Malatgliati, 2004).

In general terms, marine species are thought to disperse further, have higher gene flow, larger geographic ranges, lower levels of genetic differentiation among populations, and higher levels of genetic variation within populations (Féral, 2002). In fact, about 70% of benthic marine species are characterised in their life cycle by high dispersal and migratory capabilities through a planktonic phase. Many planktonic larvae spend several weeks, or even months in the plankton, where they can potentially be widely dispersed by currents and can cross any discernible barrier (Scheltema, 1971, 1983; Palumbi, 1994). Furthermore, marine populations tend to be large, with very high fecundities, and explosive reproductive potential. For these reasons, marine species are viewed as consisting of very widely distributed populations that are not strongly genetically structured and appear to act as large, panmictic units (Palumbi, 1994). They often represent a serious challenge to the allopatric speciation model, where a population is broken up into smaller units by a physical barrier, so

that drift, mutation and divergent selection can generate genetic differences that lead to intrinsic barriers to reproduction (Palumbi, 1994).

Genetic studies of marine invertebrates have generally provided good support for this challenge to allopatric speciation (Levinton and Koehn, 1976; Gyllensten, 1985; Waples, 1987; Palumbi and Wilson, 1990; MacMillan et al., 1992; Ward et al., 1994; Palumbi, 1995). However, an increasing number of exceptions to this idea of large-scale panmixia have been identified: several studies have reported high genetic differentiation among populations even in marine species with potentially high dispersal (Winans, 1980; Doherty et al., 1995; Johnson and Black, 1995; Lavery et al., 1996; Palumbi et al., 1997; Huang et al. 2000; Nesbø et al., 2000; Riginos and Nachman, 2001;) and many sibling species complexes, closely related with very low genetic distances, have been detected from coral reefs to the deep sea implying recent species formation (Knowlton, 1993; Palumbi, 1997; Gray, 2001). Furthermore, genetic pools of the majority of widely distributed species are rarely homogenous from one end of their geographical distribution to the other (Burton, 1983; Reeb and Avise, 1990; Watts et al., 1990; Karl and Avise, 1992; Hilbish, 1996; Neigel, 1997).

Although the predominant mechanisms leading to population subdivision and promoting genetic divergence are not always clear (Palumbi, 1994), several factors may be important either singly or in combination. Among them biological factors such as larval behaviour, selection on recruits, species interaction and local adaptation have to be considered (Schmidt and Rand, 1999; Jones et al. 1999, Swearer et al. 1999, Luttikhuizen et al. 2003, Taylor and Hellberg, 2003; Jenkins, 2005). Moreover, a large

number of mechanisms of reproductive isolation such as differences in spawning time, mate recognition, environmental tolerance and gamete compatibility have been implicated in marine speciation events (Palumbi, 1994).

Other mechanisms that might enhance genetic differentiation in the marine environment are historical environmental factors (Bert, 1986; Palumbi, 1994; Lavery *et al.*, 1996), isolation by distance (Palumbi *et al.*, 1997; Johnson and Black, 1998), habitat discontinuities (Winans, 1980; Burton and Feldman, 1981; Doherty *et al.*, 1995; Johnson and Black, 1995) and chemical-physical barriers such as gradients of temperature, salinity, nutrients and/or the presence of local eddies, gyres and current reversals (Palumbi, 1994, Neigel, 1997).

Therefore, the ocean is not as continuous as it appears, but is to some degree a fragmented habitat, with "invisible" barriers that can be complex and sometimes sharper than on land; these can affect the dispersal of the planktonic larvae and consequently the gene flow and population genetic structure of species (Quinteiro *et al.*, 2007; Palumbi, 1994).

Hence, in light of the changing paradigm of the marine environment and the genetic population structure of the species within it, there is a need for a better understanding of the geographic patterns and the spatial scales of genetic structuring in the sea and the factors that shape and maintain them. One of the main objectives of this project has been to collect information on the scales of genetic spatial differentiation in the marine environment using the barnacle *Chthamalus montagui* as the target species.

Moreover, particular consideration should be given to peripheral and marginal populations when studying the geographic pattern of genetic differentiation. Peripheral populations are here defined as those at the edge of the core distribution of the species, whereas marginal populations are those living in atypical ecological environments for that species. Peripheral and marginal populations are often genetically different from central populations living in the typical habitat of the species. They can present unique genetic characteristics due to their geographic isolation and/or selection, producing adaptation to exceptional conditions (Johannesson and Andrè, 2006). In some studies reduced genetic variation has been detected within them (Lesica and Allendorf, 1995; Palumbi, 1997; Schwartz *et al.*, 2003).

Hence, in order to develop strategies for conservation of marine genetic biodiversity conservation it is important to investigate these vulnerable populations. One of the aims of this project has been the study of peripheral and marginal populations of *Chthamalus montagui* in the UK.

1.2.2 Human factors influencing marine genetic biodiversity

Biodiversity has been defined above at several levels of biological organization, including genes, species, communities, and ecosystems (Féral, 2002; Meffe and Carroll, 1997). Human activities cause massive impacts on biodiversity at all these levels (Templeton, 2001). In particular, they can have dramatic effects on the amount and distribution of genetic diversity within species, directly altering the dynamics of evolution itself with respect

to the fundamental processes of adaptation and speciation (Templeton, 2001).

Most of the threats to marine biodiversity are in the coastal zone and are a direct result of the human population and its growth. It is estimated that more than 67% of the human population lives within 60 km of the shore line and the population is steadily increasing (Gray, 1997). This puts increasing pressure on coastal areas to provide more housing, more food, more recreation, more jobs etc.

Marine ecosystems were in the past considered effectively infinite, therefore, human activities like fishing or waste disposal were not considered as significant threats. This misconception is further exacerbated by the still quite pervasive belief that biodiversity in marine ecosystems is generally much less vulnerable to extinction caused by anthropogenic influences than biodiversity in terrestrial ecosystems (Backeljau, 2003).

Yet, compelling evidence indicates that marine ecosystems are undergoing rapid and radical degradation (as suggested by symptoms such as collapsing fisheries, coral bleaching, marine epidemics, algal blooms, invasive species, mass mortalities, etc.) (Lubchenco, 2003). Moreover, the risk of extinction in marine species may be far greater than is generally assumed due to several factors, mostly related to human activities, such as overexploitation, pollution, introduction of alien invasive species and habitat alteration and/or destruction (Roberts and Hawkins, 1999).

The last of these is one of the primary impacts of human activities. Coastal areas are a complex mosaic of habitats, variously interspersed and interconnected. Human activities often modify natural patterns of coastal landscapes, causing habitat modification or fragmentation, thus altering the level of isolation and connectivity among populations (Abbiati, 2003).

Habitat fragmentation and related impacts at both genetic and species levels have received wide attention in terrestrial habitats for predicting for example the consequences of urban development (Newman, 2000), but this is a quite new concept in the marine environment. Continuous shorelines can be interrupted by coastal cities and harbours, populations previously connected can be separated, and thus become smaller and more isolated. Small and isolated populations lose genetic variation at a high rate by genetic drift, through a reduction of the gene flow and alteration of metapopulation structure (Templeton, 2001). Habitat destruction might also lead to losses of certain biotopes and this might have adverse effects on the part of evolution guided by natural selection (Johannesson, 2003).

On the other hand, artificial substrates such as breakwaters for beach protection, jetties, seawalls, pontoons and pier pilings, which have become ubiquitous features of open coasts (Bacchiocchi and Airoldi, 2003), bays (Sammarco et al., 2004) and estuaries (Chapman and Bulleri, 2003) can provide suitable substrata for hard-bottom benthic organisms. Some of these can be non-indigenous species, which can spread in this novel habitat (Bulleri and Airoldi, 2005). In general, these artificial habitats can act as stepping stones along coastlines, increasing the gene flow among formerly isolated populations and reducing local genetic variation among populations

(Abbiati, 2003). Their effect in altering the natural genetic pattern of populations is the reverse of habitat fragmentation.

One of the objectives of this project has been to investigate the effect of the artificial substrates on the genetic biodiversity of the barnacle *Chthamalus montagui* in the Adriatic Sea.

To conclude, there is a need to acquire knowledge about patterns and processes that can affect marine biodiversity, in order to establish effective management and conservation plans. It is only by considering genetic diversity, too often neglected by stakeholders, that a given plan will have long-term success (Maltagliati, 2003). Today this is possible thanks to molecular techniques and markers developed in recent decades.

1.3 Molecular techniques and markers

The application of techniques using molecular markers to research questions in ecology and evolution delimits a recently defined discipline called "Molecular Ecology" (Schierwater *et al.*, 1994; Carvalho, 1998; Féral, 2002). Molecular markers reveal variations in the DNA nucleotide sequence among individual genomes (polymorphisms, due to point mutation, insertion, deletion or translocation etc.). They may provide useful information at different levels: population structure, phylogenetic relationships, patterns of historical biogeography, levels of gene flow, analysis of parentage and relatedness.

Before describing the different markers and molecular techniques available for the estimation of genetic variation, it is important to consider that sequence changes occur at a rate that is more or less proportional to time, and the number of mutations which differentiate two genomes is proportional to the time of disjunction between them (the molecular clock hypothesis - Zuckerkandl and Pauling, 1965). Therefore, the resolution of the molecular techniques used should match the time scale of interest.

Moreover, it is worth remembering that DNA is composed of coding regions (genes) and non-coding regions, the latter generally representing the higher percentage of the whole genome. Non-coding regions can either be functional, with a role in the regulation of transcription or can apparently lack a known function. Mutations, which can accumulate more easily in non-functional regions, together with the recombination happening during

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meiotic events, determines the existence of individual-specific "DNA fingerprints".

An ideal class of molecular marker is polymorphic, co-dominant, heritable and expressed in a stable way, distributed throughout the genome, easy to detect and score; it has to give reliable and reproducible results using a methodology that can be applied to different species.

Molecular markers can investigate both nuclear DNA and cytoplasmic genomes such as mitochondrial DNA.

Nuclear DNA

The nuclear genome is generally present in diploid condition and undergoes biparental inheritance, with recombination between homologous chromosomes during meiosis (prior to the haploid phase, typically restricted to the gametes in animals). Single-locus nuclear genes are particularly useful in detecting functional polymorphisms and population structure. Some nuclear genes have multiple copies in the genome; ribosomal DNA repeats are easily assayed and have been used extensively for systematic studies (Hillis and Dixon, 1991). Many coding gene regions are conserved but flanked by non-conserved spacer regions. The spacers often show variation at the individual and population levels offering information on population structure and levels of gene flow.

Mitochondrial DNA

The cytoplasmic mtDNA occurs in high copy numbers. It is normally inherited from the female parent so that each copy is identical. The relatively rapid rate of sequence divergence, the maternal-haploid inheritance, and the absence of recombination, which makes it a single

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heritable unit (effectively a single locus with multiple alleles) in the great majority of cases, make mitochondrial DNA valuable for examining population structure. It is now classically used in population biology and has become a major tool for investigating relationships among populations and closely related taxa (Moritz, 1994; Avise, 2000). It has greatly contributed to the establishment of phylogeography (Avise, 2000). There has been an increasing number of mtDNA studies, and these have used either coding or non-coding regions.

In the past, information at the genetic level was limited mainly by the availability of tools and techniques. In the last two decades many molecular markers have been developed and new sophisticated techniques have become increasingly available (Avise, 1994; Skibinski, 1994; Slatkin et al., 1995; Thorpe and Smartt, 1995; Burton, 1996; Ferraris and Palumbi, 1996; Carvalho, 1998). A methodological revolution came from the Polymerase Chain Reaction (PCR) (Mulis and Faloona, 1987; Sakai et al., 1988): this technique is basically a primer extension reaction for amplifying specific nucleic acids in vitro. The use of a thermostable polymerase, Taq (first isolated from the hot spring bacterium *Thermus aquaticus*), allows a short stretch of DNA (usually fewer than 300 bp) to be amplified to about a million fold so that one can determine its size, nucleotide sequence etc. The stretch of DNA to be amplified, called target sequence, is identified by specific pair of DNA primers, oligonucleotides usually about 20 nucleotides in length. The quantities of produced DNA are sufficient to be directly visualised on a gel by fluorescence after colouration with stains such as Ethidium Bromide. Furthermore, it is now possible to work with very small

initial amounts of DNA, as virtually one single cell is enough (Féral, 2002) to get about a million of copies of the target DNA.

The main markers and techniques for detecting genetic variation are described below.

1.3.1 Allozymes

Allozymes are protein markers that can be considered the precursor of the molecular markers. The analysis of enzyme variation has been in widespread use for four decades since the works of Harris (1966) and Lewontin and Hubby (1966). Allozymes are co-dominant markers and allow the investigation of variation in the expression of DNA regions codifying for specific functional proteins.

Electrophoresis is used to distinguish protein alleles by their different rates of migration through a gel in an electric field, followed by visualisation by histochemical staining. New alleles, consequence of a mutation, can be detected (Feral, 2002). This technique is simple, rapid and inexpensive with limited requirements for equipment; in this sense it is a basic method for studies of genetic variation and it has proved to be robust and applicable to most living organisms. Drawbacks are that protein studies investigate only a limited fraction of DNA, they cannot take into consideration silent mutations in the coding regions they target, and they thus underestimate the real genetic variation. Moreover enzyme isoforms can be influenced by post-translation modifications induced by the metabolic state, and they are sometimes under heavy selection (Johannesson *et al.*, 1995), which may sometimes limit their use as markers of gene flow. However, protein

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analysis is still considered a valid tool for studies of diversity at individual and population levels, in species where sufficient variability exists (Procaccini and Maltagliati, 2004).

1.3.2 DNA fingerprinting

DNA fingerprinting techniques compile individual-specific genetic fingerprints and include analyses of DNA repeated sequences and of random interspersed regions. Several techniques belong to this class, as described next.

Restriction Fragment Length Polymorphism (RFLP)

Restriction endonucleases (RE) (Linn and Arber, 1968; Avise, 1994) are highly specific enzymes that cleave DNA wherever a particular nucleotide sequence occurs (usually 4–6 bp). When the DNA is digested with such an enzyme, it is cut into fragments. Different individuals may produce a different number of restriction fragments, or homologous fragments may differ in size.

The technique is based on the comparison of the size and number of DNA fragments obtained through digestion of DNA by RE and separated by electrophoresis. (Lessa and Applebaum, 1993). Differences between individuals in size and number of restriction fragments can arise from mutations creating or destroying cleavage recognition sites; additionally, differences in size between homologous restriction fragments can be created by insertions or deletions between cleavage sites. Restriction digestion followed by RFLP analysis is typically carried out on PCR products

(PCR-RFLP), although organismal DNA (e.g. purified mtDNA) was originally used.

VNTR – Variable Number of Tandem Repeats and SSR – Simple Sequence Repeats

VNTR, referred as minisatellites, are relatively small fragments with repeat sequences from ten to a few hundred base pairs (Jeffreys *et al.*, 1985a,b; Nakamura *et al.*, 1987) and SSR, defined as microsatellites, have tandem repeats of short sequence motifs (shorter than 8-10 bp). These stretches of DNA are widely distributed throughout the genomes of plants and animals (Jarne and Lagoda, 1996). They are numerous and highly variable as there is strong variability in the number of repeats at such a given locus that corresponds to relatively high mutation rates (errors of replication, unequal crossing over, polymerase slippage, gene conversion).

They are inherited in a co-dominant, Mendelian and neutral fashion, therefore they can be present with one or two alleles in a single individual, depending on whether the locus is homo- or heterozygous. Specific primers are needed to amplify these DNA stretches, and stringent PCR reaction conditions guarantee reproducibility of the method. Several loci can be examined for each individual providing a multilocus genotype, and several alleles for each locus can be present in a single population, which makes these markers a powerful tool for population genetic studies. Detection of allele sizes was traditionally based on the use of radioactively labelled PCR primers; nowadays it is common to use fluorescent primers in automatic sequencers.

RAPD, AFLP, DALP and ISSR

These four techniques refer to the analysis of the presence/absence of multiple fragments amplified via PCR using primers of arbitrary sequence, and produce high numbers of polymorphic markers without prior knowledge of the target DNA (see Williams *et al.*, 1990; Hadrys *et al.*, 1992; O'Hanlon *et al.*, 2000). Compared to other molecular techniques, they are relatively cheap, quick and simple, and do not require the development of specific primers for the studied species. All four techniques produce multi-locus fingerprints with polymorphism represented by the presence or absence of bands. The treatment of data obtained with this class of markers (dominant data) allows the genetic relationships among individuals to be assessed, but requires assumptions to obtain estimates of within-population genetic variability, such as heterozygosity (Lynch and Milligan, 1994).

RAPD - Random Amplified Polymorphic DNA

The RAPD technique (Williams *et al.*, 1990) involves amplification of genomic DNA fragments through PCR using a single short primer of arbitrary sequence to screen the whole genome. The individual DNA fingerprint comprises a series of anonymous DNA fragments produced in the amplification that may, in combination, be highly polymorphic. There is the possibility that small fragments are not visualised, so genetic variation may be hidden. Moreover, low stringency in PCR conditions for RAPD (short primers, low annealing temperature) poses a potential problem for reproducibility (Ferraris and Palumbi, 1996). This may be controlled by careful optimisation and standardisation of the protocol to improve repeatability of results, followed by controls and evaluations of the consistency between different laboratories.

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AFLP - Amplified Fragment Length Polymorphism

This technique is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA. Polymorphism detected by the AFLP technique is usually more robust since generic primers with more stringent reaction conditions are used (Vos *et al.*, 1987).

DALP - Direct Amplification of Length Polymorphism

This technique uses longer and more stable PCR primers than RAPD (Desmarais *et al.*, 1998). All the fragments generated can be directly sequenced with the same two universal M13 sequencing primers. This strategy combines the advantages of a high-resolution fingerprint technique and the possibility of characterising the polymorphisms.

ISSR - Inter-Simple Sequence Repeats

This technique is based on the amplification of DNA fragments between two microsatellite motifs (Wolfe and Liston, 1998) using a single primer that targets the repeat itself, with 1-3 bases that anchor the primer at the 3' or 5' end of the repeated sequence. It is technically simple, provides highly reproducible results and generates abundant polymorphisms in many systems.

SSCP, TGGE and DGGE

These three techniques allow the detection of sequence differences among PCR products from a target gene by looking at changes in fragment conformation and stability through different gel separation methods. These techniques thus allow rapid detection of sequence variation without generating explicit sequence information.

SSCP - Single Strand Conformation Polymorphism

SSCP is based on the principle that changes in DNA sequences alter the folding of single-strand DNA, which affects its electrophoretic mobility (Orita *et al.*, 1989; Hayashi, 1992; Sunnucks *et al.*, 2000). The mobility of the single-strand DNA, electrophoresed under non-denaturing conditions, is determined by both fragment length and secondary structure, which is sequence-dependent. A fragment may adopt several conformations for any given set of electrophoretic conditions and these are visualised as separate bands in the gel. A single base change is sufficient to alter secondary structure and hence mobility (Ferraris and Palumbi, 1996).

TGGE - Temperature Gradient Gel Electrophoresis

TGGE is based on differences in melting temperature of double-stranded DNA or RNA sequences (Henco *et al.*, 1994). The heat is used as a source of energy to make the hydrogen bonds thermodynamically unstable. DNA or RNA fragments with point mutations will show a different melting behaviour (due to different melting temperature: Tm) and thus different conformation compared to wild type DNA. By applying a temperature gradient during the electrophoretic separation of DNA or RNA, fragments of identical length but different sequence can be separated.

DGGE - Denaturing Gradient Gel Electrophoresis

DGGE relies on the variations in the stability of DNA duplexes due to nucleotide sequence differences, this method detects mutations by separating PCR amplified DNA fragments on a denaturing gradient gel (Myers *et al.*, 1987). DGGE results in high probabilities of detection of DNA sequence differences, but requires special equipment to regulate temperature, and/or the pouring of gradient gels. For occasional use, these

requirements can be prohibitive, and for large-scale screening, the accumulated costs are high.

1.3.3 DNA sequencing

DNA sequencing, determining the exact sequence of bases, allows direct analysis of mutations in PCR amplified DNA fragments. Two methods for sequence determination have been available for more than twenty years: Maxam and Gilbert (1977; 1980) and Sanger et al. (1977). The most commonly used is the Sanger methodology (Sanger et al., 1977; Avise, 1994; Ferraris and Palumbi, 1996) which is applied in automatic sequencers: primers are labelled with fluorochromes and DNA sequences are visualized by means of specific software. DNA sequence analysis is by far the most precise genotyping method. Recent development of high-throughput new-generation sequencing, such as parallel pyrosequencing, has markedly reduced the cost and duration of large-scale sequencing projects.

A new class of marker, derived from sequence analysis, is the SNP (Single Nucleotide Polymorphism). The technique is based on the analysis of single base differences (SNPs) in genomic DNA. At each position, different sequence alternatives (alleles) can exist in individuals within populations. Large population screenings can be performed by PCR (Kuhner et al., 2000; Nielsen, 2000).

Figure 1.1 shows a qualitative representation of the relative strengths and weaknesses of different molecular markers strategies. Approaches are compared in relation to information per marker, the number of markers

(loci) per typical study, and cost per study in term of capital outlay and technical expertise (from Belfiore and Anderson, 2001).
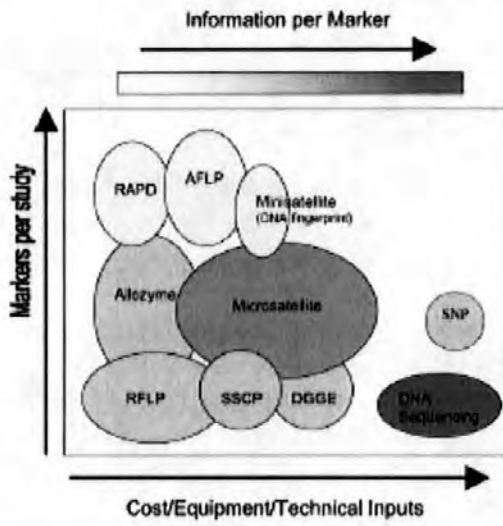
Figure 1.1 - Qualitative representation of the relative strengths and weaknesses of different molecular markers (from Belfiore and Anderson, 2001).

Chapter 2

Barnacle biology, systematics and genetics, with particular reference to

Chthamalus montagui

The target species of this study is the barnacle *Chthamalus montagui*.
Barnacles are sessile hermaphroditic crustaceans, almost ubiquitous in littoral communities and one of their dominant components; they are the most characteristic organisms of the eulittoral zone throughout the world (Stephenson and Stephenson, 1972).

This account is not intended as a review of all the literature available on the subject, but as an introduction to some aspects and selected information useful to better understand the target species and give an overview of the studies carried out on barnacle population genetics, taxonomy and phylogeny.

2.1 Taxonomy of Chthamalus montagui
(Southward, 1976)

PHYLUM Crustacea

CLASS   Maxillopoda

SUBCLASS   Cirripedia

ORDER   Thoracica

SUB-ORDER   Balanomorpha

FAMILY   Chthamalidae

GENUS   *Chthamalus* (Ranzoni 1818)

SPECIES   *Chthamalus montagui* (Southward, 1976)

Chthamalus montagui was identified as distinct species by Southward (1976); previously it was considered a variety of *Chthamalus stellatus* (Poli

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1874).   The two species, which often overlap on the shore, were distinguished (Southward, 1976) on the basis of their different morphology (in particular the shape of the opercular plates and the setation of the cirri) and distribution on the shore (vertical zonation and sheltered vs. exposed locations).   The taxonomic separation was confirmed by genetic studies employing allozymes (Dando *et al.*, 1979), where the two species showed different electrophoretic mobility for eight enzymes, four of which were classified as species-specific. Further work carried out by Dando *et al.* in 1981 and in 1987 provided strong evidence in support of this. More recently, Perez-Losada *et al.* (2008) combined DNA sequence data from 3 nuclear genes with morphological characters of different species, representing almost all the Thoracica families, to assess tempo and mode of barnacle evolution: they confirmed that *Chthamalus stellatus* and *Chthamalus montagui* are two distinct species, although very close.

2.2 External morphology

I will deal with the external morphology of *C. montagui* (Figure 2.1a), that allowed species identification during fieldwork, and then I will explain in detail how can be possible distinguished it from *C. stellatus* (Figure 2.1b).

Figure 2.1 – Photo of *C. montagui* (a) and of *C. stellatus* (b) by Prof. A.J. Southward, published on *MarLIN* web site

The shell of C. montagui, with six coarsely ridged wall plates, is brownish or greyish, usually conical to low conical, but often elongated or even columnar when barnacles live crowded together at the higher tidal levels. The surface is nearly always corroded, often punctuated, and the sutures are frequently obscure or obliterated. The opercular opening is almost always kite-shaped or subquadrangular; the joint between the tergum and scutum crosses the centre line less than one third of the way down towards the rostrum (Figure 2.2). The scutum is much longer than broad, the apex often forming an angle less than 90°. The tergum is short and wide. The tissue inside the opercular aperture is blue with brown and black markings (Southward, 1976). The shell reaches a maximum diameter of approximately 14 mm, depending on habitat, food availability and level on shore (Riley, 2002).
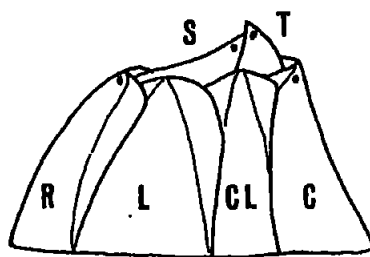
Figure 2.2 Arrangement of shell plates of Chthamalus spp.. Scutum (S); tergum (T); carena (C); rostrum (R); lateral (L); careno-lateral (CL) (Relini, 1980).

It is quite easy with practice to distinguish C. montagui and C. stellatus during fieldwork, though a good hand lens is needed for the smaller specimens. In fully grown individuals of C. stellatus the rounded form of the aperture and the position of the articulation separating the deep tergum and relatively short scutum will always separate them from C. montagui (Figure 2.3). When the shell is much corroded an additional indication is

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given by the position of the adductor muscle pit, which shows through the upper surface of the scutum as a little hump: in *C. montagui* this hump is long and narrow, and lies close to the opening between the pair of scuta, while in *C. stellatus* it is much broader and extends further across the width of the scutum, sometimes appearing almost centrally placed (Southward, 1976).
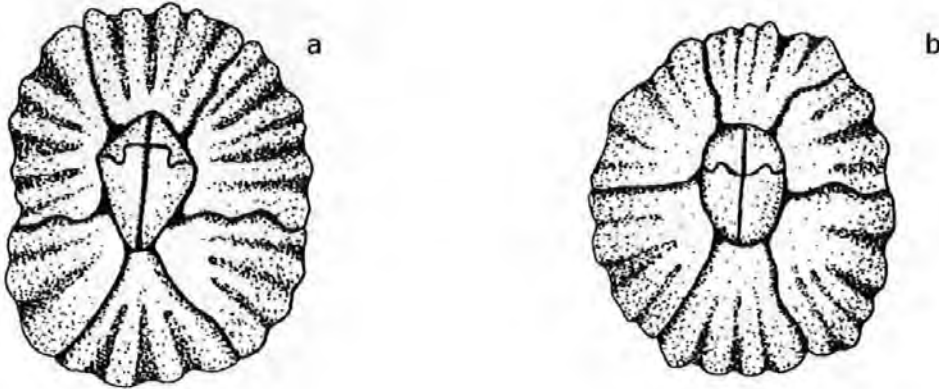
Figure 2.3 – Outline sketches of *C. montagui* (a) and *C. stellatus* (b) (Hawkins and Jones, 1992).

2.3 Distribution

Geographical distribution

Crisp *et al.* (1981) described the geographical distribution of *Chthamalus montagui*, which overlaps extensively with that of *Chthamalus stellatus*. *C. montagui* occurs abundantly on rocky shores of the Atlantic coasts of Britain, Ireland, France, Spain, North Africa, in the western and eastern Mediterranean Sea and in the Black Sea. Unlike *C. stellatus* it is rare or absent from offshore islands. The northern and southern species limits are respectively North Scotland (south of Aberdeen, Riley, 2002) and North Africa (Mauritania, Crisp *et al.*, 1981). In the English Channel the Isle of Wight represents the eastern limit of the species (Hawkins and Jones, 1992; Herbert and Hawkins, 2006).

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Horizontal and vertical distribution

With regard to the vertical and horizontal distribution on the shore, barnacles are considered the main colonizer on moderately exposed shores. They are generally limited by physical factors towards the upper limit and by biological ones at the lower (Connell, 1961, 1972; Pannacciulli and Relini, 2000). Brief immersion, and consequent problems with desiccation and poor food supply, are limiting on the high shore. Mid and low shore barnacles, instead, are constantly competing for space (Connell, 1961; 1972; Pannacciulli and Relini, 2000) and are more subjected to predation and to the destructive force of wave action.

The vertical and horizontal distribution of *C. montagui* has been extensively investigated (Southward, 1976; Crisp *et al.*, 1981; Burrows *et al.*, 1992, Pannacciulli and Relini, 2000), and compared to that of *C. stellatus*. It appears that the two species can be separated by habitat: *C. montagui* is more common in sheltered, embayed and semi-estuarine sites, while *C. stellatus* prevails on wave-beaten open coasts. Where they overlap, *C. montagui* is dominant in the upper barnacle zone (mean high water of spring tides, MHWS and mean high water of neap tides, MHWN), while *C. stellatus* is more common lower down (mean tide level, MTL, and below) (Pannacciulli and Relini, 2000).

The leading factors in producing the different adult distribution in the two *Chthamalus* species seem to be larval dispersal, development and settlement (Burrows *et al.*, 1999). It has been demonstrated (Jenkins *et al.*, 2005) that active substratum selection by cyprids at settlement determines adult vertical and horizontal distribution. The role of post-

settlement mortality (Delany *et al.*, 2003) and morphological characters (Foster, 1971) can also contribute to these differences. The morphology of the opercular plates of *C. montagui* is in fact believed to confer a better resistance to desiccation stress, which allows this species to colonise the upper shore. Moreover, it has been suggested (Burrows, 1988) that *C. montagui* juveniles may require a certain amount of exposure to air in order to consolidate and harden their shell plates, a characteristic that would make low sites on the shore relatively unsuitable for this species. This is confirmed also by a study carried out by Power *et al.* (2001), from which it appears that *C. montagui* avoids wet areas at settlement and/or suffers higher post-settlement mortality in damper sites.

2.4 Reproduction, settlement and recruitment

Barnacles are hermaphroditic organisms. They generally reproduce by cross-fertilisation by performing internal fertilisation by pseudo-copulation (Klepal, 1990), which imposes extremely restricted mating distances limited by the length of the penis (in *Semibalanus balanoides* for instance the penis is about two to three times the shell length, Stubbings, 1975).

In isolated conditions, when the nearest neighbour is too far away for copulation to occur, *Chthamalus* species may self-fertilise (Barnes and Crisp, 1956; Barnes and Barnes, 1958; Pannacciulli and Bishop, 2003). This ability allows them to survive also at very low densities. However, it has been noted that in self-fertilising individuals oviposition is delayed, and eggs can be slightly abnormal and less viable (Barnes and Crisp, 1956; Barnes and Barnes, 1958).

C. montagui is capable of breeding in its first year (Burrows, 1988), about 9-10 months after settlement (Southward and Crisp, 1954). It seems that it attains sexual maturity when the rostro-carinal diameter reaches 4.5 mm (O'Riordan *et al.*, 1992).

Barnacles then brood developing embryos within their mantle cavity before releasing larvae into the plankton. Crisp (1950), Crisp and Davies (1955), Le Reste (1965), and Achituv and Barnes (1976), suggested that *Chthamalus* produces several broods each year. Burrows (1988) from *in vivo* laboratory experiments estimated that *C. montagui* embryos at 15°C took approximately 23 days to develop completely to the stage at which larval release occurred. Taking into account the time of development of each brood and the proportion of time each individual spent brooding annually, Burrows calculated that *C. montagui* had an average of between 1 to 2 broods per year at high shore level, 2 or 3 broods per year at mid shore, and 2.5 to 4 broods per year at low level. A study carried out on experimental plates in Lough Hyne, South-West Ireland (O'Riordan et al., 1992), indicated that the number of broods not only differed with shore levels, but also with year classes (older barnacles producing more broods).

Patel and Crisp (1960) from laboratory experiments observed that *Chthamalus* (probably *C. montagui*, because specimens were collected in Anglesey where *C. stellatus* is rare) would only breed at temperatures equal to or above 15°C. According to Hines (1978), the length of the breeding season of *Chthamalus spp.* is usually broadly defined by temperature and the production of broods is limited by food availability and by the temperature-dependent development rate of the brood in the mantle cavity.

Burrows *et al.* (1992) found that the onset of breeding by *C. montagui* coincided with a rise in sea temperature above 10°C. This implies that the beginning and the length of the breeding season should vary with latitude, becoming progressively shorter going North.

It is possible to compare the breeding season throughout most of the range of distribution of the species: from February to early October in the Mediterranean (Relini, 1983), from April to early October in northern Spain (Miyares, 1986), from April to September/October in south-western England (Burrows *et al.*, 1992), from June to August/September in mid-Wales (Kendall and Bedford, 1987) and from June to August/September in south-western Scotland (Barnes, 1972). From these studies it appears that the main effect of latitude is on the onset of breeding: four months separated the beginning of the season in the Mediterranean, February, and in SW Scotland, June, whereas the cessation of breeding at all sites occurred in September or early October.

When the embryonic development in the mantle cavity is completed, *Chthamalus* releases the larvae into the plankton in the first of six naupliar instars. A hatching substance, a prostaglandin-like compound (Clare *et al.*, 1982; 1985) found only in thoracican barnacles, is produced by adult tissue only at a certain time of the year and is able to stimulate larval release. Burrows *et al.* (1992) related larval release of *Chthamalus* spp. to blooms of flagellates. From laboratory experiments (Burrows *et al.*, 1999) it emerged that larvae of *C. montagui*, cultured at 19°C, reached stage VI and were ready to settle in 11 days. Development time in culture may, however, be shorter than in natural conditions. The range of larval dispersal in barnacles

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can vary, and is likely to be influenced by both large-scale (e.g. offshore hydrodynamic processes, Barnes, 1956; Raimondi, 1990) and small-scale (e.g. local water turbulence, O'Riordan, 2004). In some cirripedes dispersal is estimated to be in the order of 15 to 20 km per generation (Strathmann, 1974; Crisp, 1976)

Jenkins (2005) demonstrated that the basic pattern of adult *Chthamalus* distribution is established at settlement, when planktonic cyprids irreversibly attach to substratum. He demonstrated that cyprids actively select the substratum and that selection is operated not only on the horizontal gradient of wave exposure, but also over the vertical gradient of the shore. However, post-settlement mortality in the early life of newly settled marine invertebrates can be extremely high and have a strong modifying effect on the characteristic adult pattern (Gosseil and Qian, 1997).

Delany *et al.* (2003) found that mortality after settlement was the most significant factor in determining the distribution of *C. montagui* and *C. stellatus* on the shores of SW Ireland. These results were confirmed also by Power *et al.* (2006) who implicated differential mortality patterns in individuals up to the age of 11 months in determining patterns of distribution of both species. This means that the recruitment phase, that determines which barnacles survive settlement and become part of the adult breeding population, is very important in structuring intertidal communities (O'Riordan *et al.*, 2004). For this reason recruitment has been used as a variable in modelling: a model of open populations with space-limited recruitment was developed with direct application to *C. montagui*

(Hyder *et al.*, 2001) and another was produced to estimate the effects of recruitment disturbance on two interacting barnacle species, *Semibalanus balanoides* and *C. montagui* (Svensson *et al.*, 2006).

Timing of settlement is linked to the reproductive period and it may be influenced by interrelated factors such as latitude, temperature and food availability as well as shore height (O'Riordan et al., 2004). In North Italy, for instance, recruitment of *C. montagui* begins in May (Pannacciulli and Relini, 1999), which is earlier than in SW Ireland but later than in NW Spain (O'Riordan et al., 2004), in agreement with the latitudinal pattern.

2.5 Growth and longevity

Once barnacles are fixed in place, they cannot detach again (Crisp, 1955). All species grow faster in early life and slower later; in addition chthamalids, like others barnacles, become tubular in shape when crowded (Southward and Crisp, 1965). The growth rate can be influenced by abiotic (temperature, light, current, exposure to wave action, tidal level and surface contour) and biotic (food supply, population density, competing organisms, parasites, reproduction, size, species and age) factors (Crisp and Bourget, 1985).

Burrows (1988) observed that rates of growth of *Chthamalus montagui* were remarkably consistent over gradients of both tidal height and exposure to wave action. Growth was found to virtually cease during the winter and begin again the following spring (Burrows, 1988). This evidence could be explained through the slowing effect of low temperatures on cirral activity and consequently on food intake.

Photographic monitoring over a twenty year period of selected groups of chthamalids along the Ligurian coast (Italy) showed that on relatively sheltered shores, specimens of *C. montagui* survived for at least 10 years (Pannacciulli, 1991).

2.6 Genetic approaches to taxonomy and phylogeny of barnacles

Phylogenetic analysis aims to discover the evolutionary relationships among organisms. The level of similarity in genetic data reflects that of relatedness among organisms, from single individuals to populations and higher taxonomic levels (Procaccini and Maltagliati, 2004). Theory predicts that in two related organisms, neutral mutations on DNA sequences accumulate linearly since their last common ancestor (Kimura, 1983). This assumption gives the basis for the molecular clock hypothesis (Zuckerkandl and Pauling, 1965; Arbogast *et al.*, 2002) which, calibrated with data coming from paleontological and morphological analyses, allows the dating of phylogenetic relationships among organisms.

Genetic analyses have been used extensively to investigate various aspects of barnacle taxonomy (Dando, 1987). In particular, some species complexes of the genus *Chthamalus* were resolved using allozyme electrophoresis. Dando *et al.* (1979) compared the electrophoretic mobility of thirteen enzymes in *Chthamalus stellatus* and *Chthamalus montagui*. They showed that the two species differed entirely for the mobility of eight enzymes and had species-specific alleles for a further four. The separation of the two species, initially based on morphological and ecological

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characters (Southward, 1976), found further support in the genetic studies. Power *et al.* (1999) using mtDNA (COI and COII)-RFLP analysis from selected adults and cyprids of *C. stellatus* and *C. montagui*, showed clear differentiation between the two species and confirmed the use of size to distinguish cyprids of the two species.

Recently, Perez-Losada *et al* (2008) combined morphological data with DNA sequencing from 3 nuclear genes (18S, 28S and H3) for almost all families of Thoracica, to assess the tempo and mode of barnacle evolution. They rearranged thoracican classification based on estimated phylogenetic relationships.

Tsang *et al.* (2007) elucidated the taxonomic status of the acorn barnacles *Tetraclita japonica* and *Tetraclita formosana* using mtDNA sequencing (COI and Control Region) combined with morphological data.

A phylogenetic study of chthamaloids (Cirripedia; Thoracica; Chthamaloidea) was carried out by Fisher *et al.* in 2004. Partial sequences of the mitochondrial genes COI and 16S rDNA were analysed in eight taxa of chthamaloid barnacles. Phylograms based on 16S rDNA were generally consistent with the traditional phylogeny of the Chthamaloidea. It is interesting to highlight that slight differences in 16S sequence diversity between *Chthamalus stellatus* from Plymouth (UK) and Israel were shown; these results are in agreement with the trend in genetic diversity, in terms of allozyme heterozygosity and allelic diversity, between Mediterranean and Atlantic populations found by Pannacciulli *et al.* (1997a).

The analyses of COI by Fisher *et al.* (2004) resulted in phylogenetically meaningless trees, with most branches stemming from the common basal node. It was suggested that in the chthamaloids, at least at inter-generic level, polymorphism in the COI gene was saturated due to the fast rate of nucleotide substitutions that did not affect the encoded protein, and thus could not resolve the relationships within this superfamily.

2.6.1 Markers for taxonomy and phylogeny of barnacles

To summarize, DNA sequencing has been the preferred technique used in phylogenetic studies. The DNA regions most examined in barnacles were: COI, COII, Control Region, 12S and 16S for the mtDNA (Power *et al.* 1999; Fisher *et al.*, 2004; Simon-Blecher *et al.*, 2007; Tsang *et al.*, 2007); 18S, 28S, H3 in the nDNA (Perez-Losada *et al.*, 2008). Earlier, allozymes were employed for taxonomical studies (Dando *et al.*, 1979; Dando, 1987).

2.7 Population genetics of barnacles

Studies of genetic variability at population level allow the understanding of genetic relationships among individuals within a population and between populations over time and space (Procaccini and Maltagliati, 2004). I focused my attention on population genetic studies of barnacles and on the markers employed to assess them.

Many projects have focused on population genetics: they studied "how Mendel's laws and other genetic principles can be applied to entire populations" (Hartl et al., 1989).

A population is a group of organisms of the same species that is relatively isolated from other such groups; individuals of the same population frequently interbreed but individuals in different populations rarely or never do. Genetic differences can thus accumulate between populations, and can be characterised in terms of allele or haplotype frequencies. The main evolutionary forces that can cause divergence in allele/haplotype frequencies are selection, genetic drift and mutation. The first of these can be related to habitat characteristics, and the second to effective population size. Gene flow between populations will reduce differentiation and will relate to migration rate and to species dispersal capabilities (Carvalho, 1998; Procaccini and Maltagliati, 2004). A general association exists thus between mode of dispersal and the degree of genetic differentiation among populations (Bohonak, 1999).

In marine systems, larval dispersal ability has often been hypothesized as the primary determinant of population genetic structure (Burton, 1983;

Hedgecock, 1986; Palumbi, 1995). As Todd (1998) argued, analyses of population genetic heterogeneity provide an indirect mean of assessing the scale of realized larval dispersal and whether or not populations are demographically open or closed over a given range.

For species with a long-lived planktonic larva, such as barnacles, the population structure on the large scale (10 to 100 km) is the product of the interaction between selection and present or historical patterns of gene flow (Hilbish, 1985; Reeb and Avise, 1990; Karl and Avise, 1992; Holm and Bourget, 1994).

A study of asymmetric migration events across the marine biogeographic boundary of Point Conception (California, USA) was carried out by Wares *et al.* (2001). Patterns of genetic diversity were investigated, using mitochondrial DNA (mtDNA) Cytochrome Oxidase I (COI) sequence data, for the barnacles *Balanus glandula* and *Chthamalus fissus*, to test the hypothesis that gene flow in a species with a pelagic larva may be limited by temperature gradients and ocean currents that can affect species distributions by erecting barriers to dispersal of planktonic larvae. The tested hypothesis was confirmed by the results of the cladistic analyses.

Many studies have investigated phylogeographic relationships among populations. A well-defined pattern of genetic variation, using mtDNA sequence data, was found in the goose barnacle *Pollicipes pollicipes* within its distribution range in the North East Atlantic (Quinteiro *et al.*, 2007); coastal currents and mesoscale hydrodynamics were identified as the main factors determining the population genetic structure.

York *et al.* (2008), using a combined approach of mtDNA sequence and microsatellite data, examined both historic and contemporary processes that shaped the biogeography of the barnacle *Catomerus polymerus* in Australia. The mtDNA data provided information regarding the history and origin of the current populations, showing a deep phylogeographical split within southern Australia, strongly correlated with a phylogeographical barrier in the Bass Strait region: the emergence of the Bassian Isthmus, during glacial periods, promoted allopatric speciation in the southern Australian marine environment. The microsatellite data, instead, gave the opportunity to examine the species' contemporary structure, indicating significant genetic divergence between the eastern and western lineages of *Catomerus polymerus* and suggesting the presence of four subregions.

A deep genetic cline between southern (from about Monterey Bay southward) and northern (from northern California through Alaska) populations of the barnacle *Balanus glandula* was described by Wares *et al.* (2005). COI mtDNA sequence data, combined with coalescence estimators of the separation time for these two regions, suggested that a late Pleistocene event, more than 100.000 years ago, could have been responsible for the initial separation that was later maintained by oceanographic mechanisms or natural selection.

Dufresne *et al.* (2002) compared patterns of genetic structure at potentially selected loci (two allozymes) and neutral molecular markers (six microsatellites) in the barnacle *Semibalanus balanoides* from the Gulf of St. Lawrence to assess the possible role of selection and gene flow in the

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genetic structuring of barnacle populations on a large geographical scale. Results supported the hypothesis that the broad scale pattern of allozyme allelic shifts is maintained by selection. Other studies confirm that selection can be strictly related to habitat characteristics (Procaccini and Maltagliati, 2004).

In *Semibalanus balanoides* a close relationship between genotype frequencies at the Mannose-6-Phospate Isomerase (MPI) locus and the degree of physical stress experienced by barnacles in distinct habitat types on the shore has been well documented (Schmidt and Rand, 1999). Furthermore, it was demonstrated that in *S. balanoides* selection acts over a short time interval within the life cycle, occurring between metamorphosis and early juvenile stages (Schmidt and Rand, 2001).

Population genetic studies have also been carried out to investigate the origins and sources of barnacle invasions in geographical areas outside the species' natural range.

Zardus *et al.* (2005) investigated the origins and incursions of the Atlantic barnacle *Chthamalus proteus* in the Pacific. They characterized genetic variation in native and introduced populations, by using direct sequencing of mtDNA (COI), and tried to match regions to determine if there were multiple geographical sources and introduction points for this barnacle.

Sources of invasion of the north eastern Pacific barnacle *Balanus glandula* in Japan and Argentina were examined by Geller *et al.* (2008) by comparing COI and EF1 (Nuclear Elongation factor 1 alpha) genotypes of native vs.

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introduced populations. The same markers were used by Simon-Blecher *et al.* (2008) to trace the origin of this barnacle species along the south western African shores.

Other population genetic studies were carried out to assess the effects of pollution on organism genotypes. Patarnello *et al.* (1991) investigated, by electrophoretic analysis of the polymorphic loci MPI, PGI and PGM, the effects of thermal and chemical pollution on the genetic structure of *Balanus amphitrite* populations from Venice lagoon. Data confirmed that selection is mainly attributable to heavy metals.

Finally, focussing the attention on our target species, *Chthamalus montagui*, it has to be mention that the existence of Atlantic and Mediterranean forms of *Chthamalus montagui* was established by analyzing the polymorphism of 15 allozymes (Dando and Southward, 1981). The results of this study were confirmed by Pannacciulli (1995) and Pannacciulli *et al.* (1997a), who investigated the population genetic structure of *Chthamalus montagui* and *Chthamalus stellatus* over their North-East Atlantic and Mediterranean distribution, using six and four allozymes respectively. Electrophoretic data were analysed using a technique based on Wright's *F*-statistics (Pannacciulli *et al.*, 1997b). In each species a single locus showed marked differentiation between Atlantic and Mediterranean localities.

2.7.1 Markers for population genetics of barnacles

Many different markers were applied in the population genetic studies mentioned above.

Allozymes, such as MPI, PGI, PGM, GOT, PEP etc., have been employed extensively in studies of geographical population differentiation and gene-flow (under the general assumption of neutrality) and taxonomy (Dando and Southward, 1981; Patarnello, 1991; Pannacciulli *et al.*, 1997a; Schmidt and Rand, 1999 and 2001).

The most common DNA-based markers utilized were microsatellites (Dufresne *et al.*, 2002; York *et al.*, 2008) and the COI locus of the mtDNA (Wares *et al.*, 2005; Zardus *et al.*, 2005; Quinteiro *et al.*, 2007; Geller *et al.*, 2008; Simon-Blecher *et al.*, 2008). MtDNA is considered to be a good marker for the examination of historical patterns, while the rapid rate of mutation of microsatellites make them ideal for investigating contemporary population structure and the ecological factors responsible (York *et al.*, 2008). In 2001 Chu *et al.*, explored the possibility of using the first internal transcribed spacer (ITS-1) of ribosomal DNA as a molecular marker for studying inter- and intra-specific genetic variation in crustaceans. The outcome of this work showed that ITS-1 is highly divergent among different crustaceans and could be an appropriate marker for molecular systematics at species and population level.

The role of selection in maintaining genetic polymorphisms at allozyme loci has been reported in many marine species. It is not clear whether the allozyme loci themselves or other loci, in close linkage with allozymes, are the targets of selection (Mitton, 1998). Dufresne *et al.* (2002) suggested comparing patterns of genetic structure at putatively selected loci with those obtained from more neutral markers such as mtDNA and/or microsatellites. Gene flow and drift should equally affect all neutral loci,

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whereas selection is more likely to be locus-specific (Lewontin and Krakauer, 1973). When they are not linked to selected genes, microsatellite markers are potentially good markers for analyses assuming that they are located mostly in non-coding regions (Queller *et al.*, 1993). Nevertheless, Dufresne *et al.* (2002) indicated that microsatellites may not always behave in a neutral way and have to be used cautiously, especially when evidence for genetic structuring relies on only a few assayed loci without a priori knowledge or a comparison with potentially selected loci. In response to these considerations, many studies have been carried out using a multi-marker approach (Schmidt and Rand, 1999, 2001; Rand *et al.*, 2001; Dufresne *et al.*, 2002; Simon-Blecher *et al.*,2008; York *et al.*, 2008).

Isolation and characterization of microsatellite markers in the acorn barnacle *Semibalanus balanoides* were carried out by Dufresne *et al.* (1999). Later, seven highly polymorphic microsatellite markers were isolated from *Chthamalus montagui* by Pannacciulli *et al.* (2005). This work was very useful for this PhD, as the markers found direct application in the population genetic studies of this project.

Chapter 3

Materials and methods

3.1 Fieldwork

In the main tasks of this project different sampling areas were selected and locations identified as explained in detail in the specific chapters. However, a common sampling strategy was adopted to collect *C. montagui* for genetic analysis.

At each location, 3 sites were randomly selected, 70-150 metres one from the other. At each site, 50 to 100 specimens were collected from that part of the shore where *C. montagui* was most abundant. However, the full number of sites and specimens sampled could not generally be analysed because of constraints on time and resources.

The experimental design is represented in Figure 3.1.
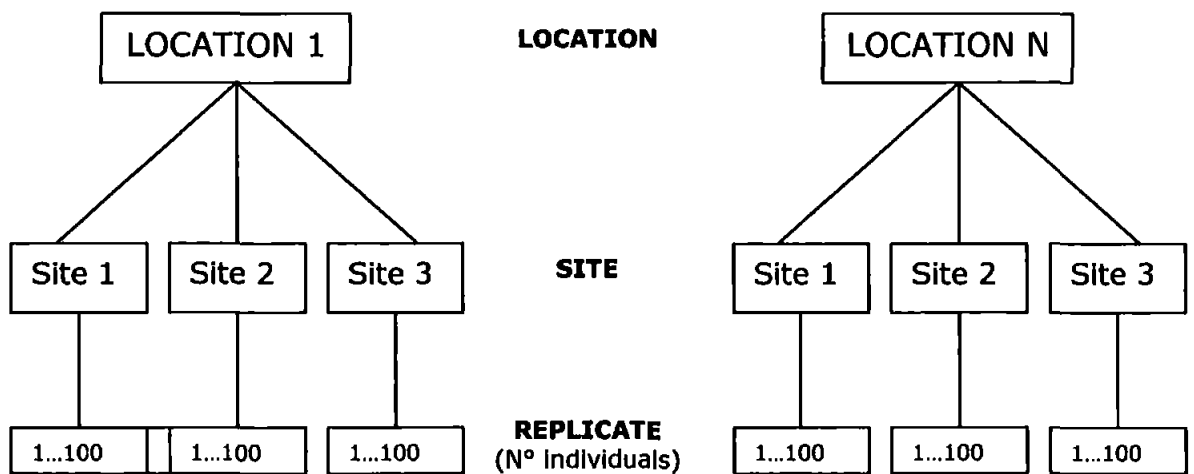
Figure 3.1- Experimental design for sampling.

During fieldwork, specimens complete with shell and soft tissue were scraped off the substratum and immediately preserved in tubes containing

ethanol (95% EtOH). Several individuals from the same site were stored in the same tube, which was labelled with the name of the location, site and date of sampling. Upon arrival in the laboratory, samples were kept at - 20°C until dissection and genetic analysis.

3.2 Laboratory work

3.2.1 DNA extraction

DNA was extracted from the soft tissue of each individual. The whole animal was pulled out with a pair of forceps from the bottom part of the shell, the side that attaches to the substratum, making sure not to include gonads or egg masses that are often present in the mantle cavity.

The following techniques for DNA extraction were tested to find the most suitable one for *C. montagui*: Phenol-Chloroform standard protocol (Sambrook *et al.*, 1989), DNA extraction kits (Qiagen DNAeasy Extraction Kit and Gentra PureGene), guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987) and Salting-Out extraction (Aljanabi and Martinez, 1997).

The quantity and quality of the extracted DNA was assessed by Tris Borate EDTA (TBE, recipe in Appendix 3.1) agarose gel electrophoresis (1% agarose) and visualised by fluorescence after staining with Ethidium Bromide (electrophoretic run of about 30 min at 100 mA). DNA concentration was roughly estimated by comparing the intensity of the DNA bands with those of a 100 bp ladder (New England BioLabs) and appropriate dilutions were established. The quality and dilution of the extracted DNA

was further tested by verifying the outcome of the PCR amplification employing microsatellite markers.

After screening, the Salting-Out extraction method (modified after Aljanabi and Martinez, 1997) was selected as the most suitable for *Chthamalus montagui* specimens as it gave very good results, it was cheap and safe to handle.

DNA extractions were carried out following the protocol listed in Appendix 3.2. DNA was extracted from 32 individuals at each site investigated in the project.

3.2.2 Microsatellite markers

Microsatellites were employed as molecular markers for the population genetic studies. The different tasks of the project employed a variable number of microsatellite loci, three to six, to detect genetic variation. These microsatellites were developped specifically for *Chthamalus montagui* by Pannacciulli *et al.* (2005) and proved to be highly polymorphic. Details of the six loci employed are reported in Table 3.1.

Table 3.1 - Microsatellite loci with core repeats, primer sequences, optimal annealing temperature (TA), GeneBank accession number (from Pannacciulli *et al.*, 2005).

Locus	Repeat in clone	Primer sequence (5'-3')	TA (°C)	GeneBank n°
CM2/15	$(CA)_{10}$	F: GTACGAGTGGCTTCGCTTG R: ATGTTTGCCATGATGTAGGC	55	AY847005
CM 4/5	$(AC)_{29}TCGC(AC)_{29}$	F: TGCTCACAAACCATGACTGG R: ACGCTAAAGAGGCACATTCG	45	AY847007
CM 5/23	$(AC)_3(GC)_8(AC)_{36}$	F: CGCTCTCCCTCAAAACTCC R: ACCGTGAGTAACAGCATACG	50	AY847010
CM 4/3	$(GT)_{16}AT(GT)_2$	F: TGCTCACAAACCATGACTGG R:ACGCTAAAGAGGCACATTCG	50	AY847006
CM 5/18	$(TG)_2TA(TG)_2TT(TG)_{13}$	F: TGATTGTCGGTTTAGCAACG R: TCACTGTGAAGTCTGACATGG	45	AY847009
CM 9/11	$(GT)_{13}AG(GT)_{10}$	F: ACAAGCCTTCGCCTCATTCC R:CCCACTTATCAACGCTTCTGC	60	AY847012

3.2.3 PCR reaction, DNA sequencing and fragment analysis

DNA amplification was performed by Polymerase Chain Reaction (PCR) using two different Master Mixes, depending on the TAQ Polymerase available. The two Master Mixes differed as one employed Qiagen HotStar Taq DNA Polymerase (Table 3.2), Q solution and appropriate buffer (with

DMSO etc.), while the other used Sigma JumpStart Taq DNA Polymerase (Table 3.3).

Table 3.2: Master Mix employing Qiagen HotStar Taq DNA Polymerase and Q solution.

	Initial concentration	Amount for sample (µl)	Final concentration
Water		6.2	
Buffer*	10 X	1	1 X
Q solution	5 X	0.7	0.35 X
dNTPs	2.5 mM	0.8	0.2 mM
Primer F	100 µM	0.1	1 µM
Primer R	100 µM	0.1	1 µM
DNA	25-50 ng/µl	1	2.5-5 ng/µl
Hot Star Taq	5 U/µl	0.05	0.025 U/µl
Final Volume		10 µl	

*Buffer contained 15 mM MgCl$_2$

Table 3.3: Master Mix employing JumpStart Taq DNA Polymerase (Sigma).

	Initial Concentration	Amount for sample (µl)	Final Concentration
Water		6.9	
Buffer*	10 X	1	1 X
dNTPs	2.5 mM	0.8	0.2 mM
Primer F	100 µM	0.1	1 µM
Primer R	100 µM	0.1	1 µM
DNA	25-50 ng/µl	1	2.5-5 ng/µl
Jump Star Taq	2.5 U/µl	0.1	0.025 U/µl
Final Volume		10 µl	

*Buffer contained 15 mM MgCl$_2$

The amplification, carried out on an ABI 3700 thermocycler, consisted of an initial denaturation at 95°C for 15 min, 33 cycles of denaturation at 95°C for 30 sec, annealing for 30 sec at a locus-specific temperature and extension at 72°C for 30 sec, followed by a final step of 72°C for 10 min.

Details of the primer pairs, including annealing temperatures, are listed in Table 3.1.

To allow later screening of the PCR products on a sequencer, one member of each primer pair employed in the PCR reaction was end-labelled with a fluorophore (6-FAM, HEX or NED, Applied Biosystems). In detail: CM 2/15 and CM 5/18 primers were labelled with NED (yellow); CM 4/5 and CM 4/3 with HEX (green) and CM 5/23 and CM 9/11 with 6-FAM (blue). The choice of fluorophore for each primer was made on the basis of the expected amplified product size. In fact, pairs of primers that delivered products of similar sizes were labelled differently. Pairs of primers that, instead, produced products of clearly different sizes could be labelled with the same colour and potentially be run in the same sequencing gel, while still allowing separate pattern interpretation.

Prior to the sequencer run, PCR products were checked and visualised on TBE agarose gel (1.5 %) after staining with Ethidium Bromide using a 500 bp ladder (New England BioLabs) as reference (electrophoretic run of about 1.30 h at 100 mA).

PCR products were then diluted (from 1:20 up to 1:70) and, for each individual, products from the following sets of primers, marked with different fluorophores, were combined in one of the 96 wells of a sequencing plate: CM 2/15 (NED), CM 4/5 (HEX) plus CM 5/23 (6-FAM); and CM 5/18 (NED), CM 4/3 (HEX) plus CM 9/11 (6-FAM). This strategy was adopted to make the best use of the 4 capillary system of the 3100 Avant ABI capillary DNA sequencer employed in this study. In this way it

was possible to get information on three different microsatellites in the same run. All runs also included a reference standard, the 500 bp ROX ladder (Applied Biosystems). Fragment lengths were detected and analysed using GENE Scan software (ABI). Single peaks were measured directly while, when stutter bands were present, only the highest peak was taken into account. For each locus, all data resulting from the analysis of all individuals in the study and from several sequencing runs were plotted on a size-frequency graph, recording values to the first decimal point. This allowed grouping of data that, despite minor differences in size, represented a single allele. In this way allele sizes were established at each locus. When an allele was represented a single time or only a very few times, the sequencing gel was checked again to confirm the existence of the allele.

3.3 Genetic data analysis

Genetic data analysis was carried out using software packages specific for population genetic studies. Table 3.4 lists the main software employed.

Table 3.4:Main software employed in the genetic data analysis.

Software	Version	Reference
GENETIX	4.03	Belkhir et al., 1996
ARLEQUIN	3.1	Excoffier et al., 2005
GENEPOP	4.0	Rousset, 2008
FSTAT	2.9.3	Goudet, 2001
RSTCAL	3.12	Goodman, 1997
MICROSAT	1.5 d	Minch et al., 1996
PHYLIP	3.68	Felsestein, 2005
HIERFSTAT	0.04-3	Goudet, 2005
STRUCTURE	2	Pritchard et al., 2000
STATISTICA	6.1	Statsoft Inc., 1997

Below is a brief overview (following Procaccini and Maltagliati, 2004) of the main parameters calculated for genetic data analysis.

Fit to Hardy-Weinberg equilibrium

The Hardy-Weinberg law (Hardy, 1908; Weinberg, 1908; Stern, 1943) predicts that under stable conditions after a generation of random mating, genotype frequencies throughout a population at a specified gene reach a specific equilibrium value. This value can be defined by the square of the allelic frequencies.

If we consider that there are only two alleles (A and a) with allele frequencies respectively of p and q, the three possible genotypes are AA, Aa, aa and their frequency follows the binomial expansion of:

$$(p+q)^2 = p^2 + 2pq + q^2 = 1$$

The frequency of the genotype AA will be p^2, Aa will be 2pq, and aa will be q^2; populations are considered to be in Hardy-Weinberg equilibrium when homozygotes and heterozygotes are in these proportions. The assumptions for equilibrium are that the population is idealized: infinite in size to eliminate genetic drift, sexually reproducing, randomly mating and diploid. Moreover, gene pool frequencies will remain unchanged if no mechanisms that can cause evolution are acting on the population: in other words mutation, selection and migration should not occur. Hence, a large, randomly-mating, non-evolving population should follow the Hardy-Weinberg law (Ayala, 1982).

Heterozygosity

Heterozygosity is often one of the first parameters calculated from a data set as it can tell a great deal about structure and even history of a population. For instance, very low heterozygosity values can indicate severe effects of small population sizes (population bottlenecks or metapopulation dynamics that severely reduce the level of genetic variation compared to what is expected). In particular the expected heterozygosity, H_E, or gene

diversity, D, as Weir (1990) called it, is the most extensively used descriptor of the within-population genetic variability. The simplest way to calculate expected heterozygosity for a single locus is as follows:

$$H_E = \sum_{i=1}^{K} p_i^2$$

where p_i is the frequency of the ith of k alleles, while across loci the mean heterozygosity is normally employed. Heterozygosities of two populations can be compared statistically by t-test after arcsin square root transformation of single-locus values (Archie, 1985).

F-statistics

In population genetics "F" stands for "fixation index", with fixation being increased homozygosity resulting from inbreeding. Wright (1951; 1978) developed three fixation indexes to evaluate population subdivision: F_{IS}, F_{ST} and F_{IT}.

F_{IS} is a measure of the deviation of genotypic frequencies from panmictic frequencies in terms of heterozygote deficiency or excess. It is what Weir and Cockerham (1984) denominated as the inbreeding coefficient (f), which is conventionally defined as the probability that two alleles in an individual are identical by descent (autozygous). It is calculated in a single population

as
$$F_{IS}= 1-\left(\frac{H\,obs}{H\,exp}\right)$$

where H_{obs} is the observed heterozygosity (the number of heterozygous individuals divided by the total number of individuals) and H_{exp} is the expected heterozygosity, calculated on the assumption of random mating. Negative F_{IS} values indicate excess (outbreeding) and positive values indicate deficit (inbreeding) of heterozygosity compared with Hardy-Weinberg law expectations.

F_{IT} is rarely used. Called by Weir and Cockerham (1984) total inbreeding coefficient, it estimates the correlation of uniting gametes relative to gametes in the pooled population.

F_{ST} measures the reduction in heterozygosity due to population subdivision. It was called the coancestry coefficient (θ) by Weir and Cockerham (1984) and is defined as a correlation of gametes within subpopulations relative to gametes drawn at random from the entire population. F_{ST} is always positive or equal to 0; 0 signifies panmixia (no subdivision, random mating occurring, and thus no genetic divergence within the population). On the contrary, $F_{ST}=1$ means complete isolation (extreme subdivision). F_{ST} values up to 0.05 indicate negligible genetic differentiation whereas >0.25 mean great genetic differentiation among the analyzed populations. Slatkin (1995) developed an estimator of F_{ST} (denominated R_{ST}) for microsatellites.

Genetic subdivision among populations was quantified using both F-statistics (Weir and Cockerham, 1984) and R-statistics (Slatkin, 1995), considering that the first is a measure of heterozygote deficiency among populations following the Infinite Allele Model (IAM) while the second is based on allele size differences and follows a Stepwise Mutation Model (SMM) more suitable for microsatellites. The use of both estimators in this project is justified by the fact that few microsatellites strictly follow the SMM while most of them fit an intermediate mutation model between IAM and SMM (Di Rienzo *et al.*, 1994).

F_{ST} can also be used to estimate gene flow among populations:

$$N_m = 0.25 \left(\frac{1 - F_{st}}{F_{st}} \right)$$

where N_m is the N number of effective migrants (those that reproduce in the population into which they migrate) per generation (Wright, 1943; Slatkin, 1987).

Linkage disequilibrium

Linkage disequilibrium (LD) will tend to arise between two loci on the same chromosome; as a result of linkage, specific alleles at the two loci are found together more or less frequently than might be expected by chance. The same situation may exist for more than two alleles. The magnitude of the effect is expressed as the delta (D) value and corresponds to the difference between the expected and the observed haplotype frequencies. It can have positive or negative values. LD is decreased by recombination. Thus, it decreases every generation of random mating unless there is some process opposing the approach to linkage equilibrium. Permanent LD may result from natural selection if some gametic combinations are in higher fitness than other combinations.

Genetic distance

Genetic distance is a measure of genetic relatedness of populations. The estimate is based on the number of allelic substitutions per locus that have occurred during the separate evolution of two populations. A number of genetic distance indices have been developed (Nei, 1972; Rogers, 1972; Tajima and Nei, 1984; Tamura, 1992; Goldstein *et al.*, 1995 etc). The need for different indices arises from different statistical assumptions, mutation models and/or genetic markers employed. In particular, the index proposed by Goldstein *et al.* (1995) is specific for DNA microsatellite markers, under the assumption of evolution according to the Stepwise Mutation Model.

Genetic distance-based methods of phylogenetic tree inference

There are numerous variations of genetic distance-based methods (UPGMA, Neighbor-joining, Fitch-Margoliash, etc.) which all rely on generating a matrix of pairwise differences (or distances) or similarities. In its simplest form, one could merely count the number of differences between all possible pairs of sequences, and then use a clustering algorithm to cluster together those sequences with the smallest number of differences.

Bootstrapping

The bootstrap (Efron, 1979) is a general re-sampling technique for estimating the distribution of statistics. The basic idea behind bootstrap concerns the estimation of variance in an unknown distribution from which data are re-sampled randomly. The bootstrapping procedure constructs a number of arbitrary data sets. Re-sampling is performed "r" times (usually $1,000 < r < 100,000$) obtaining r estimations of the parameter that is calculated. One of the applications of bootstrap in population genetics is the estimation of confidence intervals for parameters such as F_{IS}, F_{IT}, F_{ST}, N_m, etc. Another important application is the assessment of robustness of the nodes of phylogeographic trees. In this approach, instead of a set of values x1, x2..., xn, the starting point is a table of populations scored for a set of characters (e.g. type of alleles, allelic frequencies). The procedure consists of bootstrapping across characters, hence re-sampling and replacing characters of the original data matrix at random, and calculating the tree for each re-sampling. Bootstrap values annotated on the nodes of the tree representing the total data set indicate the percentage of re-samplings in which a given node has been generated.

Jackknifing

The function of jackknifing is similar to bootstrapping, to generate a distribution for statistics that permits the calculation of dispersion estimates. The distribution is generated by re-sampling the original data set with replacement to produce "pseudoreplicate" data sets; jackknifing produces a limited number of pseudoreplicates, each containing all but one of the original data elements. For a data set with 20 elements, 20 pseudoreplicate data sets will be generated, each lacking a different data element.

Mantel's test

The Mantel's test (Mantel, 1967) assesses the probability that two distance matrices are independent from one another; it has been used to detect isolation by distance among populations. In this approach the matrices of the geographic distances and of genetic distances among populations are compared.

Analysis of molecular variance

Analysis of molecular variance (AMOVA, Excoffier *et al.*, 1992) allows the partitioning of molecular variation into hierarchical levels, for which the significance of departures from the null hypothesis can be tested. AMOVA is based on the proposal that the sum of squares in conventional analysis of variance can be expressed as the sum of squared distances between pairs of phenotypes (Hoeffding, 1948).

A hierarchical AMOVA partitions the total variance into covariance components due to intra-individual, inter-individual, and/or inter-population differences.

Bayesian clustering analysis

The Bayesian clustering method developed by Pritchard *et al.*, 2000 was used to infer population structure. This approach differs from the analyses described above as individuals are assigned to clusters (populations) based exclusively on their individual genotypes. Individual genotypes are assigned to clusters on a probabilistic basis, which identifies the affinities of individual multilocus genotypes to genetic populations and aims to delineate populations that are, as far as possible, in Hardy-Weinberg and linkage equilibrium (Evanno *et al.*, 2005).

The details of the statistical data analysis carried out in the different tasks of the project are explained in the following specific chapters.

Chapter 4

The influence of habitat corridors generated by artificial substrates on the genetic pattern of *Chthamalus montagui*

same time, in the introduction of hard-bottom habitats with the occurrence of new species in the area (Airoldi et al., 2005).

These changes in species composition, abundance and diversity represent a substantial modification of the characteristics of the native biotope (Angermeier, 1994) and can have important consequences in the functioning of the coastal ecosystem (Loreau et al., 2001).

In fact, the system of artificial structures provides novel habitats for the colonization of marine organisms and often supports the invasion of non-indigenous species (Holloway and Keough 2002; Lambert and Lambert 2003; Thornber et al. 2004). Bulleri and Airoldi (2005), for instance, studied the distribution and the dynamics of the spread of a non-indigenous green alga, Codium fragile sp. tomentosoides, at a variety of spatial scales on the breakwaters along the north-east Adriatic coast. They identified some of the mechanisms of its successful establishment; experiments indicated that the sheltered habitat on the landward side of the breakwaters offered particularly favourable conditions for the growth of this macroalga, promoting its spread along formerly exposed sandy shores. Therefore, these structures offer suitable habitat for species living on hard substrata (among them also invaders) and enhance their further spread, by functioning as "corridors" across areas of unsuitable habitat (e.g. across sandy or muddy areas). Thus artificial substrates may act as stepping stones, disrupting natural barriers and facilitating the dispersal of a species across habitats and regions that naturally would be poorly connected (Abbiati, 2003).

4.1 Introduction and specific aims

In the last centuries, humans have become a major force able to change landscapes at a variety of spatial and temporal scales (Ayensu et al., 1999). This is also true for coastal areas, where the population is steadily increasing and the worldwide trend is towards urbanisation (Gray, 1997).

Artificial structures, such as pier pilings, floating pontoons, breakwaters and seawalls have become common features of landscape in shallow coastal waters of urbanised areas and in some areas they have replaced considerable portions of natural habitats (Chapman and Bulleri, 2003).

Along most European coasts hard defence structures to protect coastal areas, have proliferated leading to severe alteration of the coast. Under future global climate change scenarios, the intensity of storms and height of sea level are predicted to increase, augmenting the need for introducing these coastal defence structures (Airoldi et al., 2005). In some regions, for instance the Italian Adriatic coast, they already cover over half of the shoreline, resulting in dramatic changes to the coastal landscape and environment (Bacchiocchi and Airoldi, 2003).

The type and magnitude of the changes induced by these structures can vary considerably depending on the environmental setting where they are built. However, the construction of these hard defence structures in areas dominated by sandy shores always results in a local loss of soft-bottom habitats with the associated assemblages of animals and plants and, at the

same time, in the introduction of hard-bottom habitats with the occurrence of new species in the area (Airoldi et al., 2005).

These changes in species composition, abundance and diversity represent a substantial modification of the characteristics of the native biotope (Angermeier, 1994) and can have important consequences in the functioning of the coastal ecosystem (Loreau et al., 2001).

In fact, the system of artificial structures provides novel habitats for the colonization of marine organisms and often supports the invasion of non-indigenous species (Holloway and Keough 2002; Lambert and Lambert 2003; Thornber et al. 2004). Bulleri and Airoldi (2005), for instance, studied the distribution and the dynamics of the spread of a non-indigenous green alga, Codium fragile sp. tomentosoides, at a variety of spatial scales on the breakwaters along the north-east Adriatic coast. They identified some of the mechanisms of its successful establishment; experiments indicated that the sheltered habitat on the landward side of the breakwaters offered particularly favourable conditions for the growth of this macroalga, promoting its spread along formerly exposed sandy shores. Therefore, these structures offer suitable habitat for species living on hard substrata (among them also invaders) and enhance their further spread, by functioning as "corridors" across areas of unsuitable habitat (e.g. across sandy or muddy areas). Thus artificial substrates may act as stepping stones, disrupting natural barriers and facilitating the dispersal of a species across habitats and regions that naturally would be poorly connected (Abbiati, 2003).

Increased connectivity among populations will increase the gene flow within a species (Palumbi, 2003). Coastal defence structures could have the reverse effect compared to fragmentation, by acting as "corridors" to facilitate gene flow among previously isolated populations. Increasing gene flow can have a homogenizing effect, reducing local adaptation within a species and thus, on a larger timescale, decreasing the evolution of new species (Palumbi, 2003).

The relative dispersal ability of a species should be considered when investigating the connectivity among populations in the marine environment and the function of habitat corridors on its movement and colonization patterns (Micheli and Peterson, 1999; Darcy, 2005).

Moreover, the study of habitat "corridors", which have been well documented theoretically and empirically in terrestrial systems (Henein and Merriam 1990; Merriam and Lanoue 1990; Haddad 1999; Tewksbury *et al.* 2002), is relatively new to marine environments. Some studies revealed that the definition of corridor has to be expanded in the marine domain, where not only stationary habitat patches but also rafting on living pelagic organisms and drifting on inanimate objects can act as a corridor (Carlton, 1985; Irlandi and Crawford, 1997; Micheli and Peterson, 1999; Goodsell and Connel, 2002; Darcy, 2005). Brooks and Bell (2001), for example, demonstrated that drift algae provide a mobile corridor to epiphytic amphipods for dispersal within a seagrass landscape.

Naturally, effectiveness of artificial hard substrates as ecological corridors will depend on the suitability of the substrate as a settling environment for

rock dwelling organisms (Abbiati, 2003). Connell and Glasby (1999) examined subtidal epibiota on natural reefs and six common urban structures (sandstone retaining walls, fibreglass and concrete pontoons, concrete pilings, and wooden pilings both with and without bark) in Australia; they discovered that no one type of artificial substratum supported the same suite of epibiota as the natural reef. The differences between composition of substratum (sandstone versus concrete) and habitat type (rocky reef versus pontoon) were also explored (Connell, 2000). It was highlighted that these new artificial substrata do not function as complete analogues of natural rocky shores (Connell and Glasby, 1999).

Hence, the exact nature of artificial substrates might also affect population genetic structuring, since unsuitable substrates might not allow the establishment of viable populations. Clearly, the spacing and surface characteristics of these new substrata could both affect local or regional genetic biodiversity. Population genetic studies on this specific subject are quite rare in the literature.

In the EUMAR project the possible effects of artificial substrates on genetic biodiversity were tested on a suite of intertidal species having different presumed dispersal capabilities and life histories such as the periwinkles *Littorina littorea* and *Melarhaphe neritoides* (supposed to be long dispersed species) and the limpet *Patella caerulea* (short dispersing).

My task in the project was to investigate the "corridor effect" created by artificial substrates along the Adriatic coast on the population genetics of the barnacle *Chthamalus montagui*, by employing three microsatellite loci

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as molecular markers. Two types of locations were selected for this study: those with natural rocky shores and those offering only artificial substrata to settling barnacles. The two types of locations were interspersed to: a) allow investigations on the potential effect of artificial substrata acting as bridges between stretches of natural rocky shores, b) avoid geographical bias in the results when comparing artificial versus natural substrata. The study included also comparisons between barnacle populations from natural versus artificial shores to control for the selection that could be operated by the type of substratum.

An analogous population genetic study was carried out within EUMAR on the limpet *Patella caerulea* along the Adriatic coast, using allozymes and microsatellites. Moreover, a large-scale study on the effects of hard defence structures in the North Adriatic Sea was carried out using this limpet as target species (DELOS and EUMAR projects) combining field investigations and spatial population models (Airoldi *et al.*, 2005).

If population genetic differentiation is detected between natural shores linked by a potential artificial corridor in this study, this will imply that the artificial substrates are not acting as a corridor for genetic exchange. If, conversely, there is no genetic differentiation, this will be consistent with an effect of artificial substrates, although it will not allow a definite conclusion in the absence of historical data on levels of differentiation prior to the construction of the coastal defences.

4.2 Materials and methods

Study area

The north and central coasts of the Adriatic Sea are a flat, alluvional system that stretches almost uninterrupted for more than 300 km. The area is moderately exposed to wave action and the few limestone rocky shores occurring in the region generally offer few naturally sheltered habitats. The area is subject to a relatively large tidal excursion (about 80 cm) in comparison with other regions of the Mediterranean basin, and receives inputs of freshwater and nutrients from the Po river (Bulleri and Airoldi, 2005)

Severe erosion, together with poor coastal defence policies, has led to proliferation of artificial hard structures over more than 60% of the shoreline (Cencini, 1998). This has resulted in more than 190 km of breakwaters, groynes, seawalls and jetties. The construction of hard structures along the coast was particularly intense in the '70s and '80s, but is still ongoing (Bacchiocchi and Airoldi, 2003).

My fieldwork in the Adriatic covered a variety of coastal structures at several locations, from Trieste to Portonovo. The most frequent constructions, built with large blocks of quarried rock (long axis ranging from 1 to 3 m), are set in two different ways: (1) perpendicular to the shore (groynes), intended to retain or arrest the long-shore movement of sediment and being on average about 150 m long and (2) parallel to the coast line (breakwaters) on shallow sediments at a distance of about 220 m from the shore, intended to reduce wave action and being 100 m long on average and separated one from the other by gaps of about 20 m. Both

these defence structures extend about 2-3 m above and below mean low water level (MLLW), thus providing both intertidal and subtidal habitats for marine life (Bacchiocchi and Airoldi, 2003).

Sampling

Sample collection of *Chthamalus montagui* specimens was carried out in February - March 2003 in the central and northern part of the Adriatic Sea (Table 4.1). Figure 4.1 shows the 10 sampling locations. Figure 4.2 represents the distribution of substrata in this coastal area, which is dominated by sandy beaches and for this reason is protected by artificial substrates.

At each location 3 sites were selected (70-150 metres one from the other). At each site 50 to 100 specimens were collected from that part of the shore where they were most abundant. A single site, or sometimes two sites, was analysed per location, with 32 individuals per site (although not all individuals were successfully analysed for each locus).
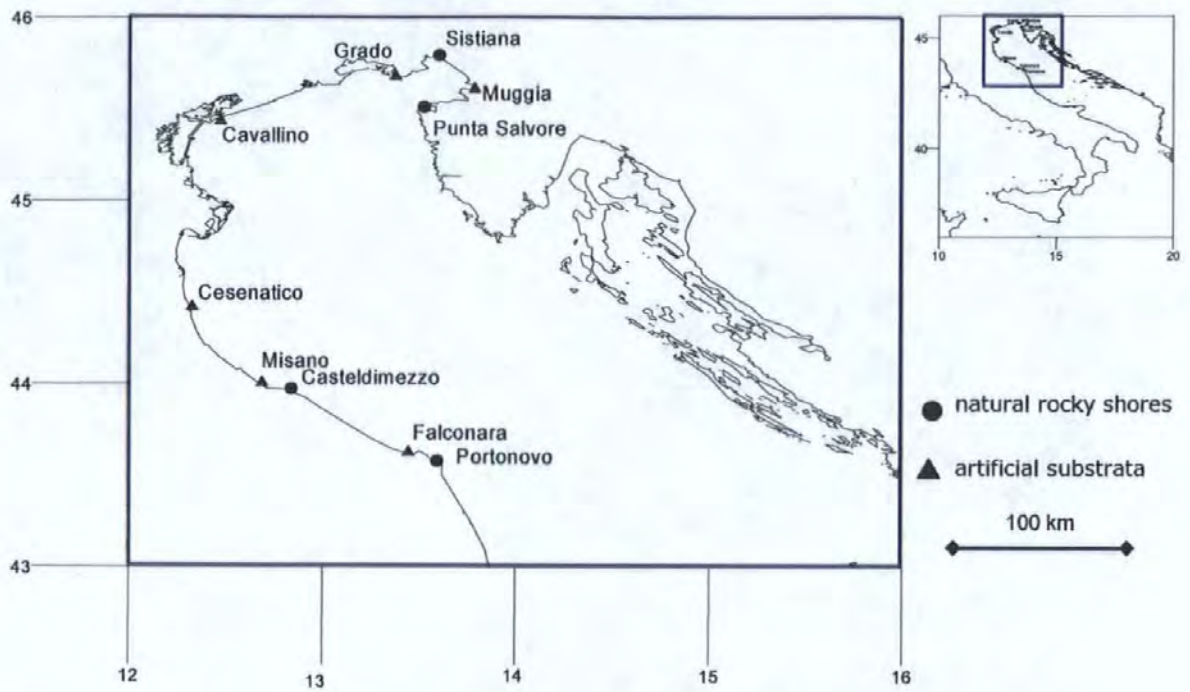
Figure 4.1 - Sampling locations in the central/northern Adriatic Sea.
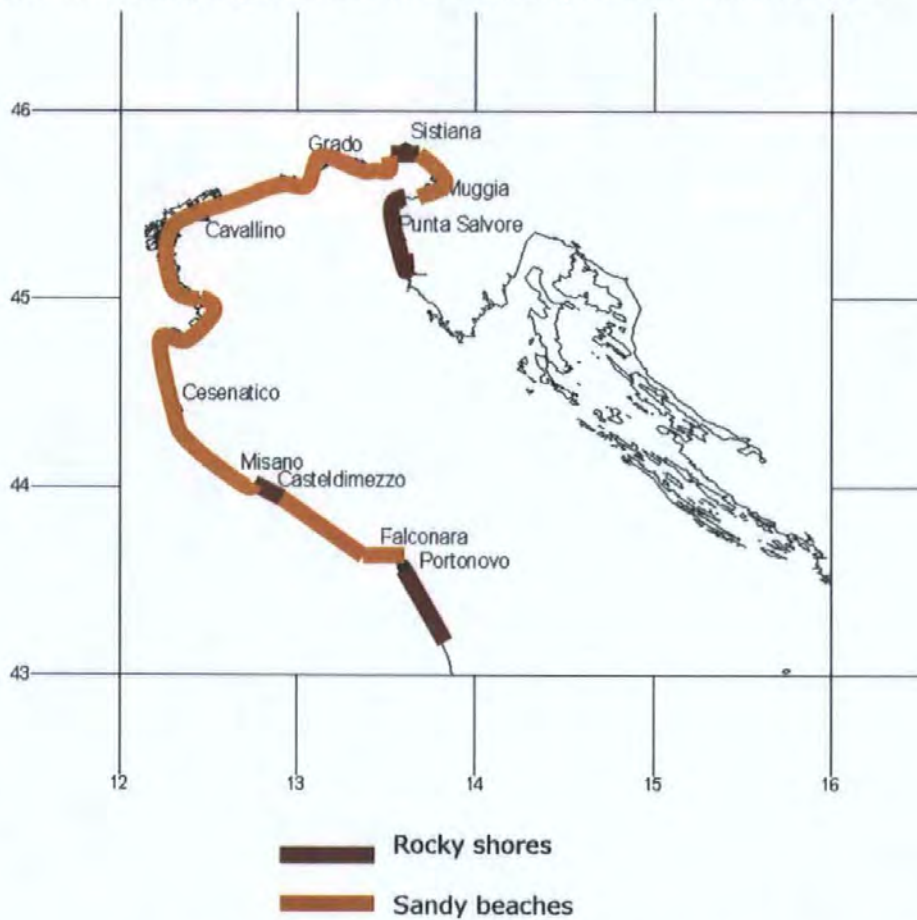
Figure 4.2 - Type of substratum characteristic of the central/northern Adriatic Sea

Table 4.1 – Sampling location, site used in the genetic analysis, abbreviation, geographical coordinates and type of substratum.

Sampling location	Site	Abbreviation	Geographical coordinates		Type of substratum
Punta Salvore	1	Pu1	13.51 E	45.51 N	Natural
Muggia	1	Mu1	13.76 E	45.61 N	Artificial
Sistiana	1	Si1	13.64 E	45.76 N	Natural
Sistiana	3	Si3	13.64 E	45.76 N	Natural
Grado	1	Gr1	13.39 E	45.67 N	Artificial
Grado	3	Gr3	13.39 E	45.67 N	Artificial
Cavallino	1	Cv1	12.55 E	45.47 N	Artificial
Cesenatico	1	Ce1	12.41 E	44.20 N	Artificial
Misano	1	Mi1	12.71 E	43.98 N	Artificial
Casteldimezzo	1	Ca1	12.80 E	43.96 N	Natural
Falconara	1	Fa1	13.39 E	43.64 N	Artificial
Portonovo	1	Po1	13.60 E	43.57 N	Natural
Portonovo	3	Po3	13.60 E	43.57 N	Natural

Laboratory work

Three microsatellites (CM 2/15; CM 4/5 and Cm 5/23) were employed as molecular markers in this population genetic study. For the specific methods (e.g. PCR conditions, GeneScan, etc.) please refer to Chapter 3 of the thesis.

Genetic data analysis

Genetic variability and Hardy-Weinberg equilibrium

Genetic diversity within populations was estimated calculating: the number of alleles per locus (N_A) and the allelic richness (A) using F-STAT version 2.9.3 (Goudet, 2001), the allele frequencies (F_A), and the observed (H_O) and Nei's 1987 unbiased expected heterozygosity (H_E) using GENETIX software package version 4.03 (Belkhir et al., 2004).

H_O and H_E values (arc-sin square root transformed) of samples living on artificial substrata and those living on artificial substrata, collected from the

nearest locations (artificial vs. natural locations), were compared using t-tests.

Single and multilocus F_{IS} were estimated using Weir and Cockerham's (1984) fixation index. Departures from Hardy-Weinberg equilibrium (HWE) were tested using Fisher's exact test, using the null hypothesis H_0 = no heterozygote deficiency, with the level of significance determined by a Markov-chain randomization (10,000 dememorizations, 100 batches and 5000 iterations per batch) using GENEPOP version 4.0 (Rousset, 2008). Significance levels for multiple comparisons of loci across samples were adjusted using a standard Bonferroni correction (Rice, 1989).

Tests for linkage disequilibrium (LD) were performed at all loci in each population and for each locus pair across all populations using GENEPOP; estimation of exact probability values was carried out by Markov-chain randomization (1,000,000 dememorizations, 1,000 batches and 50,000 iterations per batch).

Population structure

The level of population genetic differentiation was assessed using Weir and Cockerham's (1984) estimators of F-statistics (F_{IT} or F and F_{ST} or θ) calculated with the software F-STAT version 2.9.3 (Goudet, 2001). Variances of F-statistic estimators were obtained by jack-knifing over all populations according to Weir (1990). The 95% confidence interval was calculated by bootstrapping over the loci with 1000 pseudoreplicates using the software GENETIX.

The analogue *rho* of R_{ST} of Slatkin (1995) was assessed using the computer program RSTCALC (Goodman, 1997). Pairwise F_{ST}, θ (Weir and Cockerham, 1984) and R_{ST} (Slatkin, 1995) estimates, were calculated using the software package MICROSAT version 1.5 (Minch *et al.*, 1996).

Genetic subdivision among populations was quantified using both *F*-statistics (Weir and Cockerham, 1984) and *R*-statistics (Slatkin, 1995).

Global genetic differentiation per locus over all populations was tested with G tests (Goudet *et al.*, 1996) using GENEPOP, and for each pair of populations with F_{ST} permutation tests (10,000 permutations), as implemented in ARLEQUIN version 3.1 (Excoffier *et al.*, 2005).

Indirect estimates of gene flow (N_m) were obtained from the θ-values, according to Island Model formula: $N_m = \frac{1}{4}\ [(1/\ \theta) - 1]$, where N is the population size, m the migration rate and N_m the effective number of migrants per generation (Wright, 1943; Slatkin, 1987). Pairwise N_m was calculated using ARLEQUIN and a multilocus estimate of N_m was obtained using GENEPOP: three estimates of N_m were provided, using the three regression lines published in Barton and Slatkin (1986), and a corrected one was calculated using the values from the closest regression line (Barton and Slatkin, 1986).

The levels of differentiation among populations were also estimated by means of genetic distance measures: Nei's (1978) and $(\delta\mu)^2$ (Goldstein *et al.*, 1995), the last one specific for microsatellites. Genetic distance matrices were calculated using MICROSAT.

In order to provide a graphical representation of the relationships among populations as characterised by the genetic distance matrices (Nei, 1978), samples were ordinated in a bidimensional space by non-metric multidimensional scaling (nMDS, Lessa, 1990). nMDS analysis was performed using the software STATISTICA version 6.1 (Statsoft Inc., 1997).

Isolation by distance

Mantel tests of correlation between genetic and geographical distance matrices were implemented in the Isolation by Distance Web Service 3.11 (IBDWS) (Jensen *et al.* 2005). The geographical distances were calculated considering the minimal distance around the coast. The tests were conducted using two sets of genetic distances, F_{ST} and R_{ST}, in order to explore whether genetic distances based on the IAM or the SMM model were better correlated with geographical distances.

Analysis of MOlecular VAriance (AMOVA)

An analysis of molecular variance (Excoffier *et al.*, 1992), implemented in ARLEQUIN version 3.1 (Excoffier *et al.*, 2005), was carried out to assess the hierarchical partitioning of genetic variability at the location and at the sites within location levels. In addition, AMOVA was repeated to compare two groups: group 1, locations on artificial substrates; group 2, locations on natural rocky shores.

4.3 Results

A total of 406 specimens from 13 sites, with an average sample size of 30 individuals, were screened at three microsatellite loci.

Genetic variability and Hardy-Weinberg equilibrium

A summary of the genetic variability per population at each microsatellite locus and at all loci is reported in Table 4.2. The three microsatellite loci exhibited high levels of polymorphism overall, as shown from low allelic frequencies (Appendix 4.1) and high allelic richness ranging from 15.33 to 19.33 at all loci. Locus CM 2/15 exhibited the lowest number of alleles across all samples, with 7 to 11 alleles per site, while the locus CM 5/23 was the most variable ranging from 23 to 33 alleles per site.

Expected and observed heterozygosity values, averaged over all loci, ranged from 0.869 to 0.901 and from 0.306 to 0.607, respectively. Multilocus estimates of F_{IS} showed in all cases heterozygote deficiency, ranging between 0.330 and 0.639. Exact tests highlighted high significant multilocus departures from HWE in all samples. With the exception of Cv1 at the less polymorphic locus CM 2/15, significant departures from HWE were also observed for each locus in all samples (Table 4.3). The lowest value of H_O (0.120), associated with the highest of F_{IS} (0.864), was registered at the locus CM 4/5 in the Pu1 sample.

Comparisons between H_O and H_E values (arcsin square root transformed) of barnacles living on artificial substrata vs. those living on natural rocky shores from the nearest locations (artificial vs. natural locations) showed no significant differences (Mi1 vs. Ca1 P=0.344; Fa1 vs. Po1 P=0.473; Gr1+Gr3 vs. Si1+Si3 P=0.320; Mu1 vs. Pu1 P=0.060). Allelic richness was also compared between the two groups of barnacles, but the P-value after 10,000 permutations was not significant (P=0.181).

Generally no linkage disequilibrium was detected among loci across all populations (Table 4.4) and, therefore, all loci were considered genetically independent. The only exception is the Pu1 sample, where the locus CM 4/5 was significantly linked to the locus CM 2/15.

Population structure

The genetic variability, estimated by F-statistics, recorded among and within the 13 samples, showed a mean F_{ST} value of 0.001 and a mean F_{IS} of 0.481, while the R-statistics exhibited a mean R_{ST} value of −0.002 and an R_{IS} of 0.509 (Table 4.5). The results obtained with the two different estimators, F-statistics and R-statistics following the IAM and the SMM respectively, were comparable and quite similar; they both showed positive values of F_{IS} and R_{IS}, indicating deficit of heterozygosity within populations, and very low values of F_{ST} and R_{ST}, revealing very low or negligible genetic subdivision among populations at each locus, as shown by the non-significant P-values.

Pairwise R_{ST} (Slatkin, 1995) and F_{ST}, θ (Weir and Cockerham, 1984) estimates (Table 4.6) generally revealed no significant differentiation between each pair of populations as assessed with F_{ST} permutation tests (10,000 permutations).

Pairwise N_m (Wright, 1943; Slatkin, 1987) showed high values of gene flow between pairs of populations (Table 4.7) and the multilocus estimate of N_m, calculated using the values from the closest regression line (Barton and Slatkin, 1986), was 9.129 with a significant P-value (0.019), suggesting extensive genetic connectivity among samples.

Matrices of Nei's genetic distance (1978) and $(\delta\mu)^2$ (Goldstein *et al.*, 1995) are also shown in Table 4.8. A graphical representation of the relationships among populations as indicated by Nei's genetic distance was provided by the nMDS plot (Lessa, 1990) (Figure 4.3). The bi-dimensional distribution of samples did not show any clustering, samples seeming to be randomly interspersed with respect to both substrate type and geographical position.

Isolation by distance

The Mantel tests, conducted using two sets of genetic distances, F_{ST} and R_{ST}, in order to explore whether genetic differentiation based on the IAM or the SMM model were better correlated with geographical distances, were not significant. The reduced major axis regression calculated using F_{ST} showed r=-0.0725 and *P*-value=0.7580 (Figure 4.4) and the one estimated using R_{ST} exhibited r=-0.0935 and *P*-value=0.8330 (Figure 4.5). The tests were also repeated using Nei's (1978) and $(\delta\mu)^2$ (Goldstein *et al.*, 1995) genetic distance matrices; the results obtained (not shown) were in agreement with those for F_{ST} and R_{ST} (for Nei (1978) r =0.0556, *P*-value=0.2830; for $(\delta\mu)^2$ r=-0.1651, *P*-value=0.9830).

Analysis of MOlecular VAriance (AMOVA)

Hierarchical partitioning of genetic variability assessed by AMOVA (Table 4.9) showed 99.3% of variation within site, 0.58% between sites at a location and 0.12% among locations; all *P*-values were non-significant. It is evident that the major source of variation is within sites at very small spatial scale (the plot of few metres from which specimens were collected) at the inter-individual level.

AMOVA was also used to compare two groups: group 1, locations on artificial substrates; group 2, locations on natural rocky shores (Table 4.10).

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The percentages of variation between groups and within group were negligible and P-values were not significant.

Table 4.2 – Summary of genetic variability per sites at each microsatellite locus and all loci: number of sampled individuals (N), number of observed alleles per locus (N_A); allelic richness based on 25 individuals (A); Nei's 1987 unbiased expected heterozygosity (H_E); observed heterozygosity (H_O); Weir and Cockerham's (1984) estimate of Wright's (1951) fixation index (F_{IS}). F_{IS} values in bold indicate significant departures from HWE after standard Bonferroni correction. For site abbreviations see Table 4.1.

LOCUS	SITES												
	Pu1	Mu1	Si1	Si3	Gr1	Gr3	Cv1	Ce1	Mi1	Ca1	Fa1	Po1	Po3
Cm2/15													
N	30	28	31	32	29	29	32	30	29	31	32	30	28
N_A	7	9	10	7	10	7	11	10	9	10	11	9	7
A	6.33	8.77	9.54	6.73	9.69	6.85	9.81	9.77	8.55	9.53	9.99	8.66	6.77
H_E	0.681	0.725	0.814	0.768	0.771	0.757	0.778	0.815	0.748	0.784	0.789	0.816	0.708
H_O	0.267	0.429	0.548	0.531	0.621	0.552	0.625	0.667	0.483	0.484	0.500	0.400	0.429
F_{IS}	**0.612**	**0.414**	**0.330**	**0.312**	**0.198**	**0.274**	0.199	**0.185**	**0.359**	**0.387**	**0.370**	**0.514**	**0.399**
Cm 4/5													
N	25	29	27	28	27	29	26	30	30	30	29	28	29
N_A	14	18	15	11	13	13	11	15	16	17	17	15	14
A	14.00	17.10	14.69	10.84	12.70	12.67	10.96	14.40	15.08	15.89	16.12	14.73	13.64
H_E	0.869	0.904	0.874	0.845	0.881	0.856	0.793	0.908	0.823	0.896	0.908	0.869	0.890
H_O	0.120	0.310	0.222	0.214	0.333	0.241	0.154	0.500	0.267	0.400	0.379	0.250	0.241
F_{IS}	**0.864**	**0.661**	**0.749**	**0.750**	**0.626**	**0.722**	**0.809**	**0.454**	**0.680**	**0.558**	**0.587**	**0.716**	**0.732**
Cm5/23													
N	32	28	27	30	27	28	32	29	29	28	31	29	29
N_A	32	31	28	30	30	27	29	33	32	26	23	27	25
A	28.23	29.41	27.00	27.43	28.99	25.82	25.78	30.60	29.85	24.86	21.63	25.58	23.39
H_E	0.965	0.979	0.966	0.969	0.976	0.968	0.958	0.978	0.978	0.967	0.960	0.969	0.956
H_O	0.531	0.714	0.519	0.600	0.556	0.536	0.656	0.655	0.552	0.571	0.516	0.586	0.517
F_{IS}	**0.454**	**0.274**	**0.468**	**0.385**	**0.436**	**0.451**	**0.319**	**0.334**	**0.440**	**0.413**	**0.466**	**0.399**	**0.464**

Table 4.2 - continued

ALL LOCI	Pu1	Mu1	Si1	Si3	Gr1	Gr3	Cv1	Ce1	Mi1	Ca1	Fa1	Po1	Po3
A	17.67	19.33	17.67	16.00	17.67	15.67	17.00	19.33	19.00	17.67	17.00	17.00	15.33
H_E	0.838	0.869	0.885	0.861	0.876	0.860	0.843	0.901	0.850	0.882	0.886	0.885	0.851
sd	0.145	0.130	0.077	0.102	0.103	0.106	0.100	0.082	0.117	0.092	0.088	0.077	0.129
H_O	0.306	0.484	0.430	0.449	0.503	0.443	0.478	0.607	0.434	0.485	0.465	0.412	0.396
sd	0.208	0.208	0.180	0.206	0.151	0.175	0.282	0.093	0.149	0.086	0.075	0.168	0.141
F_{IS}	**0.639**	**0.447**	**0.519**	**0.483**	**0.430**	**0.490**	**0.437**	**0.330**	**0.494**	**0.454**	**0.479**	**0.539**	**0.540**

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Table 4.3 - Test for Hardy-Weinberg equilibrium (F_{IS}, Weir and Cockerham, 1984) using Fisher's method, estimation of exact probability values (P-value) by a Markov-chain randomization.

LOCUS CM 2/15 SITES	F_{IS} (W&C)	P-value
Pu1	0.612	0.000
Mu1	0.414	0.000
Si1	0.330	0.000
Si3	0.312	0.017
Gr1	0.198	0.032
Gr3	0.275	0.039
Cv1	0.200	0.087
Ce1	0.185	0.018
Mi1	0.359	0.002
Ca1	0.387	0.000
Fa1	0.370	0.009
Po1	0.514	0.000
Po3	0.399	0.001
LOCUS CM 4/5 SITES	**F_{IS} (W&C)**	**P-value**
Pu1	0.864	0.000
Mu1	0.661	0.000
Si1	0.749	0.000
Si3	0.750	0.000
Gr1	0.626	0.000
Gr3	0.722	0.000
Cv1	0.809	0.000
Ce1	0.454	0.000
Mi1	0.680	0.000
Ca1	0.558	0.000
Fa1	0.587	0.000
Po1	0.716	0.000
Po3	0.732	0.000
LOCUS CM 5/23 SITES	**F_{IS} (W&C)**	**P-value**
Pu1	0.454	0.000
Mu1	0.274	0.000
Si1	0.468	0.000
Si3	0.385	0.000
Gr1	0.436	0.000
Gr3	0.451	0.000
Cv1	0.319	0.000
Ce1	0.334	0.000
Mi1	0.440	0.000
Ca1	0.413	0.000
Fa1	0.466	0.000
Po1	0.399	0.000
Po3	0.464	0.000

Table 4.4 – Tests for linkage disequilibrium in each location and for each locus pair, estimation of exact probability values (*P*-value) and standard error (S.E.) determined by a Markov-chain randomization (1,000,000 dememorizations, 1,000 batches and 50,000 iterations per batch). Significant *P*-values in bold.

LOCATION	LOCUS 1	LOCUS 2	*P*-values	S.E.
Pu1	Cm2/15	Cm 4/5	**0.001**	0.0001
Pu1	Cm2/15	Cm5/23	1.000	0.0000
Pu1	Cm5/23	Cm 4/5	1.000	0.0000
Mu1	Cm2/15	Cm 4/5	0.405	0.0041
Mu1	Cm2/15	Cm5/23	1.000	0.0000
Mu1	Cm5/23	Cm 4/5	1.000	0.0000
Si1	Cm2/15	Cm 4/5	0.779	0.0026
Si1	Cm2/15	Cm5/23	1.000	0.0000
Si1	Cm5/23	Cm 4/5	1.000	0.0000
Si3	Cm2/15	Cm 4/5	0.851	0.0016
Si3	Cm2/15	Cm5/23	1.000	0.0000
Si3	Cm5/23	Cm 4/5	1.000	0.0000
Gr1	Cm2/15	Cm 4/5	1.000	0.0000
Gr1	Cm2/15	Cm5/23	1.000	0.0000
Gr1	Cm5/23	Cm 4/5	0.056	0.0023
Gr3	Cm2/15	Cm 4/5	0.866	0.0018
Gr3	Cm2/15	Cm5/23	1.000	0.0000
Gr3	Cm5/23	Cm 4/5	1.000	0.0000
Cv1	Cm2/15	Cm 4/5	0.250	0.0028
Cv1	Cm2/15	Cm5/23	0.055	0.0020
Cv1	Cm5/23	Cm 4/5	0.563	0.0040
Ce1	Cm2/15	Cm 4/5	1.000	0.0000
Ce1	Cm2/15	Cm5/23	1.000	0.0000
Ce1	Cm5/23	Cm 4/5	1.000	0.0000
Mi1	Cm2/15	Cm 4/5	0.723	0.0030
Mi1	Cm2/15	Cm5/23	1.000	0.0000
Mi1	Cm5/23	Cm 4/5	1.000	0.0000
Ca1	Cm2/15	Cm 4/5	0.471	0.0041
Ca1	Cm2/15	Cm5/23	0.184	0.0044
Ca1	Cm5/23	Cm 4/5	1.000	0.0000
Fa1	Cm2/15	Cm 4/5	0.676	0.0033
Fa1	Cm2/15	Cm5/23	0.052	0.0014
Fa1	Cm5/23	Cm 4/5	1.000	0.0000
Po1	Cm2/15	Cm 4/5	0.825	0.0024
Po1	Cm2/15	Cm5/23	1.000	0.0000
Po1	Cm5/23	Cm 4/5	1.000	0.0000
Po3	Cm2/15	Cm 4/5	0.802	0.0016
Po3	Cm2/15	Cm5/23	0.657	0.0020
Po3	Cm5/23	Cm 4/5	0.369	0.0043

Table 4.5 – *F*-statistics (Weir and Cockerham's, 1984) and *R*-statistics (Slatkin, 1995) for each microsatellite locus and over loci in the 13 sites. Variances of estimators were obtained by jack-knifing over all populations. The 95% confidence interval (CI) was calculated by bootstrapping over the loci. P-value of global genetic differentiation tested with G-test (Goudet *et al.*, 1996).

LOCUS	F-statistics			G test	R-statistics		
	F_{IS}	F_{IT}	F_{ST}	P-values	R_{IS}	R_{IT}	R_{ST}
Cm2/15	0.346	0.346	-0.0005	0.0979	0.300	0.306	0.0094
Cm 4/5	0.678	0.678	0.0005	0.1625	0.695	0.692	-0.0107
Cm5/23	0.407	0.409	0.0027	0.0572	0.419	0.419	0.0007
ALL LOCI	0.481	0.481	0.001	0.0596	0.509	0.507	-0.0029
CI 95%	(0.347 - 0.679)	(0.346 - 0.679)	(-0.0005 - 0.0026)				

Table 4.6 – Estimates of F_{ST} (below the diagonal) and R_{ST} (above the diagonal) for all microsatellite loci among pairs of sites. Values in bold indicate significant P-values tested with F_{ST} permutation tests (10,000 permutations).

.	Pu1	Mu1	Si1	Si3	Gr1	Gr3	Cv1	Ce1	Mi1	Ca1	Fa1	Po1	Po3
Pu1	-	0.004	0.012	**0.042**	-0.004	0.018	-0.007	0.016	0.008	0.017	-0.003	-0.005	-0.012
Mu1	0.021	-	0.039	0.012	-0.002	-0.012	-0.004	0.006	0.027	-0.014	-0.012	-0.011	0.017
Si1	0.021	0.02	-	0.117	0.021	0.035	**0.002**	0.009	0.053	0.046	0.032	0.018	0.040
Si3	0.058	0.04	0.07	-	0.018	0.020	0.057	0.036	0.110	0.007	0.040	0.008	0.043
Gr1	0.027	0.008	0.026	0.032	-	0.008	-0.001	-0.005	0.055	0.000	0.007	-0.017	0.000
Gr3	0.016	0.018	0.019	0.044	0.015	-	0.000	0.001	0.035	-0.014	-0.004	-0.003	0.037
Cv1	0.026	0.016	0.017	0.072	0.022	0.02	-	**0.001**	0.017	0.005	-0.009	-0.007	0.014
Ce1	0.025	0.012	0.026	0.024	0.009	0.016	0.026	-	0.068	0.000	0.017	-0.008	0.032
Mi1	0.012	0.014	0.014	0.057	0.017	0.012	0.014	0.018	-	0.052	0.006	0.036	0.033
Ca1	**0.029**	0.014	0.033	0.019	0.01	0.018	**0.034**	0.008	0.022	-	-0.001	-0.009	0.032
Fa1	0.022	0.026	0.03	0.031	0.024	0.017	**0.035**	0.015	0.02	0.021	-	-0.004	0.014
Po1	0.019	0.016	0.018	0.053	0.022	0.014	0.014	0.017	0.012	0.026	0.025	-	0.000
Po3	0.014	0.021	0.031	0.04	0.023	0.01	0.027	0.022	0.018	0.014	0.015	0.018	-

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Table 4.7 – Pairwise indirect estimates of gene flow (N_m) according to Wright (1943) and Slatkin (1987).

	Pu1	Mu1	Si1	Si3	Gr1	Gr3	Cv1	Ce1	Mi1	Ca1	Fa1	Po1	Po3
Pu1	-												
Mu1	15.24	-											
Si1	24.60	76.12	-										
Si3	14.46	31.34	49.56	-									
Gr1	13.54	inf	50.74	138.41	-								
Gr3	18.96	36.11	40.88	36.12	42.11	-							
Cv1	18.48	27.54	23.68	17.38	22.64	19.00	-						
Ce1	13.13	165.54	56.02	63.27	inf	46.22	15.37	-					
Mi1	20.55	80.65	33.56	24.15	32.73	153.95	24.71	34.68	-				
Ca1	11.05	64.09	30.90	126.18	inf	31.00	10.77	inf	20.93	-			
Fa1	19.13	15.08	28.24	32.05	17.86	29.78	11.79	38.36	20.76	19.86	-		
Po1	23.31	50.56	55.53	22.42	24.50	55.25	47.51	36.77	80.13	17.71	17.59	-	
Po3	27.92	27.50	20.89	25.29	22.02	376.32	14.42	24.46	33.43	63.65	46.57	38.08	-

Table 4.8 – Pairwise genetic distances. Nei (1978) values are below the diagonal and $(\delta\mu)^2$ values are above the diagonal.

	Pu1	Mu1	Si1	Si3	Gr1	Gr3	Cv1	Ce1	Mi1	Ca1	Fa1	Po1	Po3
Pu1	-	6.314	2.230	12.141	1.933	11.865	1.502	2.710	5.937	8.969	4.504	2.284	0.740
Mu1	0.067	-	14.035	3.920	5.042	1.000	2.825	3.089	5.583	0.411	0.742	2.476	7.909
Si1	0.071	0.071	-	23.749	6.899	20.760	4.574	7.842	8.788	18.098	10.228	8.099	3.964
Si3	0.327	0.228	0.462	-	5.828	5.802	10.596	4.746	17.981	2.351	7.694	4.116	10.590
Gr1	0.106	0.006	0.106	0.160	-	10.282	3.654	0.556	11.226	5.996	5.587	0.454	0.938
Gr3	0.037	0.057	0.059	0.245	0.041	-	5.968	6.975	7.318	0.895	2.150	6.417	14.457
Cv1	0.095	0.043	0.048	0.464	0.075	0.064	-	2.751	2.319	5.113	1.130	2.419	3.762
Ce1	0.084	0.014	0.111	0.108	0.008	0.038	0.099	-	9.750	3.696	3.847	0.222	2.481
Mi1	0.019	0.027	0.031	0.340	0.046	0.016	0.029	0.044	-	8.920	2.290	8.639	10.379
Ca1	0.124	0.035	0.166	0.070	0.004	0.059	0.165	0.005	0.084	-	2.233	3.222	10.016
Fa1	0.076	0.133	0.141	0.167	0.117	0.056	0.175	0.051	0.070	0.098	-	3.134	7.066
Po1	0.049	0.047	0.055	0.368	0.103	0.032	0.021	0.072	0.014	0.141	0.141	-	2.042
Po3	0.031	0.075	0.137	0.207	0.090	0.007	0.107	0.078	0.054	0.027	0.032	0.056	-
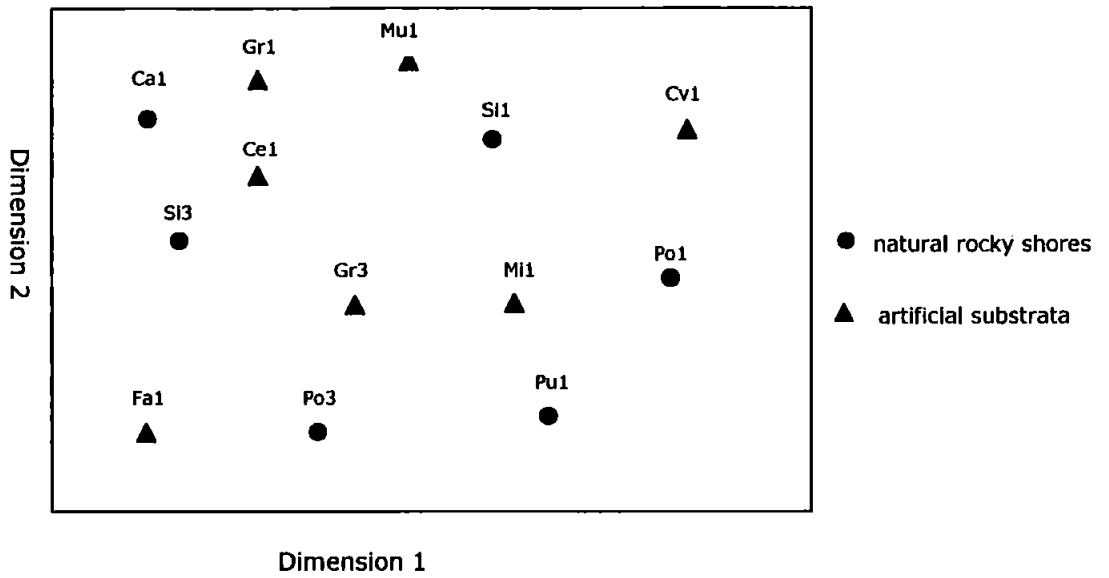
Figure 4.3 – Non-metric multidimensional scaling analysis (nMDS) of 13 samples based on Nei's (1978) genetic distance. Stress value = 0.11 (For abbreviations see Table 4.1).
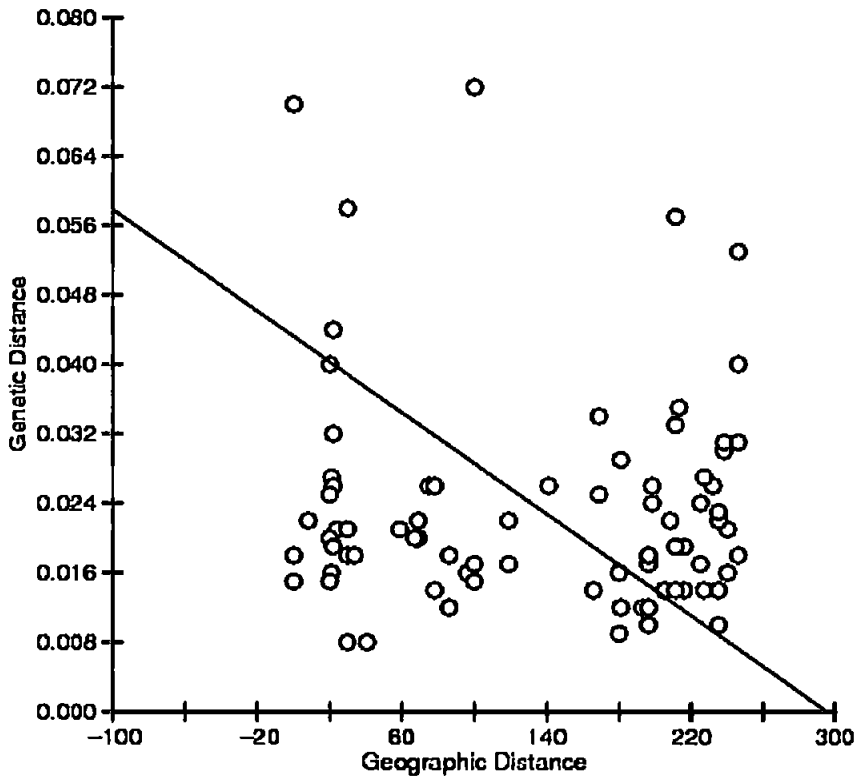
Figure 4.4 - Reduced major axis regression showing relationships between genetic and geographical distances for all 13 locations. Genetic distances based on F_{ST} (r=-0.0725 P-value= 0.7580).
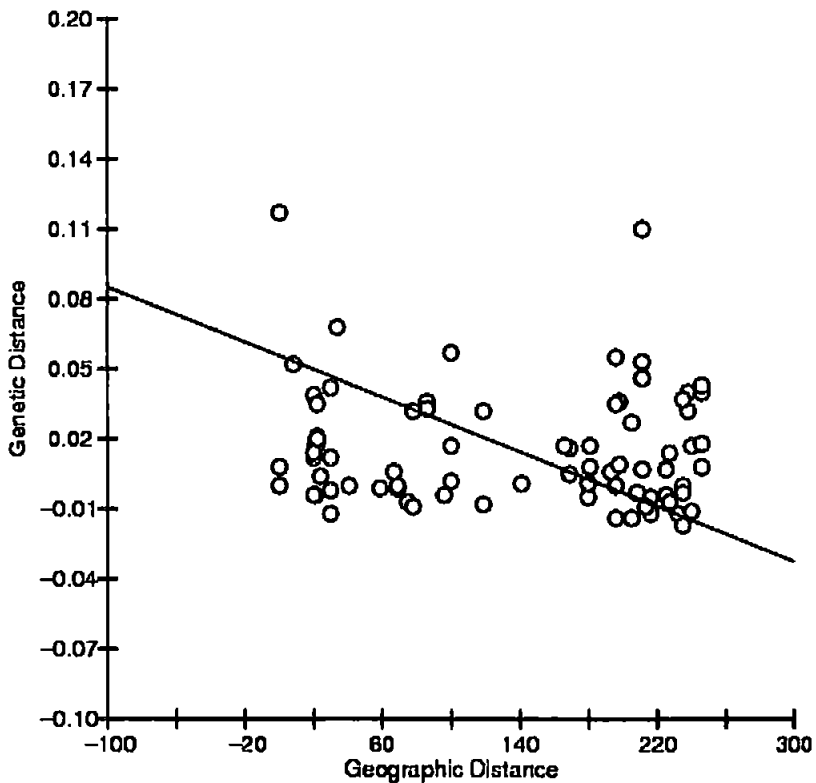
Figure 4.5 - Reduced major axis regression showing relationships between genetic and geographical distances for all 13 locations. Genetic distances based on R_{ST} (r=-0.0935; P-value= 0.8330).

Table 4.9 – Hierarchical AMOVA among locations, between 2 sites per location. Source of variation, degree of freedom (df), variance components, percentage of variation (%), fixation indices and probability value (P-value) are listed.

Source of variation	df	Variance components	%	Fixation indices	P-value
Among locations	1	0.00157	0.12	0.00118	0.335
Between sites within location	11	0.00771	0.58	0.00581	0.793
Within sites	797	1.31878	99.30	0.00699	0.758

Table 4.10 – Hierarchical AMOVA among samples grouped in two groups: group 1, locations on artificial substrates; group 2, locations on natural rocky shores. Source of variation, degree of freedom (df), variance components, percentage of variation (%), fixation indices and probability value (P-value) are listed.

Source of variation	df	Variance components	%	Fixation indices	P-value
Between groups	1	-0.00027	-0.02	-0.00021	0.528
Among populations within groups	11	0.01199	0.9	0.00902	0.352
Within populations	797	1.31654	99.12	0.00882	0.376

4.4 Discussion

In this study, the population genetic structure of the barnacle *Chthamalus montagui* was investigated by employing three highly polymorphic microsatellite loci at 13 sites along the Adriatic coast to assess the potential "corridor effect" created by artificial substrates, built to protect the coast.

Negligible levels of genetic subdivision were detected among locations, as revealed from the low and non-significant values of F_{ST} and R_{ST} and the high estimates of gene flow (N_m) between all pairs of populations. All the samples screened along the Adriatic coast seem to act as a large panmitic population. This was also confirmed by the nMDS analysis based on the genetic distances among populations and by the Mantel test that did not show any significant correlation between genetic and geographical distance matrices. These results should be viewed in relation to the dispersal capability of the species. Simulations of one-dimensional stepping-stone model of population structure showed that isolation by distance is most obvious when comparing populations separated by 2-5 times the mean larval dispersal distance (Palumbi, 2003).

The range of larval dispersal in barnacles has been estimated to be in the order of 15-20 km per generation (Strathmann, 1974; Crisp, 1976). If we consider the sampling locations of this study, the furthest-separated rocky shores without intervening natural hard substrate are Casteldimezzo (Ca1) and Sistiana (Si1), about 200 km apart. This distance is higher than the estimated potential larval dispersal of barnacles, therefore artificial substrates could act as stepping stones or "corridors" among previously isolated populations.

Support for this hypothesis comes from an investigation using a combination of field studies and spatial population models to investigate the large scale effects of the hard structures in the north Adriatic Sea on the limpet *Patella caerulea* (Airoldi *et al.*, 2005). Model simulations of the dispersal and survival of *P. caerulea* showed that the maximum dispersal distance of this species is far less than the distance between natural rocky reefs in the area. Thus, prior to the deployment of the artificial structures, the gene flow between native populations in Trieste and Ancona was probably limited. Furthermore, connectivity matrix simulations showed that if a few more structures were built in the area the result for this species would have been equivalent to a continuous rocky coast. On the contrary, if structures were removed from the system the proportion of occupied structures would decrease. Results also revealed that some structures were mainly sinks while others acted as both sources and sinks, depending on their location. In summary the number, extent and the spatial arrangement of the structures seem to influence the realised larval dispersal of *P. caerulea* (Airoldi *et al.*, 2005).

However, it is important to highlight that *P. caerula* is assumed to be a short-dispersal species, whereas *C. montagui* is a long-dispersal species. The range of larval dispersal estimated for barnacles (Strathmann, 1974; Crisp, 1976) can vary and is likely to be influenced by different factors, such as offshore hydrodynamic processes at a large scale (Barnes, 1956; Raimondi, 1990) or local water turbulence at a small scale (O'Riordan, 2004). The Adriatic Sea is a semi-enclosed elongated basin, separated from North to South into three sub-basins, characterized by a cyclonic circulation,

by eddies and gyres that lead to the formation of deep water in the Northern and Middle Adriatic (Mantziafou and Lascartos, 2004). These oceanographic processes can promote or increase species dispersal capability. Thus, the detected low genetic differentiation among populations can be related not only to the artificial substrates, but also to the effective dispersal ability of *C. montagui* possibly enhanced by local oceanographic factors.

Moreover, the AMOVA results confirmed that genetic variation among locations and between sites within a location are negligible (less than 1%) and showed that the major source of genetic variability is within sites, accounting for 99.3% of the total genetic variation. Analogous results were found in the study carried out within EUMAR in the same area, along the Adriatic coast, on the limpet *P. caerulea* using allozymes and microsatellites (Bertozzi, 2005).

In fact, the 13 barnacle populations screened in my study showed high levels of genetic variability, low allelic frequencies, high expected heterozygosity and a large number of alleles per population at each of the analysed loci (the most polymorphic one, CM 5/23, ranging from 23 to 33 alleles).

Looking at genetic variability within populations, it appears that all samples exhibited strong deviations from HWE showing highly significant values of F_{IS} at all loci. Heterozygosity deficits are frequently observed in marine invertebrates (Zouros and Foltz, 1984; Gaffney 1994; David *et al.* 1997; Ayre and Hughes, 2000; Ridgway *et al.* 2001; Addison and Hart 2004;

Duran *et al.* 2004; Le Goff-Vitry *et al.* 2004; Andrade and Solferini, 2007; Costantini *et al.*, 2007) including barnacles (Veliz *et al.*, 2006; York *et al.*, 2008). The most common explanations for the heterozygote deficiency are technical factors, such as the presence of null alleles (Foltz, 1986; Callen *et al.*, 1993; Puebla *et al.*, 2008), and (or) biological factors, such as inbreeding or Wahlund effect (Pogson *et al.*, 1995; Duran *et al.*, 2004; Plutchack *et al.*, 2006; Costantini *et al.*, 2007). In this study I do not favour the hypothesis of null alleles, because all F_{IS} estimates were positive, significant and consistent across all loci, and it seems highly improbable that all loci exhibit null alleles with such a constant frequency.

The heterozygote deficiencies observed are thus probably due to biological factors, but it is not easy to give an actual explanation on this basis. Inbreeding remains an unlikely explanation for barnacles, which have wide dispersal as larvae, and have large populations that are not subject to drastic reduction in size.

The Wahlund effect refers to reduction of heterozygosity caused by subpopulation structure, and could affect all polymorphic loci similarly (Pogson *et al.*, 1995). If two or more subpopulations have different allele frequencies then the overall heterozygosity is reduced, even if the subpopulations themselves are in HWE; the underlying causes of this population subdivision could be geographic barriers to gene flow followed by genetic drift in the subpopulations. However, no evidence of population differentiation was detected in this study.

Furthermore, comparisons between the genetic pattern of barnacles living on natural rocky shores and those living on artificial substrata were carried out to investigate the possible selection that could be operated by the type of substratum. No significant differences were detected comparing the mean number of alleles, the allele frequencies, the heterozygosity and the partitioning of genetic variance between the two groups of populations. It is important to take into account that the use of putatively neutral markers, such as microsatellites, could not detect such phenomena even if, in some cases, these markers may not behave in a neutral way (Dufresne *et al.*, 2002).

Support for these findings comes from the study on the limpet *P. caerulea* (Bertozzi, 2005): no genetic difference between samples collected from natural rocks and from artificial structures was shown by allozymes, markers considered under selection.

Moreover, other population genetic studies carried out along the European coast within EUMAR, on different sorts of natural and artificial substrates, on the periwinkles *Littorina littorea* and *Melarhaphe neritoides*, using mtDNA as a molecular marker, did not reveal any significant effect of substrate type on the population genetic pattern of these intertidal organisms.

To conclude, detecting the corridor effect in this barnacle species is not an easy task due to the possible masking effect of local spatial genetic variation and efficient larval mixing. However, the collected data do not allow rejection of the hypothesis that artificial substrates act as "corridors"

among populations favouring gene flow and promoting homogeneous genetic patterns.

Furthermore, the nature of the substratum (artificial vs. natural) did not promote genetic differentiation in barnacles, suggesting that the type of substratum does not selectively affect survival during settlement and recruitment on the basis of the tested markers.

Chapter 5

Effects of marginality and peripherality on the genetic variability of

Chthamalus montagui

5.1 Introduction and specific aims

The importance of intraspecific genetic diversity in natural populations is well established, as already explained in the general introduction of this thesis, and has been identified as a global priority for conservation (Crozier 1997; Avise and Hamrick, 1996). Genetic diversity is the clay for evolutionary adaptation and ultimately speciation, its role is fundamental to the ability of a species to persist when challenged by various environmental pressures (Allendorf and Luikart, 2007).

However, the distribution of genetic variation is often non-uniform and partitioned throughout a species' natural distribution (Lind *et al.*, 2007). Populations living at the edge of the species' geographical range, referred to here as peripheral, or/and those at the extreme of the species' environmental tolerance within its geographical range, referred to here as marginal populations, often show a different pattern of genetic variability compared to populations living in the central part of the species' geographical range, here denominated central populations (Lesica and Allendorf 1995).

Peripheral and marginal populations are often relatively small and isolated from central populations; gene flow is reduced and the probability that they are founded by a small number of individuals is high (Levin, 1970; Lawton, 1993). Reduced gene flow, small population size and founder effect will promote genetic drift and result in less genetic variation and increased differentiation of these populations (Allendorf, 1986; McCommas and Bryan, 1990; Lesica and Allendorf 1995).

Empirical data mostly support the prediction that peripheral populations are less genetically variable compared to core populations of the same species (Lesica and Allendorf, 1995; Lammi et al., 1999; Schwartz et al., 2003). For instance, a study carried out on the population genetics of a marine bivalve, *Pinctada maxima*, throughout the Indo-Australian archipelago using microsatellite markers, demonstrated that, as the geographical distance of a population increased from the centre of the species' distribution, allelic richness and observed heterozygosity correspondingly decreased, showing the lowest genetic diversity in peripheral populations (Lind et al., 2007).

In addition, many species at the edge of their range occur in unusual or atypical habitats (Lawton, 1993, Holt and Keitt, 2000; Pulliam, 2000; Maurer and Taper 2002). Peripheral and marginal populations are, therefore, under extreme selection pressure: local adaptation to exceptional conditions may result in anomalous and sometimes unique genetic patterns (Garcia-Ramos and Kirkpatrick 1997; Bouza et al., 1999). Furthermore, these populations, which are smaller and inhabit a more stressful environment than central ones, are more likely to pass through severe bottlenecks that will further reduce their genetic variability (Chakraborty and Nei 1977; Booy et al., 2000).

Given that peripheral and marginal populations are often both genetically distinct and more vulnerable, it follows that they have for long been recognized as "evolutionary laboratories" potentially favourable for speciation (Carson and Templeton, 1984; Garcia-Ramos and Kirkpatrick, 1997) and for this reason are of special interest in conservation biology

(Lesica and Allendorf, 1995). If we consider the marine environment, more data are needed to assess the importance and vulnerability of peripheral and marginal populations in order to develop adequate management strategies for marine genetic biodiversity conservation.

Within the EUMAR project, a meta-analysis of population genetic data from 29 species inhabiting the Baltic Sea was performed. The low salinity Baltic Sea is a perfect "natural laboratory", as it represents a geographically peripheral ecosystem and at the same time an ecologically extreme and marginal marine environment (Johannesson and Andrè, 2006).

My task in the EUMAR project was to compare the genetic pattern of central populations vs. peripheral/marginal populations of *Chthamalus montagui*, over the whole range of its distribution in the UK, to assess the genetic diversity of the species in these peculiar conditions, using three microsatellites as molecular markers.

The British coast is a very appropriate study area for this purpose. The geographical distribution of the target species in the UK (Figure 5.1) has its northern limit in northern Scotland, due to low water temperature, while in the English Channel the Isle of Wight represents the eastern limit of the species, due to lack of rocky substratum suitable for barnacle settlement (Hawkins and Jones, 1992; Herbert and Hawkins, 2006). Moreover, decreasing salinity and increasing sedimentation moving into the Bristol Channel provide an ecologically marginal habitat for the species.

The hypothesis to be tested was the following one: in the UK distribution range of *C. montagui*, marginal and peripheral populations show a reduction in genetic variation when compared to central ones.

When comparing peripheral vs. central populations of barnacles, loss of genetic variation could be due to reduction in larval recruitment since peripheral populations have neighbouring populations (potential sources of larvae) on only one side.   In addition, the relative isolation of both peripheral and marginal populations might promote the presence of unusual genotypes (Lesica and Allendorf, 1995; Lammi *et al.*, 1999; Sagarin and Gaines, 2002; Faugeron *et al.*, 2004).

Figure 5.1 – Geographical distribution of *C. montagui* in the UK and Ireland (from Hawkins and Jones, 1992)

5.2 Materials and methods

Sampling

Chthamalus montagui specimens were collected in September/October 2002 from the whole of the UK distribution range (Table 5.1).

Figures 5.2 and 5.3 show the 14 sampling locations: peripheral populations were sampled in Northern Scotland and the Isle of Wight; marginal populations were collected along the Bristol Channel; central populations were collected from Western Scotland, Cumbria, Wales and South-West England.

At each location barnacles were sampled from 3 sites (70-150 metres one from the other). At each site 50 to 100 specimens were collected from that part of the shore where they were most abundant. A single site, or sometimes two sites, was analysed per location, with 32 individuals per site (although not all individuals were successfully analysed for each locus).
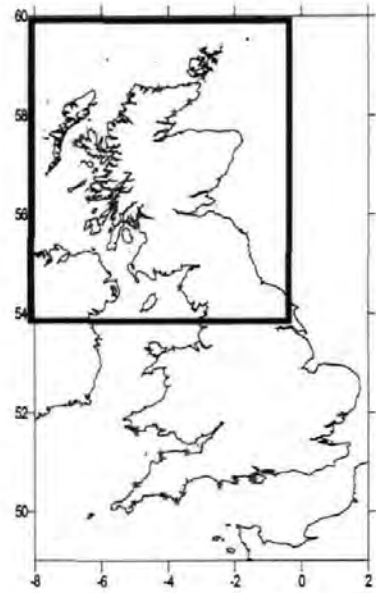
PERIPHERAL
POPULATION
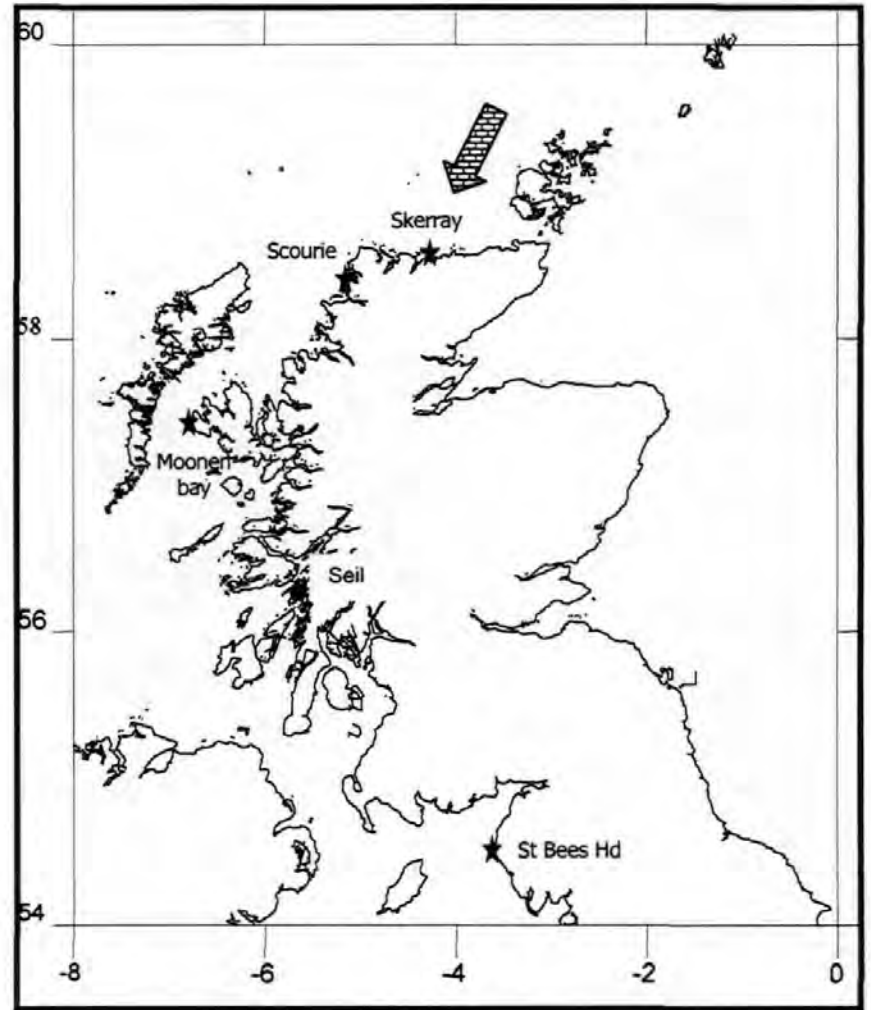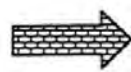
Figure 5.2 – Northern UK sampling locations

MARGINAL
POPULATION

PERIPHERAL
POPULATION
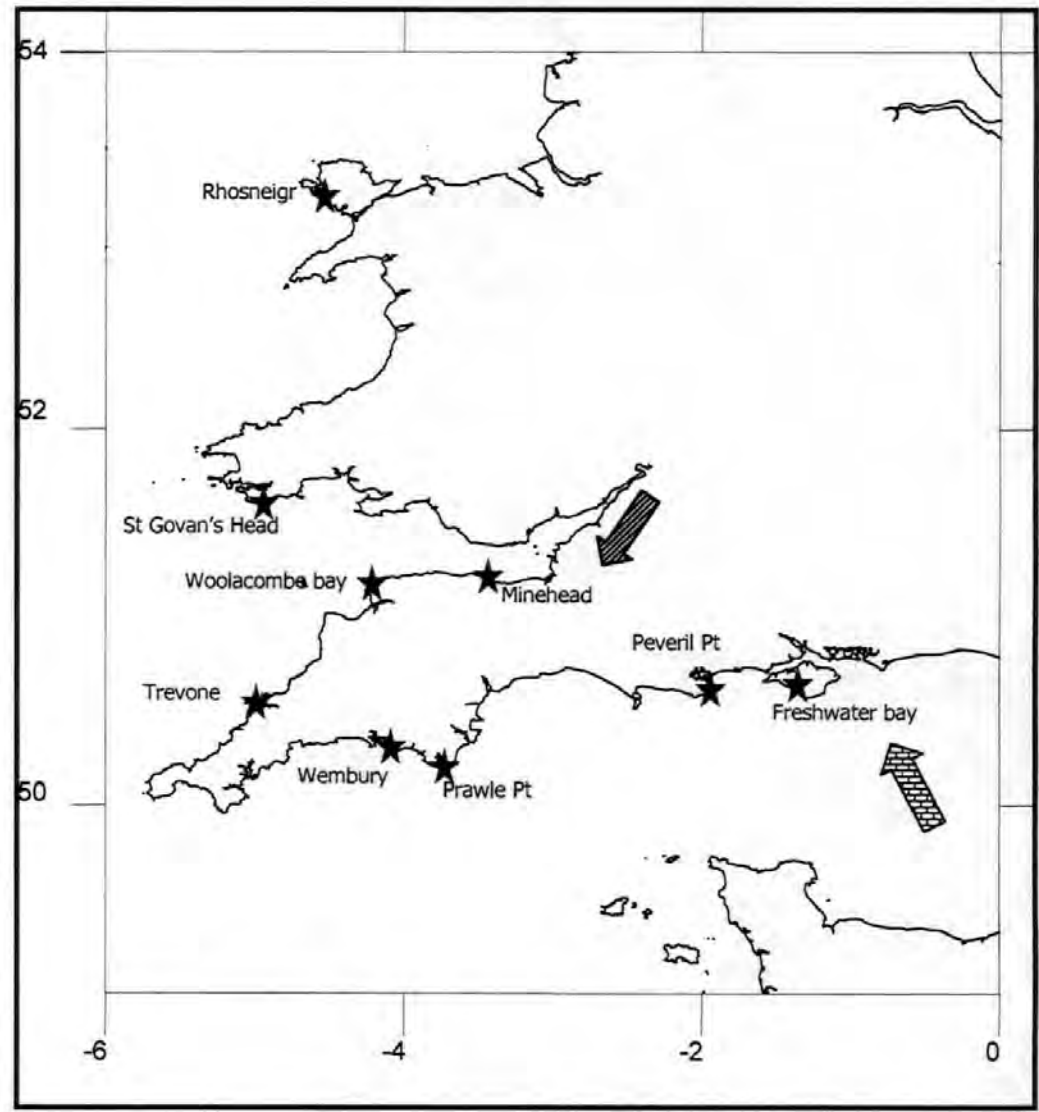
Figure 5.3 – Southern UK sampling locations

Table 5.1 – Sampling locations, sites used in the genetic analysis, geographical area, site abbreviations and geographical coordinates

Sampling location	Site	Area	Abbreviation	Geographical coordinates	
Skerray	2	Scotland	Sk2	4.28 W	58.54 N
Scourie	2	Scotland	Sc2	5.16 W	58.40 N
Moonen Bay	1	Isle of Skye	Mo1	6.79 W	57.42 N
Moonen Bay	3	Isle of Skye	Mo3	6.79 W	57.42 N
Seil	1	Isle of Seil	Se1	5.64 W	56.27 N
Seil	3	Isle of Seil	Se3	5.64 W	56.27 N
St Bees	2	Cumbria	Be2	3.62 W	54.51 N
Rhosneigr	2	Isle of Anglesey	Rh2	4.53 W	53.23 N
St Govan's Head	2	Pembrokeshire	Go2	4.94 W	51.60 N
Minehead	1	Somerset	Mi1	3.43 W	51.21 N
Woolacombe	2	Devon	Wo2	4.21 W	51.17 N
Trevone	2	Cornwall	Tr2	4.99 W	50.54 N
Wembury	2	Devon	We2	4.09 W	50.30 N
Prawle Point	1	Devon	Pr1	3.73 W	50.20 N
Prawle Point	3	Devon	Pr3	3.73 W	50.20 N
Peveril Point	2	Dorset	Pe2	1.95 W	50.61 N
Freshwater Bay	2	Isle of Wight	Fr2	1.41 W	50.63 N

Laboratory work

Three microsatellites (CM 2/15, CM 4/5 and Cm 5/23) were employed as molecular markers in this population genetic study. For the specific methods (e.g. PCR conditions, GeneScan, etc.) please refer to Chapter 3 of the thesis.

Genetic data analysis

Analysis of MOlecular VAriance (AMOVA)

An analysis of molecular variance (Excoffier *et al.*, 1992), implemented in ARLEQUIN version 3.1 (Excoffier *et al.*, 2005), was carried out to assess the hierarchical partitioning of genetic variability at the levels of location and of sites within locations.

Genetic variability and Hardy-Weinberg equilibrium

Genetic diversity within population was estimated calculating: the number of alleles per locus (N_A) and the allelic richness (A) using F-STAT version 2.9.3 (Goudet, 2001), the allele frequencies (F_A), and the observed (H_O) and Nei's 1987 unbiased expected heterozygosity (H_E) using GENETIX software package version 4.03 (Belkhir et al., 2004).

Single and multilocus F_{IS} were estimated using Weir and Cockerham's (1984) fixation index. Departures from Hardy-Weinberg equilibrium (HWE) were tested using Fisher's exact test, using the null hypothesis H_0 = no heterozygote deficiency, with the level of significance determined by Markov-chain randomization (10,000 dememorizations, 100 batches and 5,000 iterations per batch) using GENEPOP version 4.0 (Rousset, 2008). Significance levels for multiple comparisons of loci across samples were adjusted using a standard Bonferroni correction (Rice, 1989).

Tests for linkage disequilibrium (LD) were performed at all loci in each population and for each locus pair across all populations using GENEPOP; estimation of exact probability values was by Markov-chain randomization (1,000,000 dememorizations, 1,000 batches and 50,000 iterations per batch).

Population structure

The level of population genetic differentiation was assessed using Weir and Cockerham's (1984) estimators of F-statistics (F_{IT} or F and F_{ST} or θ) calculated with the software F-STAT version 2.9.3 (Goudet, 2001). Variances of F-statistic estimators were obtained by jack-knifing over all populations according to Weir (1990). The 95% confidence interval was

calculated by bootstrapping over the loci with 1,000 pseudoreplicates using the software GENETIX.

The analogue *rho* of the R_{ST} of Slatkin (1995) was assessed using the computer program RSTCALC (Goodman, 1997). Pairwise F_{ST}, θ (Weir and Cockerham, 1984) and R_{ST} (Slatkin, 1995) estimates were calculated using the software package MICROSAT version 1.5 (Minch *et al.*, 1996).

Global genetic differentiation per locus over all populations was tested with G tests (Goudet *et al.*, 1996) using GENEPOP, and for each pair of populations with F_{ST} permutation tests (10000 permutations), as implemented in ARLEQUIN version 3.1 (Excoffier *et al.*, 2005).

The level of differentiation among populations was also estimated by means of genetic distance measures: Nei's (1978) distance and $(\delta\mu)^2$ (Goldstein *et al.*, 1995), the last one specific for microsatellites. Genetic distance matrices were calculated using MICROSAT.

In order to provide a graphical representation of the relationships among populations, as characterised by the pairwise F_{ST}, θ (Weir and Cockerham, 1984) estimates and by the genetic distance matrices (Nei, 1978), samples were ordinated in a bidimensional space by means of non-metric multidimensional scaling (nMDS, Lessa, 1990). nMDS analysis was performed using the software STATISTICA version 6.1 (Statsoft Inc., 1997).

A phylogenetic tree of samples was constructed using the UPGMA method implemented in the software package PHYLIP version 3.68 (Felsenstein,

2005). The Seqboot subprogram was used to produce a large number of bootstrapped data sets (10,000 replicates). The distance matrices (Cavalli-Sforza and Edwards, 1967) of the multiple datasets were calculated with the subprogram Genedist The UPGMA trees were constructed with the Neighbor subprogram and a majority rule consensus tree, following the method of Margush and McMorris (1981), was assessed by the subprogram Consense.

Isolation by distance

Mantel tests of correlations between genetic and geographical distance matrices, were implemented in the Isolation by Distance Web Service 3.11 (IBDWS) (Jensen *et al.* 2005). The geographical distances were calculated considering the minimal distance around the coast. The tests were conducted using two sets of parameters, F_{ST} and Nei (1978), in order to explore whether genetic distances were better correlated than F_{ST} with geographical distances.

5.3 Results

A total of 525 specimens from 17 locations/sites, with an average sample size of 29 individuals, were screened at three microsatellite loci.

Analysis of MOlecular VAriance (AMOVA)

Hierarchical partitioning of genetic variability was assessed by AMOVA (Table 5.2) at the 3 locations (Moonen Bay, Seil and Prawle Point) where 2 sites per location had been analyzed (Mo1 and Mo3; Se1 and Se3; Pr1 and Pr3) to estimate spatial scales of genetic variation. Results showed that genetic variation among locations was −0.76% and not significant. Negative values of variance are not uncommon and they can be obtained when the expectation of the estimator is zero, in which case slightly positive or negative variance components can be obtained by chance (Excoffier *et al.*, 1992). Differences between sites counted for 2.62% of the total genetic variation and were highly significant. For this reason in this study, sites from the same location were considered as separate entities and dealt with as if they were independent populations. The major source of variation, 98.15%, was within-site and therefore, occurred at the very small spatial scale (from a few centimetres to a few tens of meters, where specimens were collected) at the inter-individual level.

Table 5.2 – Hierarchical AMOVA among locations and between sites within location. Source of variation, degrees of freedom (df), variance components, percentage of variation (%), fixation indices and probability values (*P*-value) are listed.

Source of variation	df	Variance components	%	Fixation indices	P-value
Among locations	2	-0.00684	-0.76	-0.00765	0.86158
Between sites within location	3	0.02341	2.62	0.02596	0.00317
Within sites	376	0.87810	98.15	0.01852	0.00802

Genetic variability and Hardy-Weinberg equilibrium

A summary of the genetic variability per population at each microsatellite locus and at all loci is reported in Table 5.3. The three microsatellite loci used as molecular markers in this population genetic study exhibited different levels of polymorphism as shown from the number of alleles and from the allelic frequencies (Appendix 5.1). The locus CM 2/15 was the least polymorphic, ranging from 1 to 5 alleles per sample; at the peripheral population Fr2 (as well as the 'central' population Se1) this locus was monomorphic, while only 2 alleles were detected at the northern peripheral population Sk2 (and at Mo1, Be2, Wo2 and Pr3). The locus CM 4/5 was the most polymorphic, showing a minimum of 12 alleles and a maximum of 22 per sample: peripheral populations Fr2 and Sk2 showed respectively 16 and 17 alleles, while the mean number of alleles in the central populations was 18. The locus CM 5/23 displayed from 6 to 12 alleles per sample: in peripheral populations Fr2 and Sk2 9 and 7 alleles, respectively, were detected. This locus was the only one to exhibit private alleles in 5 out of the 17 screened populations (Mi1; Be2; Pr1; Mo1 and Mo3); it is worth noting that the marginal population Mi1 falls among these (Appendix 5.1). Three, 18 and 10 alleles were detected in marginal population Mi1 at CM2/15, CM4/5 and CM5/23, respectively.

Expected (H_E) and observed (H_O) heterozygosity values, averaged over all loci, ranged from 0.487 to 0.618 and from 0.221 to 0.415, respectively. Differences in observed heterozygosity over all loci between peripheral and central populations were not significant, even after multiple tests adjustments (t-tests on arcsin square root transformed values). Multilocus estimates of F_{IS} indicated heterozygote deficiency in all cases, ranging

between 0.270 and 0.629. Exact tests highlighted highly significant multilocus departures from HWE in all samples (Appendix 5.2). Dealing with single loci: at CM 2/15 only Rh2 and Mi1 (the marginal population) conformed HWE expectations; significant departures from HWE were observed at all samples for the most polymorphic locus, CM 4/5, (Appendix 5.2), while at CM 5/23 almost all populations fitted HWE expectations.

Generally, no linkage disequilibrium was detected among loci across all populations (Table 5.4) and therefore all loci were considered genetically independent. One exception to this result was the peripheral population Sk2, where the locus CM 4/5 was significantly linked to the locus CM 5/23. At Se1 and Fr2 (peripheral population) it was not possible to calculate the linkage between locus CM 2/15 and the other two loci because in these populations CM 2/15 was monomorphic.

Population structure

The genetic variability, estimated by F-statistics, recorded among and within the 17 samples showed a mean F_{ST} value of 0.002 and a mean F_{IS} of 0.432, while the R-statistics exhibited a mean R_{ST} value of 0.006 and an R_{IS} of 0.554 (Table 5.5). The results obtained with the two different estimators, F-statistics and R-statistics following the IAM and the SMM respectively, were comparable and quite similar over all loci; they both showed positive values of F_{IS} and R_{IS} within populations and low values of F_{ST} and R_{ST} revealing low, but significant, genetic subdivision among populations.

Pairwise R_{ST} (Slatkin, 1995) and F_{ST}, θ (Weir and Cockerham, 1984), estimates (Table 5.6) generally revealed low differentiation between each

pair of populations, but some comparisons were significant, as assessed with F_{ST} permutation tests (10,000 permutations). A graphical representation of the relationships among populations, as reported in pairwise F_{ST}, θ (Weir and Cockerham, 1984) was provided by the nMDS (Lessa, 1990) (Figure 5.4). The bi-dimensional distribution of populations showed that the peripheral populations Sk2 and Fr2 were separated from the others (and from each other), while the marginal population Mi1 was close to the central populations.

Matrices of Nei's genetic distance (1978) and $(\delta\mu)^2$ (Goldstein *et al.*, 1995), are shown in Table 5.7. The nMDS plot (Lessa, 1990), built using the genetic distance matrix (Nei, 1978), provided a graphical representation of the relationship among populations (Figure 5.5). This result confirmed that previously obtained with pairwise F_{ST} estimates: peripheral populations, Sk2 and Fr2, were far from the others, while the marginal population Mi1 was located near the central populations.

The UPGMA phylogenetic tree (Figure 5.6), constructed using a large number of bootstrapped data sets (10,000 replicates), based on distance matrices (Cavalli-Sforza and Edwards, 1967), supported the idea that peripheral populations were genetically differentiated from central ones. It showed that the northern peripheral population Sk2 was split from the others and that Fr2, the eastern peripheral one, was clustered with the marginal population Mi1 and separated from the other samples; it has to be said that bootstrap support at the relevant nodes was quite low suggesting a cautious approach to the interpretation of these results.

Isolation by distance

Mantel tests, conducted using two sets of genetic distances, F_{ST} and Nei (1978), were not significant. The reduced major axis regression calculated using F_{ST} showed r=0.105 and P-value=0.153 (Figure 5.7) and the one estimated using Nei (1978) exhibited r=0.113 and P-value=0.115 (Figure 5.8).

Table 5.3 – Summary of genetic variability per sites at each microsatellite locus and at all loci: number of sampled individuals (N), number of observed alleles per locus (N_A); allelic richness based on 25 individuals (A); Nei's 1987 unbiased expected heterozygosity (H_E); observed heterozygosity (H_O); Weir and Cockerham's (1984) estimate of Wright's (1951) fixation index (F_{IS}). F_{IS} values in bold indicate significant departures from HWE after standard Bonferroni correction. Peripheral (*Sk2* and *Fr2*) and marginal (*Mi1*) populations are in italics.

LOCUS	SITES																
	Sk2	Sc2	Mo1	Mo3	Se1	Se3	Be2	Rh2	Go2	*Mi1*	Wo2	Tr2	We2	Pr1	Pr3	Pv2	*Fr2*
Cm2/15																	
N	*23*	27	32	32	32	29	30	31	30	*32*	31	29	31	30	30	28	*32*
N_A	*2*	5	2	3	1	3	2	3	4	*3*	2	4	4	4	2	3	*1*
A	*2*	4.79	1.92	2.64	1.00	2.96	1.77	2.48	3.30	*2.44*	2.00	3.55	3.42	3.48	1.99	2.79	*1.00*
H_E	*0.294*	0.242	0.062	0.092	0.000	0.249	0.033	0.064	0.098	*0.062*	0.151	0.134	0.125	0.129	0.097	0.105	*0.000*
H_O	*0.000*	0.037	0.000	0.031	0.000	0.000	0.033	0.065	0.067	*0.063*	0.032	0.069	0.065	0.067	0.033	0.036	*0.000*
F_{IS}	*1.000*	**0.850**	**1.000**	**0.663**	NA	**1.000**	**0.000**	-0.008	**0.326**	*-0.008*	**0.789**	**0.489**	**0.489**	**0.489**	**0.659**	**0.663**	*NA*
Cm 4/5																	
N	*23*	26	25	30	26	30	26	32	28·	*31*	30	29	27	28	30	28	*31*
N_A	*17*	15	16	17	12	16	16	21	21	*18*	18	21	22	19	20	16	*16*
A	*17*	14.62	15.50	15.75	11.71	15.03	15.63	18.40	19.23	*16.83*	16.41	19.36	20.82	18.01	18.27	14.74	*14.44*
H_E	*0.893*	0.929	0.910	0.923	0.862	0.911	0.937	0.938	0.928	*0.930*	0.922	0.947	0.955	0.916	0.934	0.912	*0.903*
H_O	*0.391*	0.346	0.320	0.167	0.269	0.233	0.423	0.406	0.393	*0.355*	0.267	0.517	0.370	0.321	0.367	0.536	*0.419*
F_{IS}	*0.567*	**0.632**	**0.653**	**0.822**	**0.692**	**0.747**	**0.554**	**0.571**	**0.581**	*0.622*	**0.714**	**0.458**	**0.617**	**0.653**	**0.611**	**0.417**	*0.540*
Cm5/23																	
N	*25*	31	32	31	32	26	30	32	28	*29*	31	30	29	32	28	28	*32*
N_A	*7*	8	6	11	9	6	7	7	7	*10*	8	9	10	10	12	6	*9*
A	*6.76*	7.40	5.36	9.96	7.72	5.87	6.25	6.29	6.29	*9.12*	7.39	8.42	9.29	8.50	10.99	5.61	*7.85*
H_E	*0.662*	0.679	0.576	0.764	0.599	0.694	0.637	0.651	0.571	*0.774*	0.657	0.694	0.750	0.680	0.690	0.640	*0.727*
H_O	*0.560*	0.710	0.344	0.613	0.500	0.462	0.500	0.563	0.357	*0.828*	0.645	0.633	0.690	0.563	0.607	0.643	*0.656*
F_{IS}	*0.157*	-0.046	**0.407**	0.201	0.167	0.339	0.218	0.138	**0.379**	*-0.071*	0.018	0.089	0.081	0.175	0.122	-0.005	*0.099*

Table 5.3 - continued

ALL LOCI	Sk2	Sc2	Mo1	Mo3	Se1	Se3	Be2	Rh2	Go2	Mi1	Wo2	Tr2	We2	Pr1	Pr3	Pv2	Fr2
N_A	8.67	9.33	8.00	10.33	7.33	8.33	8.33	10.33	10.67	10.33	9.33	11.33	12.00	11.00	11.33	8.33	8.67
H_E	0.616	0.617	0.516	0.593	0.487	0.618	0.536	0.551	0.532	0.589	0.577	0.592	0.610	0.575	0.574	0.552	0.543
sd	0.302	0.348	0.428	0.441	0.442	0.338	0.460	0.446	0.416	0.463	0.392	0.416	0.432	0.403	0.431	0.411	0.479
H_O	0.317	0.364	0.221	0.270	0.256	0.232	0.319	0.344	0.272	0.415	0.315	0.407	0.375	0.317	0.336	0.405	0.359
sd	0.287	0.337	0.192	0.304	0.250	0.231	0.250	0.255	0.179	0.386	0.309	0.298	0.313	0.248	0.288	0.324	0.332
F_{IS}	0.491	0.414	0.576	0.548	0.478	0.629	0.410	0.379	0.493	0.298	0.458	0.317	0.390	0.453	0.419	0.270	0.344

Table 5.4 – Tests for linkage disequilibrium at each sites and for each locus pair, estimation of exact probability values (*P*-value) and standard error (S.E.) determined by a Markov-chain randomization (1,000,000 dememorizations, 1,000 batches and 50,000 iterations per batch). Significant *P*-values in bold. Peripheral (*Sk2* and *Fr2*) and marginal (*Mi1*) populations are in italics.

SITES	LOCUS 1	LOCUS 2	*P*-VALUE	S.E.
Sk2	*Cm2/15*	*Cm 4/5*	*0.4644*	*0.0048*
Sk2	*Cm2/15*	*Cm5/23*	*0.0900*	*0.0017*
Sk2	*Cm5/23*	*Cm 4/5*	***0.0133***	*0.0044*
Sc2	Cm2/15	Cm 4/5	0.8296	0.0085
Sc2	Cm2/15	Cm5/23	0.5133	0.0183
Sc2	Cm5/23	Cm 4/5	1.0000	0.0000
Mo1	Cm2/15	Cm 4/5	1.0000	0.0000
Mo1	Cm2/15	Cm5/23	1.0000	0.0000
Mo1	Cm5/23	Cm 4/5	0.9985	0.0006
Mo3	Cm2/15	Cm 4/5	0.4228	0.0097
Mo3	Cm2/15	Cm5/23	0.3624	0.0096
Mo3	Cm5/23	Cm 4/5	0.7611	0.0218
Se1	Cm2/15	Cm 4/5	Not calculable	
Se1	Cm2/15	Cm5/23	Not calculable	
Se1	Cm5/23	Cm 4/5	0.9831	0.0038
Se3	Cm2/15	Cm 4/5	0.6000	0.0045
Se3	Cm2/15	Cm5/23	0.0718	0.0030
Se3	Cm5/23	Cm 4/5	0.3158	0.0198
Be2	Cm2/15	Cm 4/5	0.8836	0.0041
Be2	Cm2/15	Cm5/23	0.2078	0.0034
Be2	Cm5/23	Cm 4/5	0.6576	0.0236
Rh2	Cm2/15	Cm 4/5	0.6235	0.0103
Rh2	Cm2/15	Cm5/23	0.4160	0.0080
Rh2	Cm5/23	Cm 4/5	0.6363	0.0244
Go2	Cm2/15	Cm 4/5	0.4555	0.0116
Go2	Cm2/15	Cm5/23	0.7170	0.0093
Go2	Cm5/23	Cm 4/5	0.6100	0.0253
Mi1	*Cm2/15*	*Cm 4/5*	*0.3752*	*0.0112*
Mi1	*Cm2/15*	*Cm5/23*	*0.2370*	*0.0044*
Mi1	*Cm5/23*	*Cm 4/5*	*0.8525*	*0.0231*
Wo2	Cm2/15	Cm 4/5	0.2764	0.0091
Wo2	Cm2/15	Cm5/23	0.2675	0.0072
Wo2	Cm5/23	Cm 4/5	0.3058	0.0287
Tr2	Cm2/15	Cm 4/5	0.4696	0.0142
Tr2	Cm2/15	Cm5/23	0.7241	0.0125
Tr2	Cm5/23	Cm 4/5	1.0000	0.0000
We2	Cm2/15	Cm 4/5	0.9946	0.0013
We2	Cm2/15	Cm5/23	0.8425	0.0071
We2	Cm5/23	Cm 4/5	0.4273	0.0313
Pr1	Cm2/15	Cm 4/5	0.7680	0.0124
Pr1	Cm2/15	Cm5/23	0.0841	0.0064
Pr1	Cm5/23	Cm 4/5	0.7128	0.0326
Pr3	Cm2/15	Cm 4/5	0.5976	0.0103
Pr3	Cm2/15	Cm5/23	0.1279	0.0057
Pr3	Cm5/23	Cm 4/5	0.7980	0.0261
Pv2	Cm2/15	Cm 4/5	0.5968	0.0051

Table 5.4 - continued

Pv2	Cm2/15	Cm5/23	1.0000	0.0000
Pv2	Cm5/23	Cm 4/5	0.7466	0.0238
Fr2	*Cm2/15*	*Cm 4/5*	*Not calculable*	
Fr2	*Cm2/15*	*Cm5/23*	*Not calculable*	
Fr2	*Cm5/23*	*Cm 4/5*	*0.8881*	*0.0148*
ALL SITES	Cm2/15	Cm 4/5	0.6480	
ALL SITES	Cm2/15	Cm5/23	0.8210	
ALL SITES	Cm5/23	Cm 4/5	0.9930	

Table 5.5 – *F*-statistics (Weir and Cockerham, 1984) and *R*-statistics (Slatkin, 1995) for each microsatellite locus and over loci in the 17 sites. Variances of estimators were obtained by jack-knifing over all sites. The 95% confidence interval (CI) was calculated by bootstrapping over loci. *P*-value value of global genetic differentiation tested with G-test (Goudet *et al.*, 1996).

	F-statistics			G test	*R*-statistics		
LOCUS	F_{IS}	F_{IT}	F_{ST}	*P*-values	R_{IS}	R_{IT}	R_{ST}
Cm 2/15	0.6791	0.6846	0.0171	0.0016	0.4067	0.4038	-0.0049
Cm 4/5	0.6153	0.6163	0.0026	0.0864	0.5783	0.5809	0.0063
Cm 5/23	0.1385	0.1373	-0.0014	0.1690	0.0557	0.0510	-0.0049
ALL LOCI	0.4320	0.4332	0.0020	0.0016	0.5538	0.5564	0.0057
CI 95%	(0.1385 - 0.6791)	(0.1373 - 0.6846)	(-0.0014 - 0.0170)				

Table 5.6 – Estimates of F_{ST} (below diagonal) and R_{ST} (above diagonal) for all microsatellite loci between pairs of sites. Values in bold indicate significant P-values tested with F_{ST} permutation tests (10,000 permutations). Peripheral (*Sk2* and *Fr2*) and marginal (*Mi1*) populations are in italics.

	Sk2	Sc2	Mo1	Mo3	Se1	Se3	Be2	Rh2	Go2	*Mi1*	Wo2	Tr2	We2	Pr1	Pr3	Pv2	*Fr2*
Sk2	-	**0.028**	*0.003*	*0.009*	***0.051***	*0.065*	*0.001*	**0.020**	*0.011*	*-0.018*	*0.017*	*-0.018*	*0.043*	***0.035***	*-0.007*	***0.015***	*0.018*
Sc2	*0.027*	-	-0.011	-0.010	-0.015	-0.011	-0.008	-0.012	-0.010	*0.014*	-0.002	0.008	0.042	-0.013	-0.003	**0.010**	*-0.007*
Mo1	*0.044*	0.013	-	-0.010	-0.013	-0.002	-0.017	-0.007	-0.006	*-0.004*	0.001	-0.004	0.046	-0.010	-0.008	0.003	*-0.016*
Mo3	*0.037*	0.007	0.023	-	-0.011	-0.014	-0.009	-0.010	-0.013	*0.004*	-0.005	-0.002	0.041	-0.002	-0.012	-0.010	*-0.004*
Se1	*0.054*	**0.031**	0.021	**0.034**	-	-0.008	-0.011	-0.003	-0.003	*0.021*	0.018	0.013	0.086	-0.011	-0.001	0.008	*-0.005*
Se3	*0.015*	0.015	0.031	0.014	0.040	-	0.001	-0.001	-0.005	*0.035*	0.019	**0.021**	0.093	0.001	0.000	0.000	***0.017***
Be2	*0.031*	0.008	0.012	**0.005**	0.024	0.011	-	-0.003	-0.004	*-0.005*	0.005	-0.005	0.051	-0.009	-0.008	0.002	*-0.016*
Rh2	***0.041***	0.011	0.014	0.009	0.038	0.022	0.008	-	-0.016	*0.008*	-0.013	0.000	0.031	0.002	-0.010	0.010	*0.000*
Go2	*0.035*	0.015	0.014	0.016	0.027	0.017	0.004	0.019	-	*0.004*	-0.015	-0.004	0.027	0.005	-0.014	0.002	*0.001*
Mi1	*0.025*	*0.016*	*0.029*	*0.005*	***0.033***	*0.012*	*0.011*	*0.014*	*0.023*	-	*0.005*	*-0.016*	*0.027*	*0.017*	*-0.008*	*0.011*	***0.001***
Wo2	*0.020*	0.007	0.013	0.012	0.022	0.013	0.008	0.020	0.011	*0.015*	-	-0.005	0.022	0.018	-0.012	0.014	*0.015*
Tr2	*0.031*	0.016	0.019	0.021	0.025	0.023	0.016	0.019	0.021	*0.022*	0.015	-	0.015	0.015	-0.013	0.005	*0.000*
We2	*0.030*	0.012	0.022	0.010	0.031	0.015	0.012	0.008	0.023	*0.009*	0.016	0.009	-	0.058	0.026	0.062	***0.067***
Pr1	*0.030*	0.014	0.025	0.015	0.036	0.020	0.012	0.016	0.020	*0.014*	0.009	0.013	0.018	-	0.008	0.018	*-0.007*
Pr3	*0.026*	**0.009**	**0.016**	0.011	0.033	0.017	**0.006**	0.019	0.009	*0.014*	**0.006**	0.015	0.014	0.011	-	-0.005	*-0.003*
Pv2	***0.029***	0.011	0.015	**0.010**	**0.041**	0.010	0.004	0.011	0.008	*0.015*	0.014	**0.022**	0.016	0.018	**0.009**	-	*0.014*
Fr2	***0.038***	***0.023***	*0.019*	***0.018***	*0.046*	*0.029*	*0.018*	*0.019*	*0.036*	***0.014***	*0.024*	***0.021***	*0.016*	*0.019*	***0.022***	*0.023*	-

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Table 5.7 – Pairwise genetic distances. Nei (1978) values are below the diagonal and $(\delta\mu)^2$ values are above the diagonal. Peripheral (*Sk2* and *Fr2*) and marginal (*Mi1*) populations are in italics.

	Sk2	Sc2	Mo1	Mo3	Se1	Se3	Be2	Rh2	Go2	Mi1	Wo2	Tr2	We2	Pr1	Pr3	Pv2	Fr2
Sk2	-	2.500	1.994	3.114	4.805	4.805	2.002	0.829	0.860	0.006	0.227	0.006	3.348	3.533	0.585	5.543	1.398
Sc2	0.028	-	0.066	0.053	0.388	0.388	0.083	0.495	0.486	2.277	1.473	2.547	11.127	0.132	0.694	0.711	0.186
Mo1	0.039	-0.001	-	0.156	0.638	0.638	0.002	0.371	0.372	1.787	1.206	2.062	10.284	0.222	0.486	0.935	0.054
Mo3	0.043	-0.003	0.014	-	0.185	0.185	0.168	0.808	0.786	2.866	1.977	3.170	12.475	0.068	1.026	0.388	0.371
Se1	0.045	0.017	0.012	0.024	-	0.168	0.159	0.888	0.875	2.959	2.102	3.291	12.755	0.019	1.115	0.372	0.381
Se3	0.009	0.010	0.021	0.008	0.000	-	0.650	1.740	1.709	4.493	3.343	4.877	15.630	0.146	2.076	0.088	1.048
Be2	0.024	-0.007	0.003	-0.006	0.014	-0.003	-	0.398	0.399	1.794	1.238	2.076	10.344	0.224	0.504	0.931	0.058
Rh2	0.042	0.000	0.006	0.000	0.031	0.016	-0.001	-	0.002	0.715	0.260	0.835	6.941	1.089	0.033	2.299	0.169
Go2	0.030	0.003	0.005	0.007	0.018	0.006	-0.005	0.013	-	0.747	0.272	0.862	7.007	1.084	0.035	2.254	0.176
Mi1	0.022	0.011	0.023	-0.006	0.022	0.005	0.001	0.006	0.016	-	0.192	0.019	3.631	3.254	0.489	5.208	1.225
Wo2	0.013	-0.004	0.003	0.004	0.010	0.004	-0.002	0.015	0.002	0.009	-	0.205	4.521	2.397	0.171	4.072	0.769
Tr2	0.032	0.010	0.009	0.018	0.012	0.021	0.007	0.013	0.014	0.019	0.008	-	3.212	3.620	0.597	5.639	1.455
We2	0.033	0.004	0.011	0.002	0.018	0.010	0.001	-0.004	0.015	0.000	0.010	0.000	-	13.410	6.383	17.149	8.860
Pr1	0.029	0.007	0.017	0.009	0.027	0.015	0.003	0.010	0.013	0.008	0.001	0.006	0.013	-	1.337	0.330	0.486
Pr3	0.023	-0.001	0.006	0.003	0.023	0.010	-0.005	0.014	-0.001	0.007	-0.004	0.008	0.007	0.003	-	2.610	0.232
Pv2	0.024	0.000	0.006	0.001	0.034	-0.001	-0.006	0.003	-0.001	0.007	0.007	0.017	0.008	0.012	0.000	-	1.432
Fr2	0.036	0.015	0.012	0.011	0.040	0.025	0.012	0.013	0.033	0.006	0.020	0.016	0.008	0.013	0.016	0.018	-
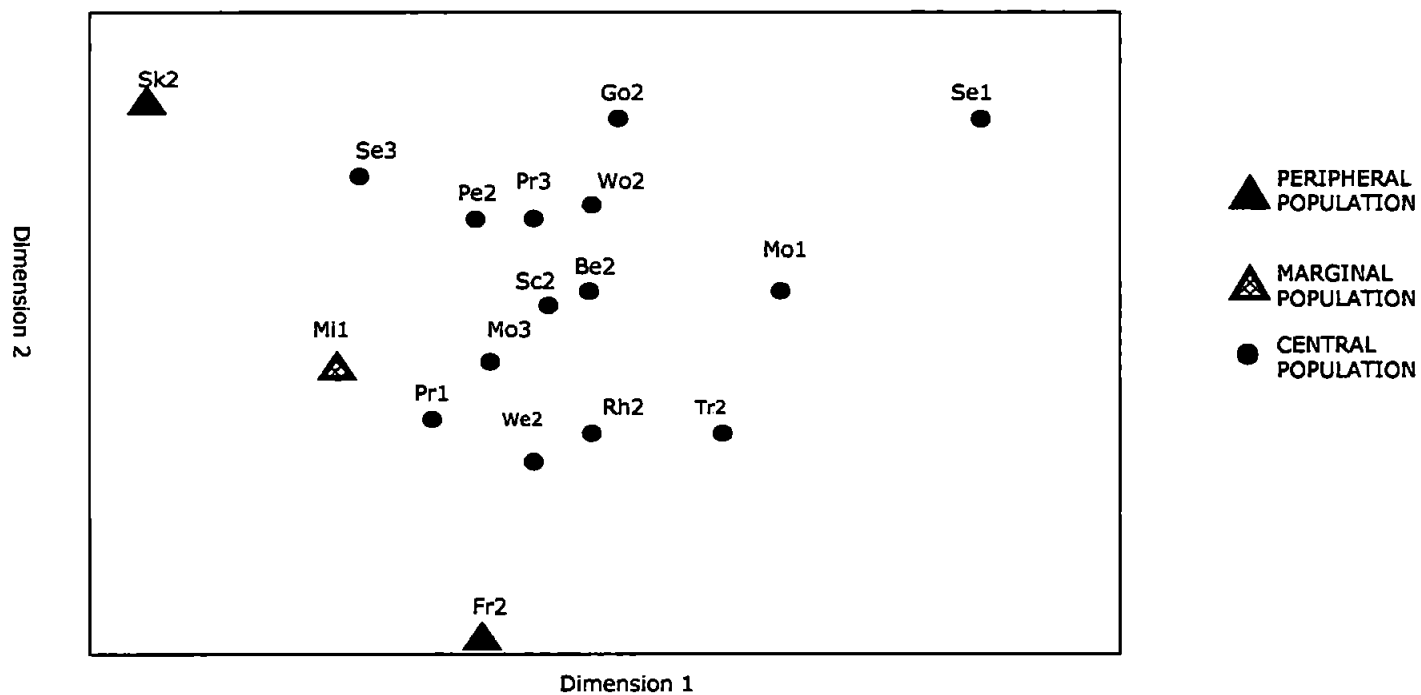
Figure 5.4 - Non-metric multidimensional scaling analysis (nMDS) among 17 samples based on F_{ST} (Weir and Cockerham, 1984). Stress value= 0.166 (for abbreviations see Table 4.1).

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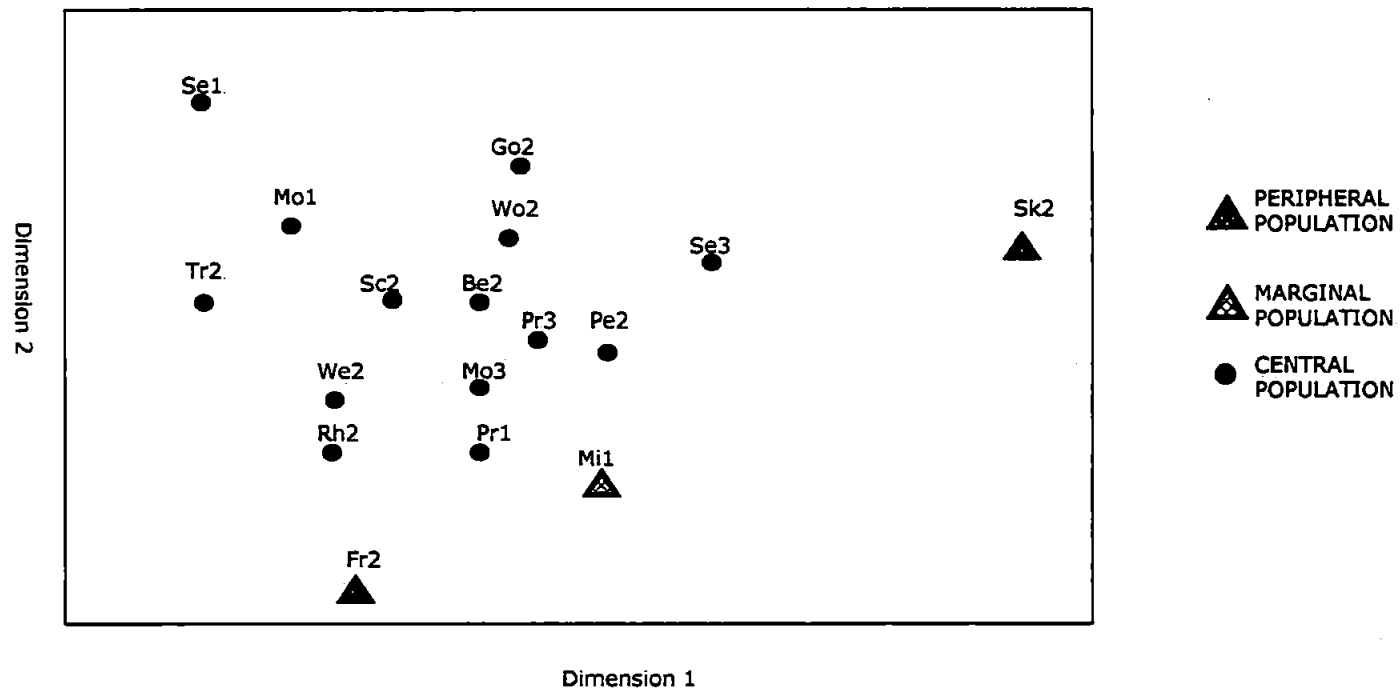
Figure 5.5 - Non-metric multidimensional scaling analysis (nMDS) among 17 sites based on Nei's (1978) genetic distance. Stress value= 0.176 (for abbreviations see Table 4.1).
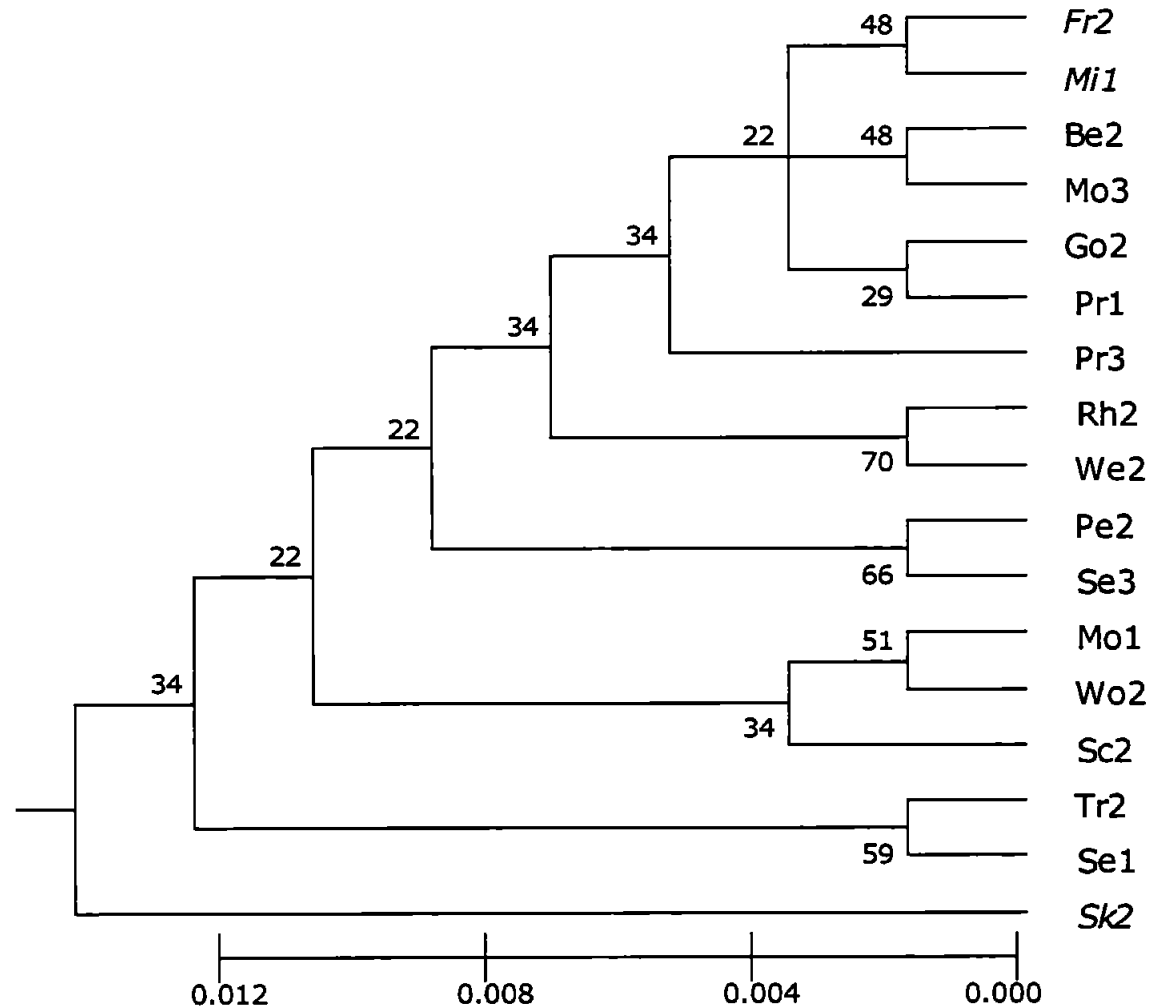
Figure 5.6 - UPGMA consensus tree based on Cavalli-Sforza and Edwards (1967) genetic distances; bootstrap (10,000 replicates) percentages are shown at nodes (for abbreviations see Table 4.1). Peripheral and marginal populations are in italics.
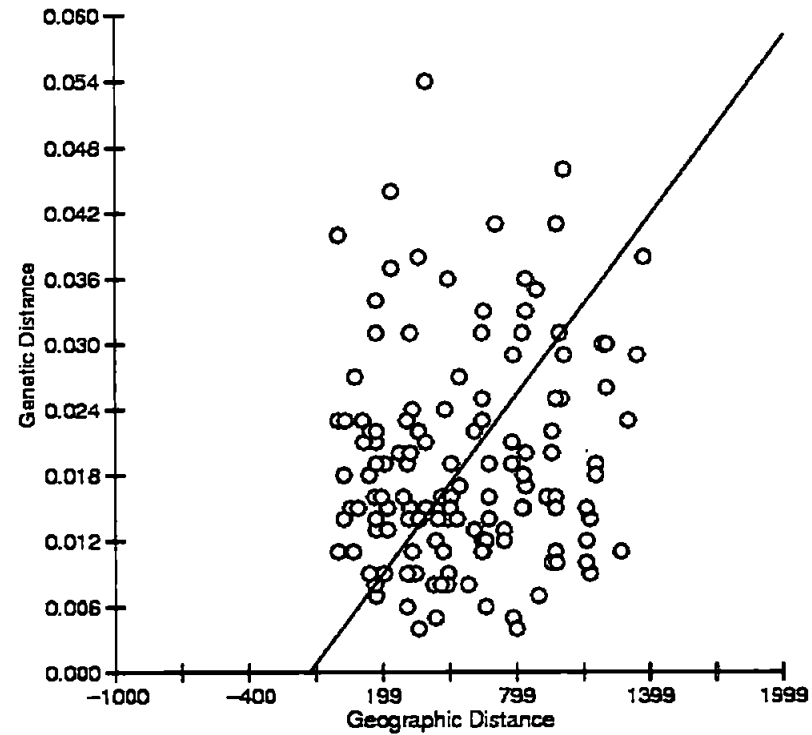
Figure 5.7 - Reduced major axis regression showing relationship between genetic and geographical distances for all 17 sites. Genetic distances based on F_{ST} (r=0.105; P-value=0.1530).
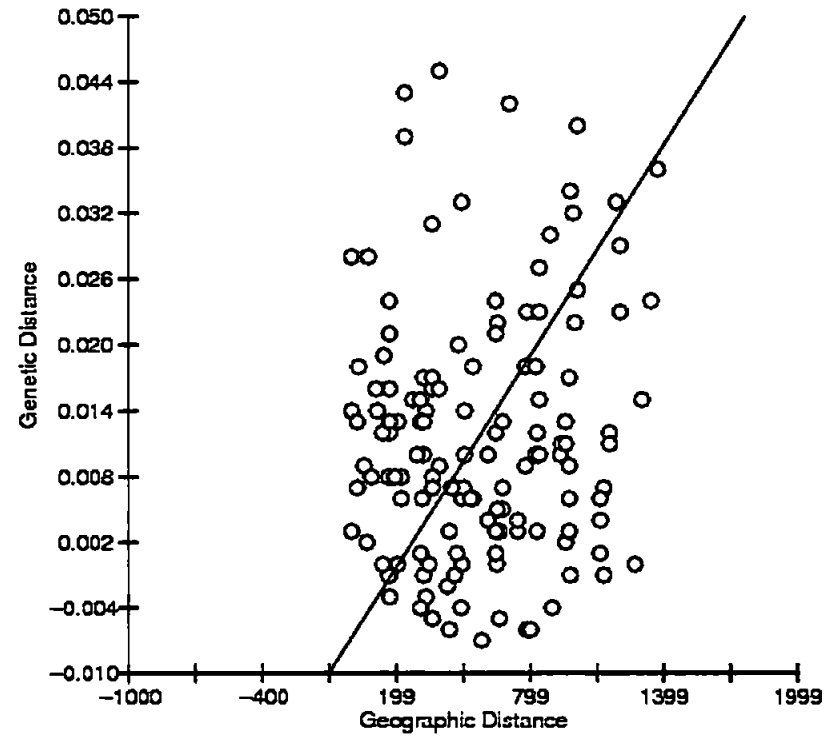
Figure 5.8 - Reduced major axis regression showing relationship between genetic and geographical distances for all 17 sites. Genetic distances based on Nei (1978) (r=0.113; *P*-value=0.115).

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5.4 Discussion

In this study the population genetic structure of the barnacle *Chthamalus montagui* was investigated over its whole distribution range in the UK, by analysing specimens from 17 locations/sites using three microsatellite markers. The aim was to assess the contribution of peripheral and marginal populations to the genetic diversity of the species.

Initially, spatial scales of genetic variation were investigated by analysing 2 sites from each of 3 locations. AMOVA results showed that differences among locations were not significant, while among sites within locations differences were still relatively small (2.62% of total variation) but significant, suggesting that, for the analysed loci, barnacle samples showed detectable differences over a distance of just a few hundred meters. An explanation for this could relate to the limited sample of the genome, three microsatellite loci, that was tested. A wider screening of the genome could provide a different pattern and possibly highlight similarities between sites of the same location. The major source of genetic variation (98.15%), instead, lays among individuals within sites therefore at a very small spatial scale (the plot of few tens of metres from which specimens were collected). This is often observed in population genetic studies of marine invertebrates employing microsatellites, suggesting that the high sensitivity and ability in detecting genetic variation of these molecular markers could be responsible for the observed pattern (e.g. Bertozzi, 1995; Costantini *et al.*, 2007; Lind *et al.*,.2007; York *et al.*, 2008). Nevertheless, the high genetic variation detected within sites could be attributed not only to the sensitivity of the molecular markers employed, but also to the biology and ecology of *C. montagui*. Barnacles brood developing embryos within the mantle cavity

before releasing larvae that feed and pass through several instars in the plankton. The released larvae spend a few weeks in the water column dispersing offshore, before finding a suitable substratum on which to settle, metamorphose and start the sessile phase. These features make it likely that barnacles originating from distant parental populations grow next to each other on the shore and partly explain the large genetic variability recorded within sites.

It is very important to bear in mind the life history and biology of *C. montagui* when focussing attention on ecologically marginal or peripheral populations. Due to the fact that these are located at the edge of the species' geographical and ecological distribution, it follows that they have neighbouring source populations in only one direction along the coast and as a result potentially receive a reduced amount of larval recruitment and gene flow.

The results obtained in this study do not disprove the hypothesis that peripheral populations are less genetically variable than central ones. Peripheral populations showed a slight lower multilocus mean number of alleles compared to the central ones. Fr2, the population at the eastern limit of the species distribution, showed a monomorphic locus (CM 2/15) and Sk2, the most northerly population, displayed linkage disequilibrium between the loci CM 4/5 and CM 5/23, a fact that was not recorded in any other sample. Linkage disequilibrium can be generated for instance by bottlenecks (Templeton, 2006).

Mi1, the marginal population, showed a number of alleles similar to the central populations.   Nevertheless, at locus CM 5/23, Mi1 exhibited an unusual genotype, showing a private allele. However, other populations also had private alleles at this locus.

Many other studies, carried out on different organisms, already demonstrated that reduced genetic variation and presence of unusual genotypes are quite common in peripheral and marginal populations, often isolated or adapted to exceptional environmental conditions (Lesica and Allendorf, 1995; Lammi et al., 1999; Schwartz et al., 2003; Johannesson and Andrè, 2006; Lind et al., 2007).

It is worth noting that no significant differences of expected and observed heterozygosity values over all loci were detected in t-tests between peripheral and central populations.   Expected heterozygosity has been widely used as a measure of genetic variation, however Allendorf (1986) compared the effects of bottlenecks on the loss of alleles and on the reduction in heterozygosity.   Population bottlenecks of short duration had little effect on heterozygosity, but reduced severely the number of alleles present.   Heterozygosity provides a good measure of the capability of a population to respond to selection immediately following a drastic event. Nevertheless, the number of alleles is a more important indicator for the long-term response to selection and survival of populations and species (Allendorf, 1986).

Furthermore, multilocus estimates of F_{IS} showed in all cases heterozygote deficiency,   and   exact   tests   highlighted   highly   significant   multilocus

departures from HWE in all samples. Heterozygosity deficits have been frequently observed in marine invertebrates (Zouros and Foltz, 1984; Gaffney, 1994; David et al., 1997; Ayre and Hughes, 2000; Ridgway et al., 2001; Addison and Hart, 2004; Duran et al., 2004; Le Goff-Vitry et al., 2004; Andrade and Solferini, 2007; Costantini et al., 2007) including barnacles (Veliz et al., 2006; York et al., 2008). The most common explanations for heterozygosity deficiency are: technical factors, such as the presence of null alleles (Foltz, 1986; Callen et al., 1993), and (or) biological factors, such as inbreeding or Wahlund effect (Pogson et al., 1995; Duran et al. 2004; Plutchack et al., 2006; Costantini et al., 2007).

It is difficult to provide an exact explanation for our findings, probably technical and biological factors could have mutually contributed. In fact, if we consider that locus CM 5/23 fitted HWE expectations while the other two loci did not, it can be suggested that some technical factors could have affected the two non-fitting loci.

Indices of genetic subdivision among populations, F_{ST} and R_{ST} estimators, showed quite low, but significant, mean values over all loci, revealing slight genetic differentiation.

Mantel tests conducted using two sets of genetic distances, F_{ST} and Nei (1978), to detect isolation by distance were not significant. These results are probably strictly related to the dispersal capability of the species; in fact, simulations using the one-dimensional stepping-stone model for population structure showed that isolation by distance is most obvious when comparing populations separated by 2-5 times the mean larval dispersal

distance (Palumbi, 2003). In our case the most distant populations (Sk2 and Fr2) were separated by nearly 1400 km of coastline that, while larval dispersal of barnacles in the open sea is in the order of 15-20 km (Strathmann, 1974; Crisp, 1976), meaning that genetic structuring should start to occur in samples that were at least 30-75 km distant. Given the fact that our sampling locations were interspersed at moderate distances (ranging from 25 to 332 km) along the coast, potentially allowing exchange of larvae between them, we should expect no genetic isolation at least in those population pairs that were geographically closer.

In conclusion, the results of this study are broadly consistent with the hypothesis that peripherality affects the genetic pattern of the barnacle *Chthamalus montagui*, in terms of reduced genetic variability and slight increased differentiation of peripheral populations compared to central ones.

Therefore, peripheral populations could be more sensitive to environmental pressures, by being less genetically variable and consequently less capable of coping with environmental changes, and should receive special attention in biodiversity conservation action plans (Crozier, 1997; Lesica and Allendorf 1995).

However, it is worth noting that the ability to draw definitive conclusions on the genetic pattern of barnacles based on the screening of just a few loci is limited.

Furthermore, it is important to highlight that the choice of molecular marker to employ seems to be of great importance for investigating loss of genetic

variability within a species. In fact, the meta-analysis carried out by Johannesson and Andrè (2006) on 29 species in the Baltic Sea showed that on average Baltic populations were less genetically variable than Atlantic ones: it emerged that this pattern was not related to dispersal capability, generation time of species and taxonomic group of organisms, but that was strongly related to the type of genetic marker employed (mitochondrial DNA loci displayed a loss of genetic diversity of about 50% while nuclear ones of only 10%).

Further investigations on this topic should be carried out using different markers in order to assess population fitness and to make predictions on the importance of maintaining genetic diversity within populations (Booy *et al*, 2000).

Chapter 6

Spatial scales of genetic variation

in *Chthamalus montagui*

6.1 Introduction and specific aims

The origin and maintenance of genetic diversity is a central issue in evolutionary biology. Managing marine genetic biodiversity requires detailed information on the spatial scales of population genetic structuring and differentiation. These scales are a function of the balance between gene flow, genetic drift, mutation and selection and are therefore strongly influenced by life history characteristics such as developmental modes (direct vs planktonic developers), larval dispersal capacity (short- vs long-lived planktonic larval stages), breeding systems (asexuality vs selfing vs outcrossing), etc. Hence, these life-history features, combined with the niche characteristics and environmental tolerance of marine organisms, will ultimately shape the geographic patterning of genetic biodiversity in marine ecosystems and the scales at which this patterning is apparent.

Obviously, the complexity of the interactions between these underlying factors makes it very difficult to generalize about patterns and scales of spatial genetic variation in the marine environment or to make predictions about them. Moreover, understanding these processes in the marine realm is particularly difficult, as barriers to gene flow are not as obvious as they can be in the terrestrial environment. It was long expected that populations of marine species could reach panmixia over a broad geographical range.

However, historical environmental factors related to habitat, currents and glaciations (Roy *et al.* 1996; Wares, 2002) combined with species-specific traits may play an important role in shaping the pattern of inter- and

intraspecific differentiation, although the relative contribution of each one remains hard to disentangle. These ancestral interactions combined with present-day environmental patterns are reflected in the biogeographical realm (Longhurst, 1998) and are the focus of marine phylogeographical investigations.

As reviewed by Patarnello *et al.* (2007), there are several documented examples of phylogeographical breaks in the marine environment. Well known cases include:

- the Southern Ocean and the formation of the Antarctic Polar Front (20–30 million years ago (Ma)) (Bargelloni *et al.*, 2000);

- the Equatorial Pacific and Atlantic Oceans and the formation of the Isthmus of Panama (3.1–3.5 Ma) (Avise, 2000);

- the northern and southern Atlantic Shelf of North America (Cape Hatteras) where the Labrador Current and Gulf Stream have met on and off during the Quaternary (Weinberg *et al.*, 2003);

- Cape Canaveral in the Florida Keys (reviewed in Avise, 1992);

- the Indo-Pacific region north and south of the Flores and Java Seas (Barber *et al.*, 2000);

- the NE Atlantic Ocean and Mediterranean Sea (Patarnello, 2007);

- the Baltic Sea and the North Sea (7500 years ago) (Olsen *et al.*, 2004).

Results of these studies showed that vicariance, separating the populations on either side of a given boundary, did not affect the different taxa in the same way over the same geographical range. Life history characteristics, e.g. dispersal capabilities, could be responsible for the different patterns, as

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generally long-dispersed species can overcome geographical barriers. A homogenizing effect can also be provided by extinction/recolonization events of a given area. In fact, extinction of one of two lineages followed by recolonisation by members of the other lineage results in a genetic homogenization over the entire distribution range of the species.

The NE Atlantic/Mediterranean area, the object of the present study, is a very young system that has been subjected to rapid changes in configuration and climate over the last six million years (Pérès, 1967; Blanc, 1968). The combination of events such as the opening and closing of the Strait of Gibraltar, advances and contractions of glaciations during the Quaternary, and changes in current patterns, quite certainly contributed towards generating diversity. The present-day Mediterranean biota is largely the result of colonization, mostly from the Atlantic Ocean (Almada *et al.*, 2001; Domingues *et al.*, 2005) and to a minor extent from the Red Sea. In fact, after an isolation and desiccation period that lasted about 0.5 million years, during the 'Messinian salinity crisis' (MSC) (up to 5.33 Ma), re-flooding of the Mediterranean basin was possible because of the inflow of Atlantic waters through the newly opened Strait of Gibraltar.

Up to 18% of the world's marine biodiversity, including more than 8500 species of macroscopic organisms, resides in this semi-enclosed basin (Longhurst, 1998; Bianchi and Morri, 2000). The high biodiversity recorded in the Mediterranean could partly be the product of the many taxonomical studies that have focused on this region, but the total diversity is still very high when considering that the Mediterranean Sea represents only 0.82% of the surface area and 0.32% of the volume of the world's oceans. In

addition, endemic species constitute 28% of the Mediterranean fauna (Fredj et al., 1992), although this value seems to decrease with increasing depth (Fredj *et al.*, 1985). *Euraphia depressa* is an example of a Mediterranean endemic (Kensler *et al.*, 1965; Crisp *et al.*, 1981), and is one of only three intertidal barnacles found in this basin.

The literature provides many instances, in different marine species, of intraspecific genetic differentiation between the NE Atlantic and Mediterranean area. Patarnello *et al.* (2007) reviewed more than 70 papers revealing patterns of partial or complete genetic isolation between Atlantic and Mediterranean populations, explored by a variety of molecular markers (allozymes, mtDNA, microsatellites, etc.), and concluded that there is no obvious relationship between species' life history and dispersal ability and the level of genetic differentiation recorded between the two basins.

Genetic structuring of intertidal barnacles in the NE Atlantic and Mediterranean area has already received some attention. Dando *et al.* (1979) compared, by allozyme studies, the populations of *Chthamalus montagui* from the Adriatic and SW England and found that the two differed. The same authors (1981), still employing electrophoretic analyses, suggested the existence of "Atlantic" and "Mediterranean" forms of *C. montagui* and hypothesised that for this barnacle speciation might be in progress. In this study they observed a sharp change in phosphoglucomutase (*PGM*) allozyme frequency along the SE coast of Spain and suggested that, during periods of glaciations and interglaciations associated with marine regression and transgression in the Pleistocene, the Atlantic and Mediterranean populations of *C. montagui* became physically

separated and then diverged genetically. The maintenance of separation of the two populations was explained by the presence of opposing coastal currents leaving long sandy beaches which created a reproductive barrier between the two populations. These results were confirmed by Pannacciulli *et al*. (1997a) in a more comprehensive study employing the same markers and including samples of *C. montagui* from most of its geographical range. Based on differences in allozyme frequencies of *PGM* and of glutamate-oxalacetate transaminase (*GOT*), the authors suggested that the Atlantic and Mediterranean forms of *C. montagui* are sibling species and that the present oceanographic conditions are the main cause for the genetic separation between the two forms as the Almeria-Oran front restricts larval dispersion.

Previous works on *Chthamalus montagui* were extended in this study, by investigating spatial scales of genetic differentiation of this barnacle species, with extensive larval dispersal, over its distribution range in the NE Atlantic and Mediterranean Sea. Six microsatellite loci were used as molecular markers, these sensitive markers allowed the investigation of fine scale spatial variation that the previous studies employing allozymes could not address.

6.2 Materials and methods

Sampling

Sample collection of *Chthamalus montagui* specimens was carried out in different periods:

- UK samples were collected in September/October 2002;

- Adriatic Sea samples were collected in February/March 2003;

- Samples from Baia, Vigo, Almeria and Molyvos were collected in August/September 2003.

The 12 sampling locations are shown in Figure 6.1 and listed in Table 6.1. At 6 locations a single sampling site was analysed. At the remaining 6, two sites (indicated by the 2 stars) 300-600 m apart were analysed. Thirty-two individuals were processed per site (although not all individuals were successfully analysed for each locus).

Figure 6.1 - Sampling locations of *Chthamalus montagui*.

Table 6.1 – Sampling location, site used in the genetic analysis, abbreviation, geographical coordinates and basin of origin.

Sampling location	Site	Abbreviation	Geographical coordinates		Basin
Skerray	2	Sk2	4.28 W	58.54 N	Atlantic
Moonen Bay	1	Mo1	6.79 W	57.42 N	Atlantic
Moonen Bay	3	Mo3	6.79 W	57.42 N	Atlantic
Seil	1	Se1	5.64 W	56.27 N	Atlantic
Seil	3	Se3	5.64 W	56.27 N	Atlantic
Prawle Point	1	Pr1	3.73 W	50.20 N	Atlantic
Prawle Point	3	Pr3	3.73 W	50.20 N	Atlantic
Freshwater Bay	2	Fr2	1.41 W	50.63 N	Atlantic
Vigo	1	Vigo	8.99 W	43.28 N	Atlantic
Almeria	1	Alme	2.19 W	36.72 N	Mediterranean
Baia Blu	1	Baia	9.89 E	44.08 N	Mediterranean
Sistiana	1	Si1	13.64 E	45.76 N	Mediterranean
Sistiana	3	Si3	13.64 E	45.76 N	Mediterranean
Grado	1	Gr1	13.39 E	45.67 N	Mediterranean
Grado	3	Gr3	13.39 E	45.67 N	Mediterranean
Portonovo	1	Po1	13.60 E	43.57 N	Mediterranean
Portonovo	3	Po3	13.60 E	43.57 N	Mediterranean
Molyvos	1	Moly	26.17 E	39.37 N	Mediterranean

Laboratory work

Six microsatellites (CM 2/15; CM 4/5; Cm 5/23; CM 4/3; CM 5/18; CM 9/11) were employed as molecular markers in this population genetic study. For the specific methods (e.g. PCR conditions, GeneScan, etc.) please refer to Chapter 3 of the thesis.

Genetic data analysis

Genetic variability and Hardy-Weinberg equilibrium

Genetic diversity within populations was estimated by calculating: the number of alleles per locus (N_A) and the allelic richness (A) using F-STAT version 2.9.3 (Goudet, 2001), the allele frequencies (F_A), and the observed (H_O) and Nei's 1987 unbiased expected heterozygosity (H_E) using GENETIX software package version 4.03 (Belkhir et al., 2004).

Single and multilocus F_{IS} were estimated using Weir and Cockerham's (1984) fixation index. Departures from Hardy-Weinberg equilibrium (HWE) were tested using Fisher's exact test, using the null hypothesis H_0 = no heterozygote deficiency, with the level of significance determined by Markov-chain randomization (10,000 dememorizations, 100 batches and 5000 iterations per batch) using GENEPOP version 4.0 (Rousset, 2008). Significance levels for multiple comparisons of loci across samples were adjusted using a standard Bonferroni correction (Rice, 1989).

Tests for linkage disequilibrium were performed at all loci in each population and for each locus pair across all populations using GENEPOP; estimation of exact probability values was by Markov-chain randomization (10,000 dememorizations, 100 batches and 5,000 iterations per batch).

Population structure

The level of population genetic differentiation was assessed using Weir and Cockerham's (1984) estimators of F-statistics (F_{IT} or F and F_{ST} or θ) calculated with the software F-STAT version 2.9.3 (Goudet, 2001). Variances of F-statistic estimators were obtained by jack-knifing over all populations according to Weir (1990). The 95% confidence interval was calculated by bootstrapping over the loci with 1,000 pseudoreplicates using the software GENETIX.

The analogue *rho* of R_{ST} of Slatkin (1995) was assessed using the computer program RSTCALC (Goodman, 1997). Pairwise F_{ST}, θ (Weir and Cockerham, 1984) and R_{ST} (Slatkin, 1995) estimates, were calculated using the software package MICROSAT version 1.5 (Minch *et al.*, 1996).

Global genetic differentiation per locus over all populations was tested with G tests (Goudet *et al.*, 1996) using GENEPOP, and for each pair of populations with F_{ST} permutation tests (10,000 permutations), as implemented in ARLEQUIN version 3.1 (Excoffier *et al.*, 2005).

The levels of differentiation among populations were also estimated by means of genetic distance measures: Nei's (1978) distance and $(\delta\mu)^2$ (Goldstein *et al.*, 1995), the latter specific for microsatellites. Genetic distance matrices were calculated using MICROSAT.

In order to provide a graphical representation of the relationships among populations as characterised by the pairwise R_{ST} and genetic distance matrices (Nei, 1978), samples were ordinated in a bidimensional space by non-metric multidimensional scaling (nMDS, Lessa, 1990). nMDS analysis was performed using the software STATISTICA version 6.1 (Statsoft Inc., 1997).

A phylogenetic tree was constructed using the UPGMA method implemented in the software package PHYLIP version 3.68 (Felsenstein, 2005). The Seqboot subprogram was used to produce a large number of bootstrapped data sets (10,000 replicates). The distance matrices (Cavalli-Sforza and Edwards, 1967) of the multiple datasets were calculated with the subprogram Genedist. The UPGMA trees were constructed with the Neighbor subprogram and a majority rule consensus tree, following the method of Margush and McMorris (1981), was assessed by the subprogram Consense.

Isolation by distance

Mantel tests of correlation between genetic and geographical distance matrices were implemented in the Isolation by Distance Web Service 3.11 (IBDWS) (Jensen *et al.* 2005). Nei's (1978) genetic distance was used. The geographical distances were calculated as the minimal distance around the coast.

Analysis of MOlecular VAriance (AMOVA)

An analysis of molecular variance (Excoffier *et al.*, 1992), implemented in ARLEQUIN version 3.1 (Excoffier *et al.*, 2005), was carried out to assess the hierarchical partitioning of genetic variability between basins, among locations within basins and within locations. For this purpose samples were divided into two groups, each one representing a basin, according to the results of the UPGMA phylogenetic tree.

A further AMOVA was carried out as implemented in HIERFSTAT (Goudet, 2005), to allow the assessment of the hierarchical partitioning of genetic variation at three different levels (ARLEQUIN only allows two). Pairwise F_{ST} estimates and *P*-values between basins, among locations within basins, between sites within locations and within sites were calculated.

Moreover, AMOVA was repeated in ARLEQUIN, analysing Atlantic and Mediterranean datasets separately, and only using data from locations with two sites, to assess the hierarchical partitioning of genetic variability among locations and between sites within locations in the two separate basins.

Bayesian clustering analysis

Population structure was further investigated using Bayesian clustering analysis implemented in STRUCTURE 2.2 (Pritchard *et al.*, 2000). Each

individual was assigned to probable common clusters based on the similarity of their multilocus genotypes at the six microsatellite loci. Mean and variance of log likelihoods of the number of clusters for $K = 1$ to $K = 18$ were inferred from multilocus genotypes by running STRUCTURE five times with 1,000,000 repetitions each (burn-in=100,000 iterations) under the admixture ancestry model and the assumption of correlated allele frequencies among samples as suggested in Falush *et al.* (2003). The mean membership of each individual described the likelihood of that individual's belonging to the respective clusters.

6.3 Results

A total of 534 specimens from 18 sites, with an average sample size of 30 individuals, were screened at six microsatellite loci.

Genetic variability and Hardy-Weinberg equilibrium

A summary of the genetic variability per population at each microsatellite locus and at all loci is reported in Table 6.2. The six microsatellite loci used as molecular markers in this population genetic study exhibited different levels of polymorphism as shown by the number of alleles (N_A) or, better, by the allelic richness (A), which accounts for sample size biases, as well as by the expected heterozygosity (H_E) and the allelic frequencies (Appendix 6.1).

The locus CM 2/15 and the locus CM 5/23 were the least and the most polymorphic microsatellites, respectively, ranging from 1 to 10 alleles per sample and 6 to 30 alleles per sample. Allelic richness and expected heterozygosity at these two loci (Figure 6.2 and 6.3) showed a wide difference between Atlantic and Mediterranean sites. CM 2/15 was monomorphic at Fr2 and Se1 and CM 5/23 exhibited its minimum value of A

(4.44) at Mo1 and the maximum (19.77) at Moly. Samples from Alme showed the lowest values of A (2.805 at CM 2/15 and 8.06 at CM 5/23) and H_E (0.273 at Cm 2/15 and 0.637 at Cm 5/23) amongst the Mediterranean samples, and were comparable to the Atlantic sites.

The locus CM 4/3, at which A ranged from 2.99 to 11.86 and H_E from 0.522 to 0.912, exhibited private alleles in 7 out of the 18 screened populations (Sk2, Se1, Pr1, Fr2, Alme, Baia and Moly); Fr2 showed 4 private alleles at this locus (Appendix 6.1). The highest A (14.45) and H_E (0.943) values at the locus CM 4/5 were registered in Alme samples and the lowest ones in Moly (A=5.84; H_E=0.624).

A and H_E values per site averaged over all loci are shown in Figure 6.4. A ranged from 8.50 (Se1) to 14.33 (Si1), H_E from 0.608 (Se1) to 0.849 (Si1). Atlantic sites exhibited lower A and H_E values compared to the Mediterranean ones; Alme samples showed the lowest values of the Mediterranean sites (A=10.67; HE=0.692).

Observed heterozygosity (H_O) over all loci ranged between 0.262 (SK2) and 0.524 (Baia); among Atlantic samples Pr1, Pr3 and Mo1 showed higher values compared to the others of 0.387, 0.463 and 0.442, respectively. Multilocus estimates of F_{IS} were all positive, ranging from 0.314 to 0.629 (Table 6.2). Exact tests revealed highly significant multilocus departures from HWE in all samples (Appendix 6.2).

Generally no linkage disequilibrium was detected among loci across all populations (Appendix 6.3) and, therefore, all loci were considered

genetically independent. Nevertheless, three exceptions to this rule were registered: CM 5/23 and CM 4/5 were significantly linked in Sk2 samples; CM 4/5 and CM 5/18 were significantly linked in Mo3 samples; and CM 5/23 and CM 9/11 were significantly linked in Pr3 samples.

Population structure

The genetic variability, estimated by F-statistics, recorded among and within the 18 samples, showed a mean F_{ST} value of 0.097 and a mean F_{IS} of 0.515, while the R-statistics exhibited a mean R_{ST} value of 0.390 and an R_{IS} of 0.438 (Table 6.3). Positive and high values of F_{IS} and R_{IS} indicated deficit of heterozygosity within populations. The highest F_{ST} value (0.212) was registered at the locus CM 2/15 and the highest R_{ST} value (0.622) was recorded at locus CM 5/23. The two different estimators, F-statistics and R-statistics following the IAM and the SMM respectively, revealed genetic subdivision among populations, as shown by the highly significant P-values of global genetic differentiation.

Pairwise R_{ST} (Slatkin, 1995) and F_{ST}, θ (Weir and Cockerham, 1984) estimates (Table 6.4) generally revealed significant differentiation between each pair of populations, as assessed with F_{ST} permutation tests (10,000 permutations)

Matrices of Nei's genetic distance (1978) and $(\delta\mu)^2$ (Goldstein et al., 1995), are reported in Table 6.5.

Graphical representations of the relationships among populations, as indicated by pairwise R_{ST} (Slatkin, 1995) and Nei's genetic distance (1978) matrices, were provided by the nMDS plots (Lessa, 1990). The bi-

dimensional distribution of populations based on R_{ST} (Figure 6.5) showed three main clusters: 1. Adriatic samples, with Moly very close to them; 2. most of the Atlantic samples (Sk2, Fr2, Se1, Se3, Mo3 and Vigo), with Alme very close to them; 3. three Atlantic sites (Pr1, Pr3 and Mo1). Baia was located between clusters 1 and 2.

The nMDS plot based on Nei's genetic distance (Figure 6.6) showed the same three main clusters as noted for R_{ST}, but Moly was separated from the Adriatic samples, Alme was placed closer to cluster 3 than cluster 2, and Baia was no longer between clusters 1 and 2.

The UPGMA phylogenetic tree (Figure 6.7), constructed using a large number of bootstrapped data sets (10,000 replicates) based on distance matrices (Cavalli-Sforza and Edwards, 1967), showed two main clusters very well supported by high bootstrap percentages at the nodes: 1. Mediterranean samples (bootstrap 99%); 2. Atlantic samples plus Almeria (bootstrap 88%). A further separation into two sub-clusters was revealed within cluster 2: as previously observed in the nMDS plots, three sites among the Atlantic samples (Pr1, Pr3 and Mo1) were clustered together and separated from the others; Mo1 and Mo3 thus appeared in different sub-clusters. Baia was separated from the other Mediterranean samples within cluster 1, and the bootstrap percentage at the relevant node was very high (99%). Moreover, separation between Molyvos and the Adriatic samples was suggested, but it was not very well supported by the bootstrap percentage at the node (43%). It is worth noting that within the cluster of Adriatic samples, the 2 sites within the same location (Si1 and Si3; Gr1 and Gr3; Po1 and Po3) were clustered together.

Isolation by distance

The Mantel test showed a highly significant correlation between genetic and geographical distance matrices (Figure 6.8). The reduced major axis regression calculated using Nei's (1978) genetic distance matrix showed r= 0.774 and P-value= 0.001.

Analysis of MOlecular VAriance (AMOVA)

Initially, AMOVA was carried out to assess the hierarchical partitioning of genetic variability between basins, among locations within basins and within locations (Table 6.6). Samples were grouped in two groups according to the results of the UPGMA phylogenetic tree: group 1 (Mediterranean samples); group 2 (Atlantic samples + Almeria). Results showed that differences between groups were highly significant (P-value=0.000) and counted for 9.35% of the total genetic variation. Genetic variability among locations within groups was 3.61 % and was highly significant. The major source of variation laid within locations (87.05%).

Further investigation of the hierarchical partitioning of genetic variation was carried out, as implemented in HIERFSTAT, considering one more level: sites within location. Only locations with two sites were analysed (Se1 and Se3; Mo1 and Mo3, Pr1 and Pr3; Si1 and Si3; Gr1 and Gr3; Po1 and Po3). Pairwise F_{ST} estimates and P-values were calculated between basins, among locations within basins, between sites within locations and within sites (Table 6.7). The F_{ST} value between basins was 0.123 and the P-value 0.05; genetic difference among locations within basins was significant (P-value=0.016) and the F_{ST} estimate was 0.014; genetic variability between sites within locations was highly significant and corresponded to an F_{ST} value of 0.019; the major source of genetic differentiation was within sites

and had an F_{ST} estimate of 0.454. To further investigate the highly significant genetic differentiation between sites within locations, AMOVA was repeated for the Mediterranean and Atlantic locations separately.

Results for the Mediterranean locations (Sistiana, Grado and Portonovo) showed that genetic variation among locations was not significant (P-value=0.068) and counted for only 0.94% of the total genetic variability (Table 6.8). Differences between sites (Si1 and Si3; Gr1 and Gr3; Po1 and Po3) counted for 0.58% of the total genetic variation and were not significant (P-value=0.764). The major source of variation 98.48% laid within site and therefore resided in the very small spatial scale (the plot of few metres from which specimens were collected) at the inter-individual level.

Results for the Atlantic locations (Seil, Moonen Bay, Prawle Pt) showed that genetic variation among locations was not significant (P-value=0.457) and counted for 1.5% of the total genetic variability (Table 6.9). In this case differences between sites (Se1 and Se3; Mo1 and Mo3; Pr1 and Pr3) were highly significant and counted for 3.19% of the total genetic variation. The major source of variation was, as all the other cases, within sites: 95.32% of the total genetic variability.

Bayesian clustering analysis

STRUCTURE analyses recovered the highest posterior probabilities of the data for two (K=2; ln P of data=-12279.2) and three (K=3; ln P of data=-11887.7) clusters. The runs performed for K>3 did not show any different structure.

For K = 2, samples were separated into two clear clusters (Figure 6.9) (cluster 1: Baia, Sl1, Sl3, Gr1, Gr3, Po1, Po3, Moly; cluster 2: Sk2, Mo1, Mo3, Se1, Se3, Pr1, Pr3, Fr2, Vigo, Alme). Mediterranean and Atlantic samples were clustered separately, except for that the Almeria sample behaved as an Atlantic sample, although it showed the lowest proportion of membership (0.85) of the samples included in cluster 1 (Table 6.10). The highest probability of assignment of a member to a cluster was 0.99 for Mo1 samples in cluster 2 and 0.98 for Gr3 samples in cluster 1 (Table 6.10).

The subdivision of samples into three clusters (K=3) (Figure 6.10) confirmed the separation between Mediterranean (cluster 1) and Atlantic samples. The latter were further split into two clusters (cluster 2: Sk2, Mo3, Se1, Se3, Fr2, Vigo, Alme; cluster 3: Mo1, Pr1, Pr3). The mean proportion of membership of samples to each cluster is shown in Table 6.11. This confirmed the results obtained with the UPGMA phylogenetic tree and nMDS plots. Note that Mo1 and Mo3 again fall into different clusters in the K=3 analysis.

Table 6.2 – Summary of genetic variability per site at each microsatellite locus and all loci: number of sampled individuals (N); number of observed alleles per locus (N_A); allelic richness based on 13 individuals (A); Nei's 1987 unbiased expected heterozygosity (H_E); observed heterozygosity (H_O); Weir and Cockerham's (1984) estimate of Wright's (1951) fixation index (F_{IS}). F_{IS} values in bold indicate significant departures from HWE after standard Bonferroni correction. For site abbreviations see Table 6.1.

LOCUS	SITES																	
CM2/15	Sk2	Mo1	Mo3	Se1	Se3	Pr1	Pr3	Fr2	Vigo	Alme	Baia	Si1	Si3	Gr1	Gr3	Po1	Po3	Moly
N	23	32	32	32	29	30	30	32	24	23	23	31	32	29	29	30	28	22
N_A	2	2	3	1	3	4	2	1	2	3	9	10	7	10	7	9	7	8
A	2	1.651	2.058	1	2.678	2.55	1.825	1	1.911	2.805	7.295	7.772	5.904	7.882	5.973	7.492	5.36	7.261
H_E	0.294	0.062	0.092	0.000	0.249	0.129	0.097	0.000	0.120	0.273	0.787	0.814	0.768	0.771	0.757	0.816	0.708	0.801
H_O	0.000	0.000	0.031	0.000	0.000	0.067	0.033	0.000	0.042	0.044	0.391	0.548	0.531	0.621	0.552	0.400	0.429	0.591
F_{IS}	**1.000**	**1.000**	**0.663**	-	**1.000**	**0.489**	0.659	-	0.657	**0.844**	**0.508**	**0.330**	**0.312**	**0.198**	**0.274**	**0.514**	**0.399**	**0.267**
CM4/5																		
N	23	25	30	26	30	28	30	31	22	23	24	27	28	27	29	28	29	23
N_A	17	16	17	12	16	19	20	16	14	18	11	15	11	13	13	15	14	7
A	12.79	12.04	12.54	9.65	12.17	13.80	14.05	11.39	12.03	14.45	9.33	11.53	8.90	10.14	10.32	11.64	11.04	5.842
H_E	0.893	0.910	0.923	0.862	0.911	0.916	0.934	0.903	0.929	0.943	0.872	0.874	0.845	0.881	0.856	0.870	0.890	0.624
H_O	0.391	0.320	0.167	0.269	0.233	0.321	0.367	0.419	0.318	0.391	0.208	0.222	0.214	0.333	0.241	0.250	0.241	0.348
F_{IS}	**0.567**	**0.653**	**0.822**	**0.692**	**0.747**	**0.653**	**0.611**	**0.540**	**0.663**	**0.590**	**0.765**	**0.749**	**0.750**	**0.626**	**0.722**	**0.716**	**0.732**	**0.448**
CM5/23																		
N	25	32	31	32	26	32	28	32	24	22	24	27	30	27	28	29	29	24
N_A	7	6	11	9	6	10	12	9	7	11	14	28	30	30	27	27	25	29
A	5.41	4.44	7.73	5.90	5.14	6.41	8.15	6.32	5.64	8.06	9.63	18.28	18.44	19.53	18.02	18.04	16.52	19.77
H_E	0.662	0.576	0.764	0.599	0.694	0.680	0.690	0.727	0.690	0.637	0.746	0.967	0.970	0.976	0.968	0.969	0.956	0.978
H_O	0.560	0.344	0.613	0.500	0.462	0.563	0.607	0.656	0.542	0.364	0.792	0.519	0.600	0.556	0.536	0.586	0.517	0.500
F_{IS}	0.157	**0.407**	**0.201**	**0.167**	**0.339**	0.175	0.122	0.099	0.218	**0.435**	-0.063	**0.468**	**0.385**	**0.436**	**0.451**	**0.399**	**0.464**	**0.494**

Table 6.2 - continued

CM4/3	Sk2	Mo1	Mo3	Se1	Se3	Pr1	Pr3	Fr2	Vigo	Alme	Baia	Si1	Si3	Gr1	Gr3	Po1	Po3	Moly
N	16	21	15	13	21	21	21	21	15	22	25	18	19	25	24	22	25	24
N_A	7	13	3	5	10	10	14	10	7	7	9	8	10	8	9	10	9	9
A	6.72	10.94	2.99	5.00	9.24	9.02	11.86	8.87	6.92	6.10	7.41	7.15	8.86	6.94	7.73	8.79	7.10	7.699
H_E	0.796	0.899	0.522	0.625	0.870	0.855	0.912	0.775	0.635	0.648	0.825	0.824	0.873	0.786	0.836	0.876	0.769	0.847
H_O	0.063	0.429	0.067	0.077	0.143	0.143	0.333	0.048	0.000	0.136	0.440	0.333	0.632	0.280	0.333	0.273	0.480	0.292
F_{IS}	0.879	0.397	0.987	0.806	0.867	0.885	0.577	0.930	1.071	0.800	0.339	0.396	0.169	0.558	0.575	0.628	0.200	0.572
LOCUS	**SITES**																	
CM5/18	Sk2	Mo1	Mo3	Se1	Se3	Pr1	Pr3	Fr2	Vigo	Alme	Baia	Si1	Si3	Gr1	Gr3	Po1	Po3	Moly
N	25	25	24	26	23	29	25	31	24	25	25	20	21	25	25	23	19	24
N_A	8	8	11	8	8	8	6	11	10	9	9	11	11	11	6	8	5	9
A	6.01	5.38	7.89	6.46	6.61	5.19	4.59	7.38	7.34	6.79	6.51	8.96	8.28	7.60	4.70	6.24	4.56	6.299
H_E	0.595	0.605	0.661	0.621	0.651	0.549	0.614	0.627	0.598	0.716	0.530	0.735	0.612	0.606	0.441	0.536	0.538	0.614
H_O	0.320	0.880	0.542	0.346	0.435	0.379	0.720	0.484	0.375	0.400	0.440	0.700	0.429	0.360	0.360	0.391	0.105	0.333
F_{IS}	0.467	-0.469	0.183	0.447	0.337	0.313	-0.177	0.231	0.377	0.446	0.172	0.048	0.305	0.411	0.186	0.275	0.809	0.463
CM9/11																		
N	25	25	22	22	22	27	25	31	22	22	24	20	21	25	25	22	25	25
N_A	16	11	16	16	16	17	16	17	17	16	17	14	15	12	17	12	17	23
A	13.18	9.01	13.06	13.81	13.49	12.34	11.78	12.50	14.15	12.98	12.81	11.49	11.87	9.09	11.79	8.80	11.86	16.3
H_E	0.937	0.791	0.932	0.944	0.939	0.869	0.851	0.927	0.933	0.933	0.901	0.880	0.826	0.780	0.873	0.727	0.825	0.958
H_O	0.240	0.680	0.455	0.409	0.364	0.852	0.720	0.323	0.364	0.364	0.875	0.600	0.476	0.280	0.920	0.591	0.560	0.880
F_{IS}	0.748	0.143	0.518	0.572	0.618	0.020	0.156	0.656	0.616	0.616	0.029	0.323	0.429	0.646	-0.055	0.191	0.326	0.083
ALL LOCI																		
A	9.50	9.33	10.17	8.50	9.83	11.33	11.67	10.67	9.50	10.67	11.50	14.33	14.00	14.00	13.17	13.50	12.83	14.17
H_E	0.696	0.641	0.649	0.608	0.719	0.666	0.683	0.660	0.651	0.692	0.777	0.849	0.816	0.800	0.788	0.799	0.781	0.804
sd	0.237	0.317	0.315	0.331	0.258	0.297	0.314	0.342	0.298	0.246	0.133	0.078	0.120	0.124	0.183	0.151	0.148	0.157
H_O	0.262	0.442	0.312	0.267	0.273	0.387	0.463	0.322	0.273	0.283	0.524	0.487	0.480	0.405	0.490	0.415	0.389	0.491
sd	0.209	0.306	0.255	0.194	0.181	0.288	0.270	0.256	0.210	0.153	0.255	0.177	0.151	0.147	0.243	0.147	0.178	0.222
F_{IS}	0.629	0.314	0.524	0.568	0.626	0.424	0.326	0.517	0.586	0.596	0.330	0.431	0.416	0.499	0.383	0.485	0.507	0.395
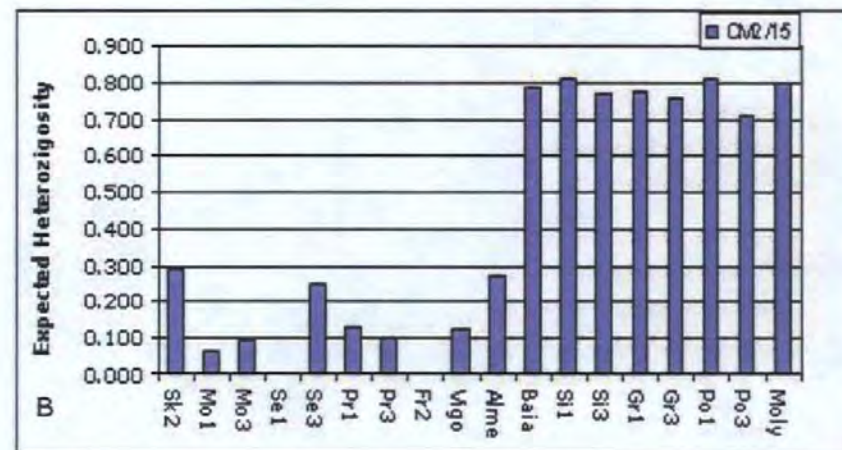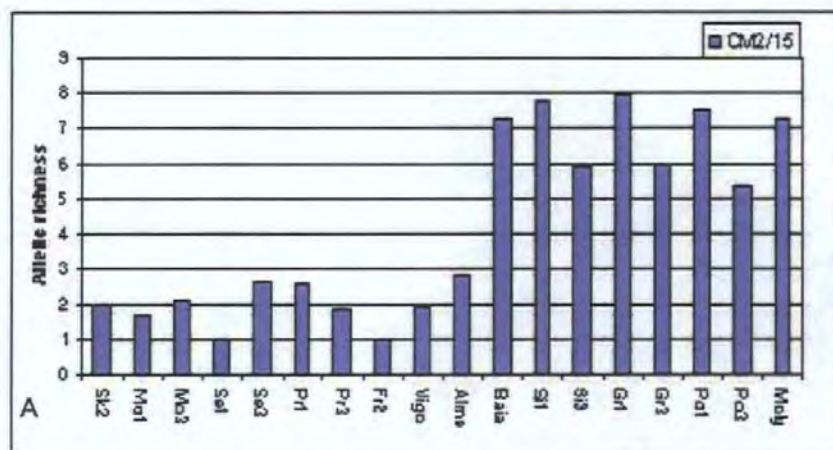
Figure 6.2 – Allelic richness (A) and expected heterozygosity (B) at locus CM 2/15 per site. For site abbreviations see Table 6.1
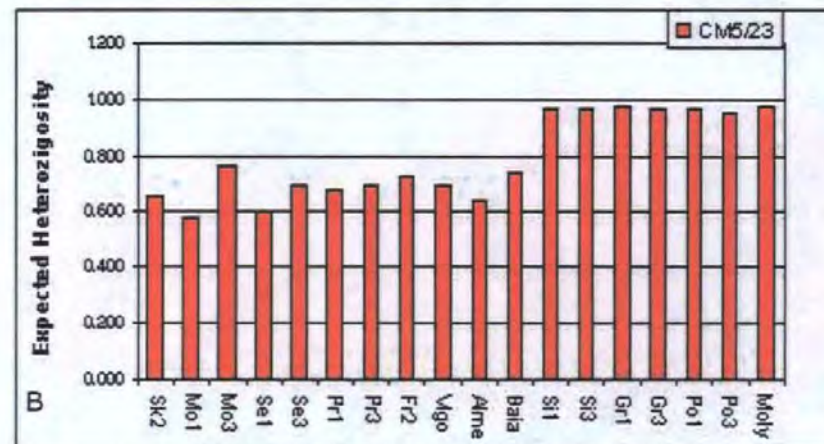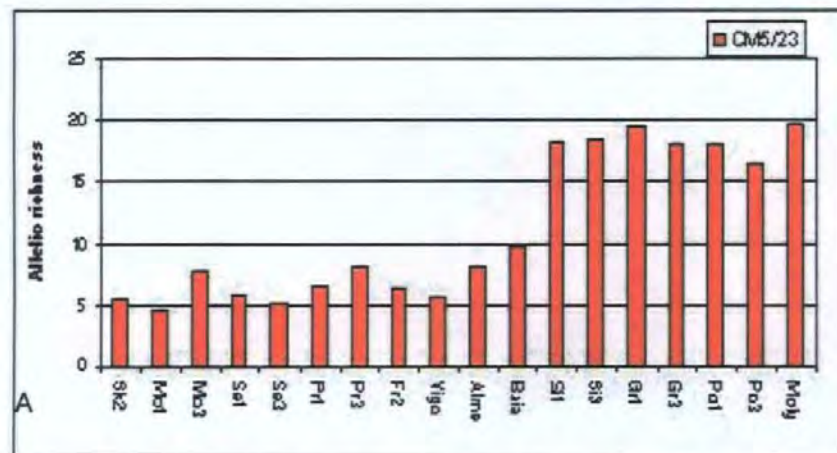
Figure 6.3 – Allelic richness (A) and expected heterozygosity (B) per site at locus CM 5/23. For site abbreviations see Table 6.1
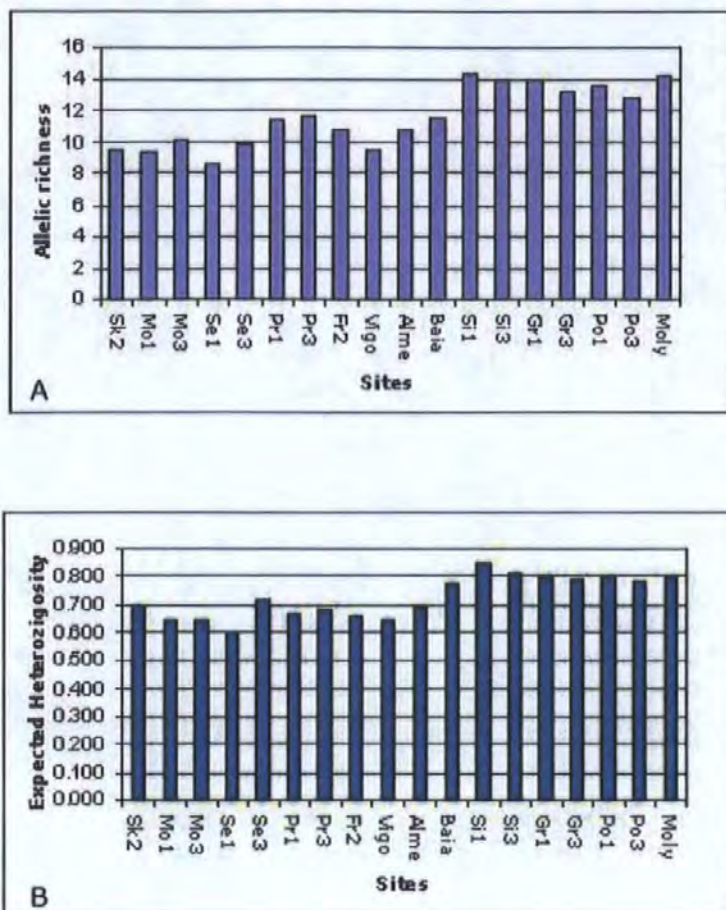
Figure 6.4 – Allelic richness (A), expected heterozygosity (B) per site over all loci. For site abbreviations see Table 6.1.

Table 6.3 – F-statistics (Weir and Cockerham, 1984) and R-statistics (Slatkin, 1995) for each microsatellite locus and over loci in the 18 populations. Variances of estimators were obtained by jack-knifing over all populations. The 95% confidence interval (CI) was calculated by bootstrapping over the loci. P-value of global genetic differentiation tested with G-test (Goudet *et al.*, 1996).

	F-statistics			G test	R-statistics		
LOCUS	F_{IS}	F_{IT}	F_{ST}	P-values	R_{IS}	R_{IT}	R_{ST}
Cm2/15	0.430	0.551	0.212	0.000	0.352	0.438	0.133
Cm 4/5	0.675	0.689	0.043	0.000	0.649	0.679	0.085
Cm5/23	0.313	0.386	0.106	0.000	0.461	0.796	0.622
Cm 4/3	0.672	0.701	0.089	0.000	0.673	0.836	0.499
Cm 5/18	0.262	0.342	0.109	0.000	0.176	0.268	0.111
Cm 9/11	0.373	0.416	0.068	0.000	0.283	0.474	0.266
ALL LOCI	0.463	0.515	0.097	0.000	0.438	0.657	0.390
CI 95%	0.329-0.599	0.399-0.643	0.066-0.138				

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Table 6.4 – Estimates of F_{ST} (below the diagonal) and R_{ST} (above the diagonal) for all microsatellite loci among pairs of sites. Values in bold indicate significant P-values tested with F_{ST} permutation tests (10,000 permutations).

	Sk2	Mo1	Mo3	Se1	Se3	Pr1	Pr3	Fr2	Vigo	Alme	Baia	Si1	Si3	Gr1	Gr3	Po1	Po3	Moly
Sk2	-	0.437	0.011	0.030	0.019	0.443	0.386	0.012	0.036	0.011	0.193	0.328	0.398	0.345	0.375	0.299	0.389	0.433
Mo1	0.120	-	0.324	0.447	0.328	0.019	-0.009	0.423	0.379	0.295	0.436	0.477	0.528	0.517	0.528	0.480	0.546	0.514
Mo3	0.034	0.134	-	0.011	-0.018	0.329	0.283	0.026	0.023	-0.011	0.161	0.286	0.338	0.306	0.346	0.267	0.331	0.361
Se1	0.040	0.128	0.021	-	0.003	0.452	0.401	0.012	-0.008	0.008	0.157	0.319	0.395	0.344	0.377	0.304	0.373	0.412
Se3	0.026	0.107	0.032	0.036	-	0.325	0.286	0.029	0.020	-0.011	0.156	0.288	0.341	0.307	0.347	0.268	0.329	0.358
Pr1	0.130	0.028	0.151	0.154	0.111	-	-0.001	0.436	0.387	0.294	0.443	0.488	0.537	0.524	0.531	0.480	0.552	0.527
Pr3	0.092	0.018	0.113	0.115	0.077	0.013	-	0.388	0.340	0.255	0.410	0.454	0.503	0.489	0.504	0.451	0.518	0.498
Fr2	0.028	0.115	0.022	0.030	0.027	0.133	0.096	-	0.020	0.028	0.138	0.277	0.330	0.289	0.329	0.264	0.309	0.361
Vigo	0.034	0.129	0.027	0.032	0.040	0.155	0.118	0.020	-	0.009	0.136	0.287	0.355	0.303	0.346	0.282	0.320	0.370
Alme	0.044	0.131	0.059	0.063	0.044	0.126	0.092	0.068	0.094	-	0.112	0.221	0.268	0.231	0.281	0.203	0.251	0.292
Baia	0.134	0.237	0.187	0.198	0.132	0.230	0.194	0.180	0.175	0.137	-	0.073	0.163	0.101	0.149	0.116	0.117	0.133
Si1	0.121	0.208	0.160	0.183	0.115	0.193	0.167	0.158	0.160	0.129	0.039	-	0.024	-0.005	0.014	0.011	0.029	0.031
Si3	0.121	0.215	0.151	0.169	0.115	0.207	0.178	0.153	0.153	0.138	0.060	0.017	-	0.005	0.018	0.007	0.013	0.042
Gr1	0.118	0.216	0.161	0.179	0.123	0.198	0.172	0.156	0.163	0.124	0.058	0.024	0.012	-	0.001	-0.004	0.002	0.060
Gr3	0.129	0.233	0.182	0.198	0.134	0.222	0.192	0.172	0.174	0.141	0.050	0.025	0.019	0.019	-	0.003	0.031	0.072
Po1	0.140	0.234	0.188	0.205	0.135	0.224	0.194	0.178	0.179	0.159	0.050	0.021	0.017	0.023	0.023	-	0.027	0.075
Po3	0.134	0.234	0.174	0.191	0.128	0.222	0.192	0.175	0.178	0.132	0.047	0.028	0.024	0.021	0.027	0.026	-	0.063
Moly	0.140	0.234	0.192	0.206	0.140	0.217	0.192	0.181	0.181	0.157	0.061	0.029	0.050	0.053	0.042	0.042	0.044	-

Table 6.5 – Pairwise genetic distances. Nei (1978) values are below the diagonal and $(\delta\mu)^2$ values are above the diagonal.

	Sk2	Mo1	Mo3	Se1	Se3	Pr1	Pr3	Fr2	Vigo	Alme	Baia	Si1	Si3	Gr1	Gr3	Po1	Po3	Moly
Sk2	0	190.21	4.57	3.82	5.68	202.05	176.68	4.72	6.84	6.41	29.19	99.41	132.90	106.15	159.67	109.98	104.86	132.83
Mo1	0.260	0	144.89	173.60	144.69	11.31	3.00	220.30	156.78	140.61	240.45	299.68	330.74	321.81	406.82	328.14	309.96	301.05
Mo3	0.046	0.275	0	3.53	0.42	156.24	134.38	10.81	5.59	2.14	31.74	102.85	136.31	113.27	171.18	117.01	109.78	127.30
Se1	0.048	0.233	0.015	0	2.99	183.39	162.77	5.99	1.50	4.22	18.68	91.79	127.68	100.88	155.40	108.21	95.80	117.91
Se3	0.036	0.233	0.042	0.038	0	152.44	133.25	12.59	5.18	2.00	30.20	102.58	136.65	112.89	170.21	116.26	108.53	126.50
Pr1	0.315	0.033	0.346	0.316	0.269	0	4.73	241.79	168.34	149.12	254.42	317.66	349.67	336.35	417.34	335.54	323.15	324.15
Pr3	0.209	0.013	0.246	0.221	0.174	0.006	0	211.81	147.72	129.66	234.26	296.25	327.52	315.58	398.31	317.38	304.84	304.28
Fr2	0.037	0.232	0.024	0.033	0.036	0.301	0.204	0	9.48	13.98	19.73	90.82	125.49	99.51	152.25	108.81	97.23	118.93
Vigo	0.046	0.258	0.029	0.033	0.059	0.357	0.258	0.018	0	4.72	20.38	91.26	127.63	100.52	156.37	110.48	94.50	117.40
Alme	0.078	0.289	0.105	0.095	0.082	0.298	0.206	0.131	0.191	0	25.37	81.44	111.27	89.98	142.21	93.16	86.60	105.54
Baia	0.444	0.901	0.620	0.575	0.469	0.974	0.757	0.605	0.566	0.454	0	41.11	70.61	49.22	87.13	60.76	43.42	57.00
Si1	0.449	0.902	0.570	0.588	0.461	0.895	0.742	0.583	0.583	0.493	0.111	0	4.54	1.49	12.53	6.80	1.48	7.81
Si3	0.423	0.882	0.488	0.487	0.431	0.931	0.757	0.521	0.509	0.513	0.216	0.026	0	3.72	6.88	5.25	6.03	9.71
Gr1	0.394	0.836	0.522	0.516	0.459	0.800	0.673	0.518	0.542	0.418	0.200	0.057	0.008	0	6.84	3.39	1.16	13.79
Gr3	0.442	0.934	0.623	0.602	0.506	0.966	0.788	0.591	0.589	0.500	0.159	0.055	0.033	0.036	0	6.94	10.06	24.84
Po1	0.518	0.988	0.683	0.662	0.529	1.033	0.840	0.653	0.637	0.625	0.161	0.039	0.028	0.053	0.053	0	6.73	21.30
Po3	0.458	0.919	0.567	0.556	0.463	0.933	0.768	0.597	0.601	0.444	0.144	0.063	0.054	0.043	0.064	0.061	0	11.081
Moly	0.509	0.963	0.700	0.653	0.558	0.948	0.818	0.652	0.637	0.605	0.217	0.084	0.186	0.194	0.134	0.139	0.141	0
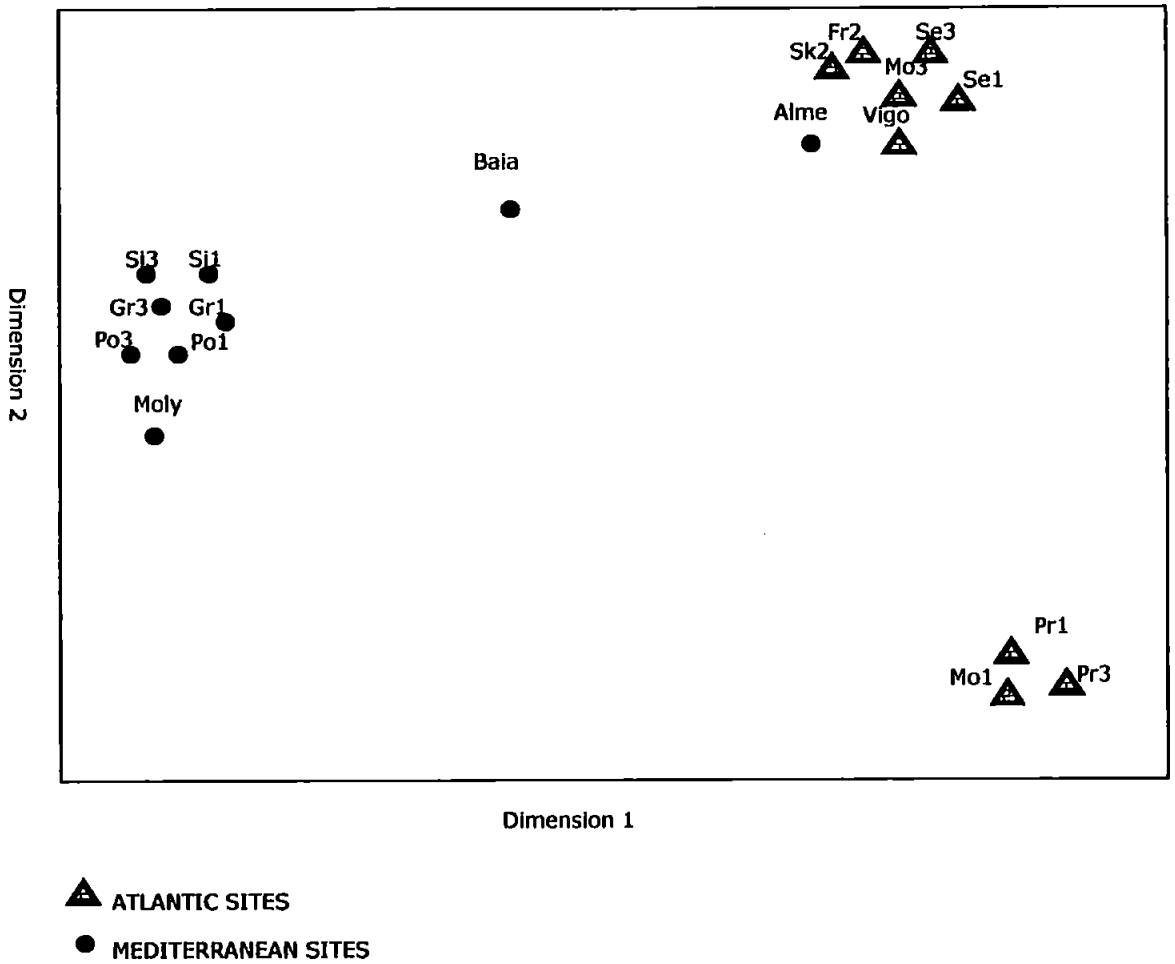
Figure 6.5 – Non-metric multidimensional scaling analysis (nMDS) of 18 sites based on R_{ST} (Weir and Cockerham, 1984). Stress value= 0.026 (for abbreviations see Table 6.1).
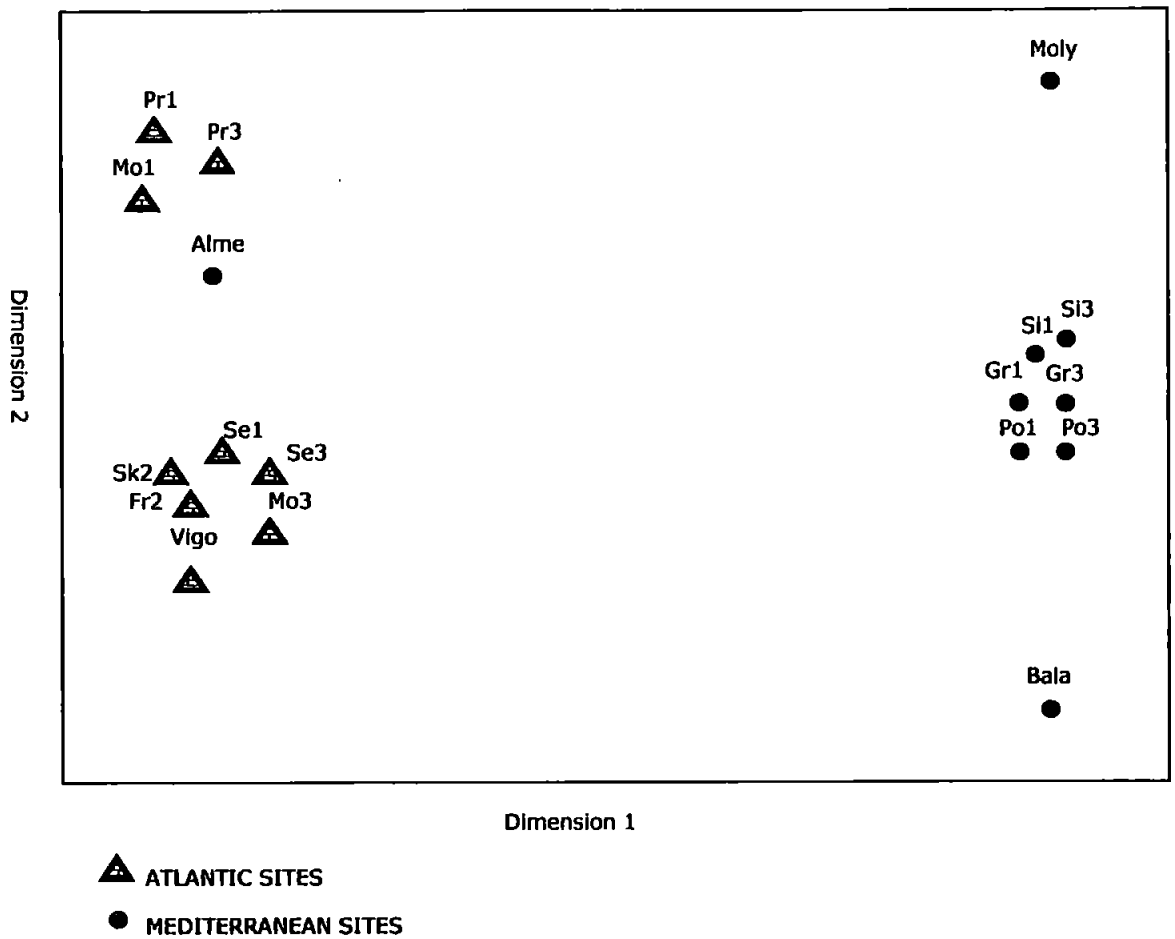
Figure 6.6 - Non metric multidimensional scaling analysis (nMDS) of 18 sites based on Nei's (1978) genetic distance. Stress value= 0.005 (for abbreviations see Table 6.1).
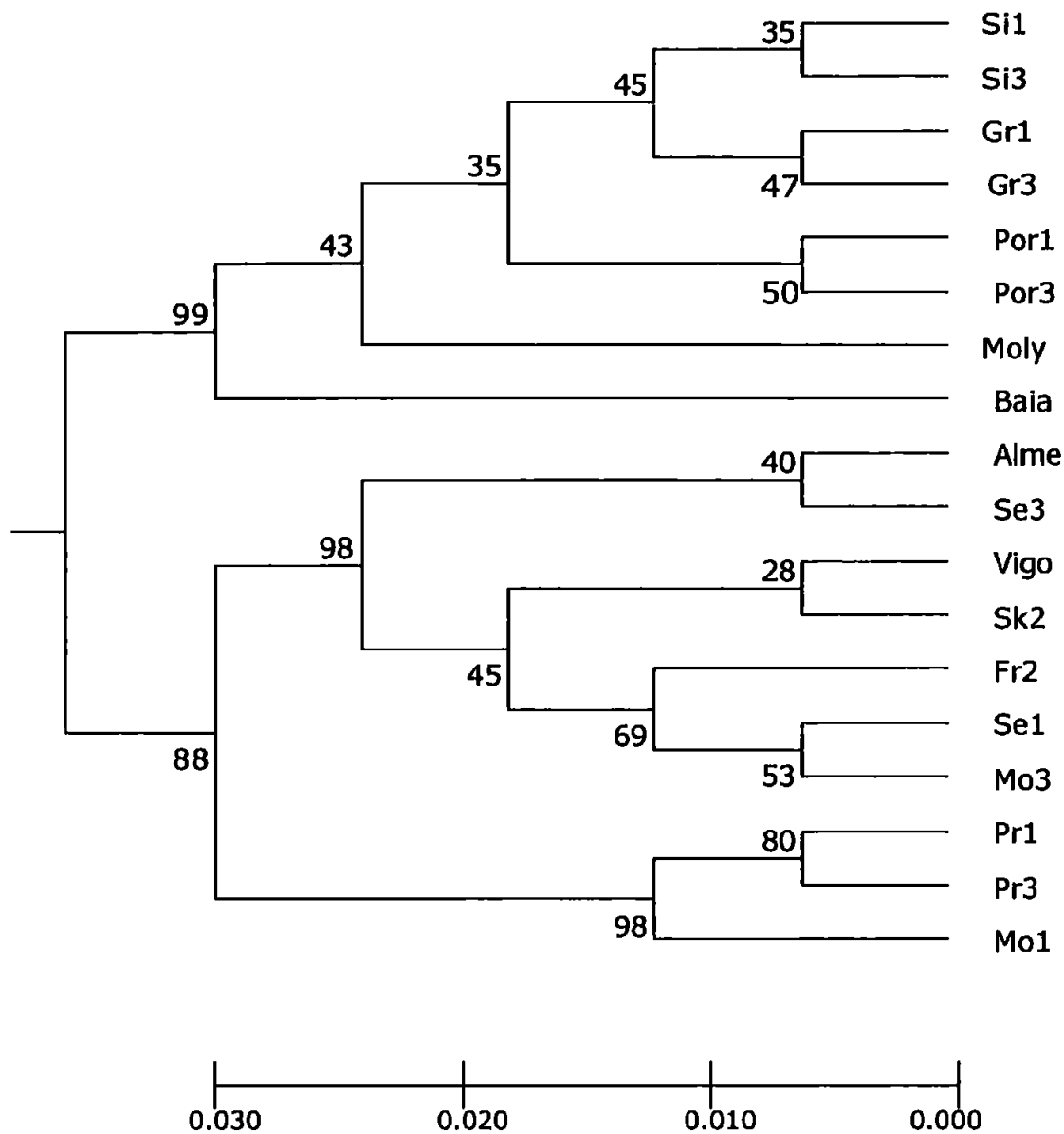
Figure 6.7 - UPGMA consensus tree based on Cavalli-Sforza and Edwards (1967) genetic distances; bootstrap (10,000 replicates) percentages are shown at nodes (for abbreviations see Table 6.1).
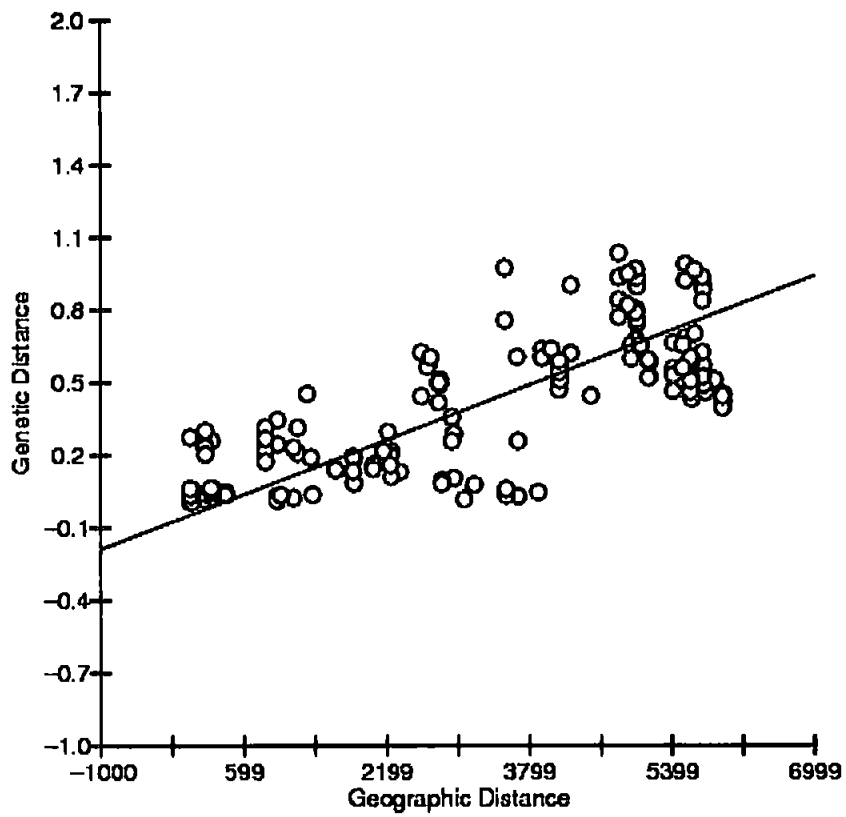
Figure 6.8 - Reduced major axis regression showing relationships between genetic and geographical distances for all 18 samples. Genetic distances based on Nei (1978) (r= 0.774; P-value= 0.001).

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Table 6.6 – Hierarchical AMOVA among samples grouped in two groups ('basins'): group 1, Atlantic locations; group 2, Mediterranean locations. Source of variation, degrees of freedom (df), variance components, percentage of variation (%), fixation indices and probability value (P-value) are listed.

Source of variation	df	Variance components	%	Fixation indices	P-value
Between basins	1	0.24126	9.35	0.09346	0.000
Among locations within basin	16	0.09313	3.61	0.0398	0.000
Within locations	1050	2.24709	87.05	0.12953	0.000

Table 6.7 – Hierarchical AMOVA among samples as implemented in HIERFSTAT. Pairwise F_{ST} estimates and P-value between basins, among locations within basins, among sites within locations and within sites.

F_{ST}	P-value	Between basins	Among locations within basins	Between sites within locations	Within sites
Total		0.123	0.135	0.152	0.537
Between basins	0.050		0.014	0.032	0.472
Among locations within basins	0.016			0.019	0.464
Between sites within locations	0.005				0.454

Table 6.8 – Hierarchical AMOVA among the Mediterranean locations with two sites per location. Source of variation, degrees of freedom (df), variance components, percentage of variation (%), fixation indices and probability value (P-value) are listed.

Source of variation	df	Variance components	%	Fixation indices	P-value
Among locations	2	0.02378	0.94	0.00941	0.068
Between sites within location	3	0.01455	0.58	0.00581	0.764
Within sites	364	2.48927	98.48	0.01516	0.118

Table 6.9 – Hierarchical AMOVA among the Atlantic locations with two sites per location. Source of variation, degrees of freedom (df), variance components, percentage of variation (%), fixation indices and probability value (P-value) are listed.

Source of variation	df	Variance components	%	Fixation indices	P-value
Among locations	2	0.03213	1.5	0.01495	0.457
Between sites within location	3	0.0685	3.19	0.03236	0.000
Within sites	376	2.04815	95.32	0.04683	0.000

Figure 6.9: Clustering analysis conducted in STRUCTURE 2.2 (K=2). In the bar plot, each vertical bar along the x axis represents one of 534 individuals grouped by location/site (see abbreviation in Table 6.1); the Y-axis represents the estimated proportion of membership of each individual to each cluster (represented by different colours).

Table 6.10: Mean proportion of membership of each sample (location/site) to each cluster (K=2).

	Sk2	Mo1	Mo3	Se1	Se3	Pr1	Pr3	Fr2	Vigo	Alme	Baia	Si1	Si3	Gr1	Gr3	Po1	Po3	Moly
Cluster 1	0.13	0.01	0.06	0.04	0.13	0.04	0.08	0.02	0.06	0.16	0.96	0.96	0.95	0.93	0.98	0.94	0.92	0.94
Cluster 2	0.87	0.99	0.94	0.97	0.87	0.96	0.92	0.98	0.94	0.85	0.04	0.04	0.06	0.07	0.02	0.06	0.08	0.06

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Proportion of individual membership
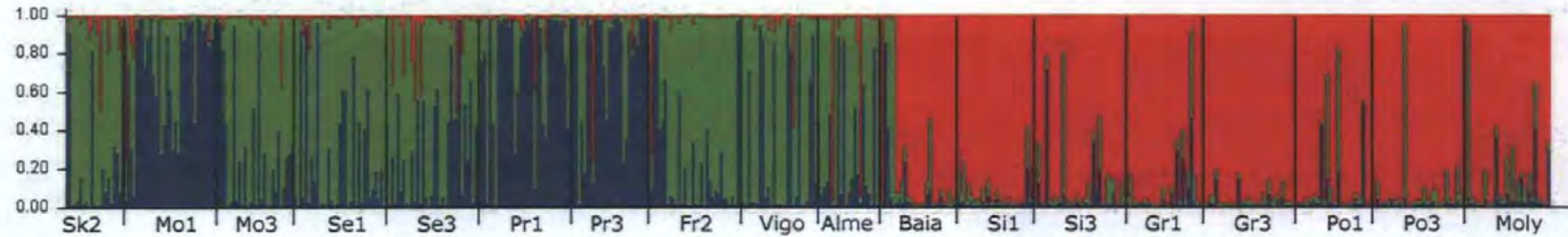
Figure 6.10: Clustering analysis conducted in STRUCTURE 2.2 (K=3). In the bar plot, each vertical bar along the x axis represents one of 534 individuals grouped by location/site (see abbreviation in Table 6.1); the Y-axis represents the estimated proportion of membership of each individual to each cluster (represented by different colours).

Table 6.11: Mean proportion of membership of each sample (location/site) to each cluster (K=3).

	Sk2	Mo1	Mo3	Se1	Se3	Pr1	Pr3	Fr2	Vigo	Alme	Baia	Si1	Si3	Gr1	Gr3	Po1	Po3	Moly
Cluster 1	0.10	0.02	0.03	0.03	0.08	0.03	0.08	0.02	0.05	0.14	0.92	0.91	0.90	0.90	0.96	0.89	0.90	0.88
Cluster 2	0.66	0.19	0.72	0.74	0.58	0.20	0.20	0.84	0.63	0.59	0.05	0.04	0.07	0.05	0.02	0.06	0.09	0.06
Cluster 3	0.24	0.80	0.25	0.24	0.34	0.77	0.73	0.15	0.33	0.27	0.03	0.05	0.03	0.05	0.02	0.06	0.02	0.06

6.4 Discussion

In this study spatial scales of genetic differentiation of the barnacle *Chthamalus montagui* were investigated over its distribution range in the NE Atlantic and Mediterranean Sea using six microsatellite loci as molecular markers.

The results showed that Atlantic and Mediterranean populations are genetically different and confirmed previous work on this subject carried out using allozymes (Dando and Southward, 1981; Pannacciulli *et al.*, 1997a). The analysis of molecular variance revealed that differences between Atlantic and Mediterranean samples were highly significant and counted for 9.35% of the total genetic variation.

Dando and Southward (1981) suggested that the history of the Mediterranean, combined with the present hydrographic pattern, might have promoted and maintained differentiation of Mediterranean populations of *C. montagui*. Following the Messinian salinity crisis (ca 5.5 million years ago) (Hsu *et al.* 1977; McCullach and De-Deckker, 1989), communication between the Atlantic and Mediterranean was re-established, and fully marine conditions were restored during the Pliocene (5.4 to 1.8 million years ago). In the Pleistocene (1.8 to 0 million years ago), and particularly during the Quaternary, a series of glacial and interglacial periods with associated marine regressions and transgressions affected the area (Blanc, 1968). Atlantic and Mediterranean populations of *Chthamalus* spp. may have become physically isolated during these cycles and differentiated genetically.

Mediterranean populations exhibited higher genetic variability than Atlantic ones, as already indicated by allozyme studies (Pannacciulli *et al.*, 1997a). In the present study allelic richness and mean expected heterozygosity, averaged over all microsatellite loci, was higher in the samples from the Mediterranean basin than in those from the Atlantic (Table 6.2). The loci CM 2/15 and CM 5/23 were the most variable among populations and showed a wide difference between Atlantic and Mediterranean sites (Figure 6.2 and 6.3). CM 2/15 was monomorphic at two Atlantic sites (Fr2 and Se1) and the allelic richness of CM 5/23 ranged from 4.44 at the Atlantic site Mo1 to 19.77 at the Eastern Mediterranean site Moly (Table 6.2).

It is important to highlight that the Almeria sample behaved, at all loci, as an Atlantic sample. In fact, looking at the population genetic subdivision, as shown by the nMDS plots or by the UPGMA phylogenetic tree, Almeria was always clustered with the Atlantic populations. This evidence was also supported by the results of the Bayesian clustering analysis, showing high mean proportion of membership (85%) of the Almeria individuals to the Atlantic population cluster.

These results supported the hypothesis that the Almeria-Oran front is the major barrier to gene flow between the Atlantic and Mediterranean basins. This front has been suggested as the location of a phylogeographical break (Avise *et al.*, 1987), not only for *Chthamalus montagui* (Dando and Southward, 1981; Pannacciulli et al., 1997a), but also for other organisms like *Mytilus galloprovincialis* (Quesada *et al.*, 1995; Sanjuan *et al.*, 1996), *Ostrea edulis* (Saavedra *et al.*, 1993), and *Meganyctiphanes norvegica* (Zane *et al.*, 2000). The Almeria-Oran front is a zone of turbulence in the

Alboran Sea (Tintore et al., 1988) that may restrict larval dispersal in both directions. The Alboran Sea is a mixture of Atlantic waters flowing into the Mediterranean basin from the North Atlantic (occupying the upper layer of the Alboran Sea), and Mediterranean waters flowing westward (in the lower layer). If the hydrographic barrier persists, genetic divergence could conceivably lead to allopatric or parapatric speciation.

The higher genetic variability of the Mediterranean populations compared to the Atlantic ones could also be related to hydrographic patterns. The unidirectional surface circulation from the Atlantic to the Mediterranean through the Strait of Gibraltar might produce asymmetrical gene flow in this direction and promote differentiation (Endler, 1977). Alleles originating in the Mediterranean population could be prevented from spreading to the Atlantic, while those arising in the Atlantic could spread readily into the Mediterranean Sea. This explanation has been put forward by Saavedra et al. (1993) for a similar pattern of allelic diversity in the oyster Ostrea edulis.

Moreover, selection under different environmental regimes could be considered a likely cause of genetic differentiation between Atlantic and Mediterranean basins in Chthamalus. For instance, salinity, summer and winter water temperatures, and tidal range differ between the NE Atlantic and the Mediterranean (McLellan, 1965; Fairbridge, 1966). Intertidal barnacles in the Mediterranean undergo extreme environmental conditions, particularly in the summer when they experience long periods of exposure to the air as wave action, the only way for upper shore barnacles to get water, is reduced. Hence, the higher genetic variability recorded in populations from this basin could be a reponse to the difficult environment

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In which they have to survive as it provides greater plasticity to a constantly changing environment.

Focussing attention at the basin scale, a genetic subdivision was detected between the western and eastern Mediterranean samples. The population from the Ligurian Sea (Baia) was separated from the others, as shown by nMDS plots and the UPGMA phylogenetic tree, and in terms of genetic variability (allelic richness and expected heterozygosity) exhibited an intermediate pattern (Figure 6.4) between the Atlantic and the eastern Mediterranean samples.

The Strait of Sicily, which is well recognised in oceanography as a physico-chemical and morphological threshold between the two sub-basins of the Mediterranean Sea, could act as a hydrographical barrier to the gene flow. Moreover, the different physical-chemical characteristics (e.g. water temperature and salinity) of the western and eastern Mediterranean could also affect the genetic pattern of barnacle populations.

Furthermore, in the eastern sub-basin, the samples from Molyvos showed a slight genetic differentiation from those of the Adriatic Sea, as seen in the nMDS plot and the UPGMA tree. It could be interesting to compare their pattern with those of samples from the Black Sea. Pannacciulli *et al.*, 1997a analysed samples of *Chthamalus montagui* from the Black Sea using allozymes, and found a great genetic similarity to Mediterranean populations, despite the geographical separation and the hydrographic barrier of the Bosphorus. The predominant movement of water is from the Black Sea into the Mediterranean, but, during autumn, surface waters can

flow in the opposite direction (Pektas, 1958). Barnacle larvae could be transported with these currents, allowing an exchange of larvae between the two seas (Pannacciulli *et al.*, 1997a); for this reason Black Sea samples could be genetically very close to the Molyvos samples.

Focussing attention on the Atlantic basin, population genetic subdivision featured two clusters, as indicated by nMDS plots, UPGMA phylogenetic tree and also confirmed by Bayesian clustering analysis. The first cluster included most of the screened populations and the second one was represented by three populations (Mo1, Pr1 and Pr3). This result was quite surprising considering that the Mo1 sample did not cluster with Mo3: the two sites, within the same location, were 200-300 apart. Moonen Bay and Prawle Point are both wide and open rocky shores within the core distribution of the species, and no unusual environmental feature was noted in either place during collection of the samples. Moreover, they are about 1000 km far apart and the IBD results showed a significant overall correlation between genetic and geographical distances.

Before offering ecological speculations on this matter, it is worth noting that samples from Mo1, Pr1 and Pr3 (but not Mo3) were run on the sequencer in the same plate; some technical factors could be responsible for the observed genetic pattern. However, further investigations on these samples are needed.

The different genetic pattern detected at Mo1 and Mo3 provides the opportunity to discuss the small spatial scale of genetic variation: differences between sites within the same location. AMOVA results for the

Atlantic locations (Seil, Moonen Bay, Prawle Pt) showed that genetic variation among locations (hundreds of km apart from one another) counted for 1.5% of the total genetic variability, while differences between sites (from 100 to 300 m apart) were highly significant and counted for 3.19% of the total genetic variation. Otherwise, it could be related to the presence of the Mo1 samples that exhibited a divergent pattern from the others.

AMOVA results for the Mediterranean locations (Sistiana; Grado; Portonovo) showed that differences between sites accounted for just 0.58% of the total genetic variation and were not significant. Nevertheless, the major source of variation, 95.32% of the total genetic variability in the Atlantic sites and 98.48% in the Mediterranean ones, laid within sites and therefore, resided in the very small spatial scale (the plot of few meters from which specimens were collected) at the inter-individual level. The life history of this barnacle with high dispersal capabilities might be responsible for this pattern: the released larvae spend a few weeks in the water column dispersing offshore, before finding a suitable substratum on which to settle, metamorphose and start the sessile phase. For this reason it could be possible to have barnacles originating from distant parental locations settling next to each other on the shore, which could partly explain the large genetic variability recorded within sites.

Furthermore, differentiation at a very small scale has been often observed in population genetic studies of marine invertebrates employing microsatellites, suggesting that the high sensitivity and ability in detecting genetic variation of these molecular markers could also affect the observed

pattern (e.g. Bertozzi, 1995; Costantini *et al.*, 2007; Lind *et al.*,.2007; York *et al.*, 2008).

Moreover, it is worth mentioning that all samples exhibited highly significant multilocus departures from HWE, while using allozymes *Chthamalus montagui* samples were in conformity to HWE (Pannacciulli *et al.*, 1997a). Heterozygosity deficits have frequently been observed in marine invertebrates (Zouros and Foltz, 1984; Gaffney, 1994; David *et al.*, 1997; Ayre and Hughes, 2000; Ridgway *et al.*, 2001; Addison and Hart, 2004; Duran *et al.*, 2004; Le Goff-Vitry *et al.*, 2004; Andrade and Solferini, 2007; Costantini *et al.*, 2007) including barnacles and using microsatellite markers (Veliz *et al.*, 2006; York *et al.*, 2008). The most common explanations for heterozygote deficiency are: technical factors, such as the presence of null alleles and or stutter peaks (Foltz, 1986; Callen *et al.*, 1993; Puebla *et al.*, 2008), and biological factors, such as inbreeding or the Wahlund effect (Pogson *et al.*, 1995; Duran *et al.* 2004; Plutchack *et al.*, 2006; Costantini *et al.*, 2007). It is difficult to provide an exact explanation for our findings, probably technical and biological factors could have both contributed.

To conclude, we can assert that the marine environment is far from the homogeneous habitat for which it has been taken, and expectations of marine genetic biodiversity have potentially been grossly underestimated, particularly in widely dispersing species. The investigation of spatial scales of genetic diversity provides useful insights on species population genetic structure. This information is relevant to marine biodiversity management and conservation action plans.

Chapter 7

Final Discussion

Briefly re-state the results of the three data chapters (Ch. 4, 5, and 6).

The results of the sampling of artificial and natural substrates in the Adriatic Sea were consistent with the hypothesis that man-made structures can act as corridors for genetic exchange between distant rocky shores, since very little genetic differentiation was detected between distant localities. However, in the absence of historical genetic data from the region, firm conclusions could not be drawn.

A study of localities around the UK lent some support to the idea that edge-of-range populations tend to have reduced genetic variability and may also harbour unusual genotypes. However, the small number of peripheral (2) and marginal (1) populations that could be studied rendered such conclusions provisional, and some populations designated as mid-range shared similar characteristics.

The above two studies utilized three microsatellite loci. A broader survey of the NE Atlantic and Mediterranean range of *C. montagui* using six microsatellite loci documented clear differentiation between Atlantic and Mediterranean populations; the Mediterranean sample from Almeria in southern Spain was Atlantic in character, supporting the suggestion that the Almeria-Oran front, rather than the Strait of Gibraltar, is the main biogeographical boundary between the two basins. Genetic diversity was consistently higher in the Mediterranean than in the Atlantic.

Although a general pattern of isolation by distance was apparent in the broad-scale survey, two replicate samples from one Atlantic shore, taken a

few hundred metres apart, were clearly distinct. The natural or artefactual cause of this differentiation requires investigation. Throughout the project, the great majority of genetic variation was present within sampling sites, suggesting efficient larval mixing and the dominance of inter-individual variation over geographical differentiation, but spatial patchiness within sites on a scale of centimetres cannot be discounted from the data obtained.

Modern techniques offer a number of molecular markers for population genetic studies. In this project microsatellites were employed as suitable markers to investigate the genetic pattern of the barnacle *Chthamalus montagui*. Their rapid rate of mutation and their position in the non-coding region of the DNA make microsatellites ideal for investigating contemporary population structure (York *et al.*, 2008) as putatively neutral markers (Dufresne *et al.*, 2002).

The six microsatellite loci screened in this study were highly polymorphic. At the locus Cm 5/23, which was the most variable, 41 alleles were scored over all samples and a maximum of 33 alleles was detected in one population. This is not uncommon: Puebla *et al.* (2008), investigating the genetic structure of the snow crab *Chionecetes opilio* in the NW Atlantic, employed eight microsatellites, one of which showed 54 alleles in one single population. However, many other population genetic studies have employed microsatellite loci that were much less polymorphic (e.g. Costantini *et al.*, 2007; Lind *et al.*, 2007; Lukoschek *et al.*, 2008).

A high number of alleles implies low frequency of individual alleles and high genetic variability among individuals. In fact, as revealed from the analyses of molecular variance carried out in the different tasks of this project, in *C. montagui* the main source of variation lies always within sites, at a spatial scale of few tens of meters. This is often observed in population genetic studies of marine invertebrates employing microsatellites, suggesting the high sensitivity and ability of these molecular markers in detecting inter-individual genetic variation (e.g. Bertozzi, 1995; Costantini *et al.*, 2007; Lind *et al.*, 2007; York *et al.*, 2008). Nevertheless, the biology and ecology of *C. montagui* should be also taken into account. Barnacles brood developing embryos within the mantle cavity before releasing larvae that feed and pass through several instars in the plankton. The released larvae spend a few weeks in the water column dispersing offshore, before finding a suitable substratum on which to settle, metamorphose and start the sessile phase. These features make it likely that barnacles originating from distant parental populations grow next to each other on the shore and partly explain the large genetic variability recorded within sites.

In all tasks of the project the screened populations exhibited large positive F_{IS} values over all loci reflecting departures from HWE and exact tests highlighted highly significant multilocus heterozygote deficiency. (An exception to this general pattern was the Cm5/23 locus in the UK-based study of peripherality and marginality). Heterozygosity deficits have frequently been observed in marine invertebrates (Ayre and Hughes, 2000; Ridgway *et al.*, 2001; Addison and Hart, 2004; Duran *et al.*, 2004; Le Goff-Vitry *et al.*, 2004; Andrade and Solferini, 2007; Costantini *et al.*, 2007; Puebla *et al.*, 2008) including barnacles (Veliz *et al.*, 2006; York *et al.*,

2008) and attempts to explain these deficits have involved a range of technical and/or biological factors (Zouros and Foltz, 1984; Gaffney, 1994; David *et al.*, 1997; Pudovskis *et al.*, 2001; Addison and Hart, 2005; Plutchack *et al.*, 2006; Brownlow *et al.*, 2008).

One of the most commonly suggested contributors to heterozygote deficiency in microsatellites is the presence of null alleles (Foltz, 1986; Callen *et al.*, 1993). Null alleles represent base-pair mutations in the primer regions which cause primer binding to weaken and/or fail, resulting in a failure to amplify certain alleles (Paetkau and Strobeck, 1995). While microsatellite regions are often highly polymorphic due to a high rate of mutation through replication slippage and proofreading events, the flanking regions surrounding microsatellite repeat regions are generally considered to be more conserved. However, as hypothesized by Brownlow *et al.* (2008), when the microsatellite loci exhibit very high levels of polymorphism it is also possible that the flanking sequences are undergoing increased levels of mutation, reducing the effectiveness of primer binding. This explanation could apply to the microsatellites screened in this project, or at least to the most polymorphic ones, such as Cm 5/23. Nevertheless, I do not favour the hypothesis of null alleles, because heterozygote deficiency was significant and consistent across all loci, and it seems highly improbable that all loci exhibit null alleles with such a constant frequency.

Another technical factor could be the presence of stutter peaks (Puebla *et al.*, 2008). Stutter peaks were observed at two loci (Cm 4/5 and Cm 4/3); however, in the case of doubts or scoring difficulties I repeated the analysis at least two times randomly after a period of time to avoid scoring bias.

Artifacts of scoring genotypes are an obvious possibility, but I am confident of the scoring. Moreover, some loci did not exhibit stutter peaks making straightforward the interpretation of the electropherogram.

Among the biological factors, it is necessary to consider the Wahlund effect, which refers to reduction of heterozygosity due to the inadvertent sampling of individuals from two different sympatric subpopulations with different allele frequencies, and could (but would not necessarily) affect all polymorphic loci similarly (Pogson *et al.*, 1995; Plutchack *et al.*, 2006). However, no evidence of subpopulation genetic subdivision was detected in this study.

Addison and Hart (2005) documented an unexpected correlation between life history traits (such as dispersal ability and breeding systems) and deviations from the HWE. Barnacles generally reproduce by cross-fertilisation by pseudo-copulation (Klepal, 1990), which can only take place when one individual is within penile distance of potential mates (Anderson, 1994). Nevertheless, they are hermaphroditic organisms and self-fertilisation has been inferred in *Chthamalus* (Barnes and Crisp, 1956; Barnes and Barnes, 1958; Pannacciulli and Bishop, 2003). Inbreeding, in the sense of selfing, could be a possible explanation of multilocus heterozygote deficiency observed in the analysed samples. Nevertheless, it is difficult to provide an exact explanation for our findings, and technical and biological factors could both have contributed.

It is important to highlight that the ability to draw definitive conclusions concerning the genetic pattern of barnacles based on the screening of just a

few loci is limited. In particular, for those tasks of the project where just three microsatellite loci were examined due to constraints of time and money, it could be worth increasing the number of microsatellite loci and/or the number of different classes of marker employed.

In general, a multi-marker approach should be preferred, as Dufresne *et al.* (2002) suggested. Microsatellites may not always behave in a neutral way and have to be used cautiously, especially when evidence for genetic structuring relies on only a few assayed loci without *a priori* knowledge or comparison with potentially selected loci. Many studies are carried out using more than one marker investigating different parts of the genome and allowing useful comparisons among them (e.g. Schmidt and Rand, 1999 and 2001; Rand *et al.*, 2001; Simon-Blecher *et al.*, 2008; York *et al.*, 2008). A wider screening of the genetic pattern could provide a more comprehensive assessment and, therefore, more robust conclusions.

Previous population genetic studies carried out on *Chthamalus montagui* employed allozymes (Dando and Southward, 1981; Pannacciulli *et al.*, 1997a). Future studies on this species could, for instance, employ markers that target genes of the mtDNA. Specific primers have been developed to amplify *Cytochrome oxydase I* and the *Control Region* of the congeneric species *Chthamalus stellatus* (Milana, 2005): the same primers could perhaps find application also in *C. montagui*. Moreover, ISSRs (Inter Simple Sequence Repeats) could be considered as alternative markers: they were also employed in a population genetic study involving two barnacle species, *C. stellatus* and *Tesseropora atlantica*, in the archipelago of the Azores (Pannacciulli *et al.*, in preparation) and they were used in a number

of recent population genetic investigations on marine invertebrates (Casu *et al.*, 2005, 2006 and 2008; Maltagliati *et al.*, 2006; De Aranzamendi *et al.*, 2008).

It is worth noting that further investigations are needed for the three UK populations (Mo1, Pr1 and Pr3) that were clustered together and differentiated from the other Atlantic ones. It is suggested to repeat the screening of the six analysed microsatellite loci and/or to proceed to investigate a wider part of the genome employing other markers.

To conclude, it has to be mentioned that *C. montagui* has proved to be a very good model organism for genetic studies. This barnacle species is very easy to sample, due to its abundance and accessibility on the shore; DNA extraction can easily be carried out using a simple, safe and cheap method (Salting-Out extraction) that provides very good quality DNA; and the microsatellite markers employed amplified well and were relatively easy to score.

Appendices

Appendix 3.1: TBE (10x) electrophoresis buffer recipe

TBE – Tris Borate EDTA (10x)

Reagents	Amount for 1 liter of solution
– TRIZMA base	108 g
– Boric Acid	55 g
– EDTA (0.5 M, pH 8)	40 ml

Procedure

Add all ingredients. Fill up with distilled water to reach 1 litre.

Mix on magnetic stirrer and bring the pH to 8.3. Store at room temperature.

Appendix 3.2: DNA Salting-Out extraction protocol (modified after Aljanabi & Martinez, 1997)

Reagents
  – TNE (Tris-NaCl-EDTA) buffer: 50 mM Tris-HCl, 100 mM NaCl, 5 mM EDTA (pH 8.0)
  – 1.5% Sodium Dodecyl Sulfate (SDS)
  – Proteinase K [10mg/ml]
  – 6 M NaCl
  – 70% and 100% Ethanol
  – TE buffer: 10 mM Tris-HCl, 0.1 mM EDTA (pH 8.0)

Procedure
  1. In a 1.5 ml eppendorf tube, resuspend the soft tissue of the specimen in 270 µl of TNE buffer, 1.5% SDS and 30 µl of Proteinase K
  2. Vortex and briefly centrifuge
  3. Incubate at 55°C for 2-3 hours
  4. Add 100 µl NaCl 6M
  5. Vortex for 15 sec
  6. Centrifuge for 18 min at 1300 rpm
  7. Prepare a new set of 1.5 ml eppendorf vials containing 2 volumes (800 µl) of absolute ethanol
  8. Remove the solution (approximately 300 µl) from the first set of eppendorfs and add to the second set of vials containing absolute ethanol. During this step make sure not to touch the white pellet on the bottom of the vial nor the white foam, if present, on the surface
  9. Mix manually by inverting vials
  10. Keep the solution at -20°C for at least 2 hours.
  11. Centrifuge at 1300 rpm for 15 min
  12. Remove absolute ethanol (by pipetting or pouring), resuspend the pellet in 300 µl of 70% Ethanol
  13. Vortex and centrifuge for 5 min at 1300 rpm
  14. Remove the ethanol and repeat the washing procedure, as from step 12, 2-3 times

15. Dry the pellet in air at room temperature or in a thermoblock set at 37°C leaving the top of the vial open

16. Resuspend the pellet in 30 μl of TE buffer

17. Vortex and centrifuge briefly

18. Keep the resuspended pellet at 4°C overnight to facilitate dissolving

19. Store the extracted DNA at -20°C.

Appendix 4.1 - Allele frequencies of the Adriatic samples of *Chthamalus montagui* at three microsatellite loci. For abbreviations see Table 4.1.

	SITES												
LOCUS	**Pu1**	**Mu1**	**Si1**	**Si3**	**Gr1**	**Gr3**	**Cv1**	**Ce1**	**Mi1**	**Ca1**	**Fa1**	**Po1**	**Po3**
CM 2/15													
N	30	28	31	32	29	29	32	30	29	31	32	30	28
142	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
146	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.03	0.00	0.00
150	0.00	0.00	0.02	0.06	0.00	0.02	0.02	0.05	0.10	0.08	0.02	0.08	0.04
152	0.40	0.48	0.32	0.38	0.43	0.34	0.36	0.35	0.40	0.40	0.25	0.32	0.38
154	0.38	0.16	0.19	0.20	0.16	0.33	0.25	0.18	0.29	0.18	0.34	0.25	0.38
156	0.15	0.14	0.21	0.22	0.14	0.14	0.19	0.17	0.09	0.15	0.19	0.10	0.14
158	0.02	0.05	0.06	0.09	0.07	0.07	0.03	0.05	0.03	0.00	0.06	0.10	0.02
160	0.00	0.04	0.05	0.00	0.05	0.00	0.02	0.00	0.03	0.03	0.03	0.00	0.00
162	0.00	0.05	0.03	0.03	0.02	0.00	0.03	0.07	0.00	0.05	0.03	0.07	0.04
164	0.00	0.02	0.00	0.00	0.05	0.07	0.02	0.00	0.00	0.00	0.02	0.00	0.00
166	0.00	0.02	0.00	0.00	0.03	0.00	0.06	0.05	0.02	0.02	0.02	0.02	0.00
168	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.02
170	0.00	0.04	0.06	0.00	0.00	0.00	0.00	0.00	0.02	0.03	0.02	0.00	0.00
172	0.02	0.00	0.03	0.02	0.03	0.00	0.02	0.00	0.00	0.05	0.00	0.00	0.00
176	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.02	0.00	0.00	0.00
180	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.02	0.00
184	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00
198	0.00	0.00	0.02	0.00	0.00	0.03	0.00	0.02	0.00	0.00	0.00	0.00	0.00

Appendix 4.1 - continued

LOCUS	SITES												
	Pu1	Mu1	Si1	Si3	Gr1	Gr3	Cv1	Ce1	Mi1	Ca1	Fa1	Po1	Po3
CM 4/5													
N	25	29	27	28	27	29	26	30	30	30	29	28	29
74	0.32	0.26	0.31	0.29	0.26	0.33	0.42	0.18	0.40	0.18	0.21	0.32	0.26
78	0.04	0.02	0.00	0.00	0.02	0.00	0.08	0.07	0.07	0.02	0.09	0.00	0.00
80	0.10	0.07	0.02	0.07	0.00	0.00	0.00	0.03	0.05	0.05	0.05	0.05	0.07
82	0.00	0.02	0.06	0.00	0.00	0.00	0.00	0.00	0.05	0.02	0.09	0.00	0.03
94	0.00	0.00	0.00	0.07	0.00	0.03	0.00	0.00	0.00	0.00	0.02	0.00	0.00
98	0.04	0.05	0.04	0.16	0.11	0.10	0.06	0.07	0.05	0.18	0.07	0.05	0.14
100	0.08	0.03	0.07	0.00	0.06	0.07	0.04	0.08	0.07	0.05	0.05	0.04	0.00
102	0.00	0.03	0.06	0.02	0.00	0.00	0.00	0.02	0.02	0.00	0.03	0.04	0.00
104	0.04	0.10	0.07	0.00	0.04	0.07	0.13	0.08	0.05	0.00	0.00	0.13	0.09
106	0.06	0.07	0.11	0.21	0.15	0.14	0.00	0.18	0.08	0.18	0.17	0.04	0.10
108	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.03	0.00	0.03	0.02	0.00	0.05
110	0.02	0.05	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.05	0.00
112	0.04	0.02	0.02	0.00	0.09	0.05	0.00	0.08	0.03	0.07	0.02	0.04	0.03
114	0.04	0.09	0.02	0.04	0.11	0.02	0.06	0.07	0.03	0.05	0.07	0.00	0.03
116	0.04	0.02	0.00	0.04	0.00	0.00	0.04	0.03	0.02	0.00	0.00	0.07	0.02
118	0.02	0.02	0.00	0.00	0.04	0.05	0.00	0.03	0.00	0.02	0.00	0.04	0.00
120	0.04	0.07	0.04	0.04	0.07	0.00	0.00	0.00	0.02	0.02	0.03	0.00	0.00
122	0.00	0.03	0.04	0.00	0.02	0.05	0.04	0.00	0.00	0.02	0.00	0.00	0.09
124	0.00	0.00	0.00	0.04	0.00	0.00	0.04	0.00	0.03	0.03	0.02	0.07	0.03
126	0.12	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02
128	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
130	0.00	0.00	0.07	0.00	0.00	0.03	0.00	0.00	0.02	0.00	0.00	0.00	0.00

	Pu1	Mu1	Si1	Si3	Gr1	Gr3	Cv1	Ce1	Mi1	Ca1	Fa1	Po1	Po3
132	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.03
134	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00
136	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00
138	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00
140	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.03	0.00	0.00	0.00
146	0.00	0.03	0.00	0.00	0.00	0.00	0.08	0.00	0.00	0.00	0.00	0.00	0.00
150	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.00
158	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.04	0.00
160	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
164	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00
166	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00
172	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00
176	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00

POPULATIONS

LOCUS	Pu1	Mu1	Si1	Si3	Gr1	Gr3	Cv1	Ce1	Mi1	Ca1	Fa1	Po1	Po3
CM 5/23													
N	32	28	27	30	27	28	32	29	29	28	31	29	29
162	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02
172	0.02	0.00	0.02	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
174	0.14	0.00	0.13	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.02
176	0.06	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
178	0.00	0.00	0.00	0.00	0.06	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00
180	0.00	0.05	0.00	0.02	0.00	0.02	0.03	0.02	0.00	0.04	0.08	0.05	0.07
182	0.02	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00
184	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00
186	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00

Appendix 4.1 - continued

188	0.00	0.02	0.04	0.00	0.02	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00
190	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.05	0.02
192	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.03	0.00	0.00	0.03	0.00
194	0.02	0.00	0.00	0.00	0.02	0.04	0.00	0.02	0.00	0.00	0.00	0.00	0.07
196	0.00	0.00	0.02	0.02	0.02	0.00	0.00	0.02	0.00	0.05	0.00	0.00	0.05
198	0.00	0.00	0.00	0.03	0.06	0.00	0.00	0.03	0.02	0.02	0.02	0.00	0.05
200	0.05	0.00	0.00	0.02	0.00	0.04	0.02	0.05	0.02	0.00	0.05	0.02	0.00
202	0.03	0.04	0.00	0.02	0.02	0.00	0.02	0.02	0.02	0.02	0.02	0.03	0.02
204	0.02	0.00	0.00	0.03	0.04	0.02	0.05	0.02	0.02	0.00	0.03	0.00	0.00
206	0.00	0.00	0.02	0.02	0.04	0.00	0.02	0.03	0.00	0.00	0.00	0.00	0.02
208	0.00	0.02	0.00	0.00	0.02	0.02	0.05	0.02	0.03	0.00	0.00	0.02	0.02
210	0.02	0.02	0.04	0.07	0.00	0.04	0.02	0.05	0.02	0.02	0.00	0.05	0.03
212	0.02	0.05	0.06	0.02	0.00	0.02	0.05	0.00	0.03	0.05	0.00	0.00	0.00
214	0.02	0.04	0.00	0.03	0.06	0.00	0.03	0.00	0.07	0.00	0.06	0.02	0.00
216	0.05	0.05	0.00	0.02	0.00	0.04	0.02	0.03	0.03	0.05	0.00	0.03	0.00
218	0.02	0.05	0.04	0.02	0.00	0.04	0.02	0.03	0.05	0.00	0.02	0.00	0.00
220	0.00	0.02	0.06	0.05	0.00	0.00	0.02	0.09	0.02	0.05	0.05	0.00	0.00
222	0.00	0.04	0.00	0.02	0.09	0.04	0.13	0.05	0.02	0.09	0.00	0.03	0.02
224	0.00	0.04	0.06	0.03	0.02	0.02	0.03	0.03	0.02	0.00	0.08	0.02	0.00
226	0.05	0.02	0.04	0.08	0.04	0.00	0.02	0.03	0.03	0.05	0.05	0.00	0.07
228	0.06	0.04	0.04	0.00	0.00	0.07	0.02	0.00	0.00	0.09	0.06	0.03	0.14
230	0.00	0.02	0.02	0.05	0.04	0.00	0.13	0.00	0.00	0.00	0.00	0.10	0.02
232	0.00	0.00	0.07	0.03	0.02	0.02	0.03	0.03	0.00	0.00	0.05	0.02	0.00
234	0.05	0.02	0.04	0.05	0.04	0.04	0.05	0.02	0.07	0.04	0.00	0.00	0.05
236	0.02	0.05	0.06	0.02	0.02	0.07	0.05	0.03	0.03	0.07	0.00	0.03	0.02
238	0.00	0.00	0.00	0.02	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.00

240	0.00	0.00	0.04	0.03	0.04	0.05	0.00	0.00	0.03	0.04	0.05	0.00	0.03
242	0.02	0.00	0.00	0.08	0.04	0.09	0.00	0.00	0.03	0.04	0.05	0.03	0.03
244	0.03	0.05	0.04	0.00	0.00	0.04	0.05	0.05	0.03	0.00	0.02	0.02	0.00
246	0.02	0.04	0.02	0.03	0.00	0.05	0.02	0.00	0.02	0.05	0.03	0.02	0.07
248	0.03	0.00	0.02	0.02	0.02	0.04	0.00	0.02	0.03	0.00	0.00	0.00	0.00
250	0.02	0.05	0.00	0.00	0.04	0.00	0.02	0.00	0.00	0.02	0.03	0.00	0.03
252	0.00	0.00	0.02	0.00	0.04	0.02	0.05	0.00	0.02	0.02	0.02	0.05	0.00
254	0.02	0.05	0.02	0.08	0.04	0.00	0.03	0.00	0.03	0.00	0.00	0.00	0.00
256	0.03	0.02	0.02	0.00	0.02	0.00	0.00	0.02	0.05	0.04	0.10	0.03	0.07
258	0.03	0.00	0.02	0.00	0.06	0.00	0.00	0.03	0.03	0.04	0.00	0.03	0.02
262	0.03	0.02	0.04	0.00	0.00	0.00	0.00	0.05	0.07	0.00	0.03	0.03	0.00
266	0.00	0.04	0.02	0.00	0.04	0.09	0.00	0.03	0.00	0.00	0.02	0.07	0.02
268	0.03	0.02	0.00	0.02	0.00	0.04	0.02	0.00	0.00	0.00	0.00	0.00	0.00
272	0.03	0.02	0.00	0.03	0.02	0.00	0.00	0.02	0.02	0.02	0.03	0.02	0.00
274	0.03	0.02	0.02	0.02	0.02	0.02	0.00	0.02	0.00	0.02	0.06	0.00	0.03
278	0.02	0.00	0.00	0.00	0.00	0.00	0.03	0.02	0.00	0.02	0.00	0.00	0.00
282	0.03	0.02	0.00	0.03	0.00	0.04	0.02	0.02	0.00	0.02	0.00	0.00	0.00
284	0.00	0.02	0.00	0.00	0.00	0.02	0.00	0.02	0.00	0.02	0.00	0.00	0.00
288	0.02	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.04	0.00	0.05	0.00
292	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00

Appendix 5.1 - Allele frequencies of the UK samples of *C. montagui* at three microsatellite loci. Peripheral (*Sk2* and *Fr2*) and marginal (*Mi1*) population are in italics. For abbreviations see Table 5.1.

LOCUS	SITES																
CM 2/15	*Sk2*	Sc2	Mo1	Mo3	Se1	Se3	Be2	Rh2	Go2	*Mi1*	Wo2	Tr2	We2	Pr1	Pr3	Pv2	*Fr2*
138	*0.00*	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.00*	0.00	0.00	0.00	0.02	0.00	0.00	*0.00*
142	*0.00*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.00*	0.00	0.00	0.02	0.00	0.00	0.00	*0.00*
148	*0.00*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	*0.00*	0.00	0.00	0.00	0.03	0.00	0.00	*0.00*
150	*0.00*	0.04	0.00	0.03	0.00	0.03	0.00	0.02	0.00	*0.00*	0.00	0.03	0.03	0.00	0.00	0.02	*0.00*
152	*0.83*	0.87	0.97	0.95	1.00	0.86	0.98	0.97	0.95	*0.97*	0.92	0.93	0.94	0.93	0.95	0.95	*1.00*
154	*0.17*	0.04	0.03	0.00	0.00	0.10	0.00	0.00	0.00	*0.00*	0.08	0.00	0.00	0.00	0.00	0.00	*0.00*
156	*0.00*	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.00*	0.00	0.00	0.00	0.00	0.00	0.00	*0.00*
158	*0.00*	0.00	0.00	0.00	0.00	0.00	0.02	0.02	0.02	*0.00*	0.00	0.00	0.02	0.02	0.00	0.00	*0.00*
160	*0.00*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.02*	0.00	0.00	0.00	0.00	0.05	0.00	*0.00*
162	*0.00*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	*0.02*	0.00	0.00	0.00	0.00	0.00	0.00	*0.00*
164	*0.00*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.00*	0.00	0.02	0.00	0.00	0.00	0.00	*0.00*
166	*0.00*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.00*	0.00	0.02	0.00	0.00	0.00	0.00	*0.00*
176	*0.00*	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	*0.00*	0.00	0.00	0.00	0.00	0.00	0.04	*0.00*

LOCUS	SITES																
CM 4/5	*Sk2*	Sc2	Mo1	Mo3	Se1	Se3	Be2	Rh2	Go2	*Mi1*	Wo2	Tr2	We2	Pr1	Pr3	Pv2	*Fr2*
74	*0.00*	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.04	*0.00*	0.00	0.00	0.00	0.02	0.00	0.00	*0.00*
78	*0.00*	0.00	0.00	0.02	0.00	0.03	0.00	0.03	0.02	*0.00*	0.00	0.05	0.00	0.05	0.03	0.02	*0.00*
80	*0.00*	0.13	0.14	0.17	0.00	0.10	0.12	0.14	0.16	*0.08*	0.05	0.00	0.04	0.04	0.08	0.16	*0.08*
82	*0.00*	0.08	0.00	0.00	0.00	0.02	0.02	0.00	0.00	*0.00*	0.00	0.03	0.00	0.00	0.02	0.05	*0.00*
94	*0.00*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.00*	0.00	0.00	0.02	0.00	0.00	0.00	*0.00*
98	*0.04*	0.08	0.10	0.10	0.29	0.07	0.06	0.08	0.05	*0.11*	0.08	0.07	0.07	0.04	0.00	0.02	*0.02*
100	*0.04*	0.13	0.14	0.13	0.13	0.08	0.12	0.13	0.09	*0.11*	0.17	0.07	0.06	0.23	0.13	0.11	*0.15*
102	*0.28*	0.12	0.06	0.12	0.15	0.22	0.13	0.05	0.18	*0.18*	0.17	0.10	0.09	0.14	0.17	0.18	*0.08*
104	*0.09*	0.06	0.20	0.02	0.13	0.07	0.06	0.08	0.04	*0.05*	0.05	0.14	0.11	0.04	0.07	0.11	*0.19*
106	*0.02*	0.00	0.00	0.03	0.04	0.13	0.08	0.03	0.02	*0.05*	0.00	0.05	0.04	0.05	0.02	0.00	*0.08*
108	*0.04*	0.00	0.02	0.03	0.04	0.00	0.06	0.02	0.07	*0.02*	0.03	0.05	0.00	0.05	0.00	0.00	*0.00*
110	*0.02*	0.02	0.00	0.07	0.00	0.07	0.06	0.03	0.02	*0.02*	0.05	0.07	0.02	0.07	0.03	0.07	*0.03*

Appendix 5.1 - continued

112	0.09	0.00	0.02	0.03	0.00	0.05	0.08	0.06	0.02	0.05	0.02	0.00	0.06	0.04	0.05	0.09	0.02
114	0.13	0.10	0.08	0.08	0.06	0.00	0.06	0.02	0.04	0.06	0.08	0.03	0.02	0.04	0.08	0.00	0.16
116	0.00	0.06	0.00	0.07	0.04	0.00	0.04	0.09	0.00	0.00	0.02	0.05	0.11	0.00	0.02	0.02	0.00
118	0.04	0.00	0.02	0.00	0.00	0.00	0.00	0.02	0.00	0.03	0.00	0.02	0.02	0.04	0.07	0.00	0.00
120	0.04	0.00	0.00	0.03	0.00	0.03	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.04	0.03	0.02	0.03
122	0.00	0.00	0.04	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.08	0.00	0.02	0.00	0.05	0.00	0.00
124	0.00	0.08	0.02	0.00	0.04	0.00	0.06	0.00	0.00	0.00	0.02	0.02	0.00	0.04	0.00	0.00	0.02
126	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.05
128	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.03	0.02	0.00
130	0.00	0.04	0.00	0.00	0.00	0.03	0.00	0.02	0.00	0.00	0.00	0.00	0.06	0.00	0.00	0.00	0.00
132	0.00	0.04	0.04	0.02	0.00	0.00	0.00	0.05	0.00	0.00	0.03	0.09	0.02	0.02	0.00	0.00	0.02
134	0.00	0.00	0.04	0.00	0.02	0.03	0.00	0.02	0.05	0.03	0.00	0.03	0.04	0.04	0.02	0.02	0.03
136	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.02	0.03	0.02	0.02	0.00	0.00	0.00	0.00	0.02
138	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.02	0.00	0.03	0.02	0.00	0.00	0.00	0.05	0.00
140	0.02	0.00	0.02	0.02	0.04	0.00	0.00	0.00	0.05	0.00	0.00	0.02	0.04	0.02	0.02	0.00	0.03
144	0.02	0.02	0.00	0.00	0.00	0.00	0.02	0.03	0.00	0.03	0.00	0.00	0.04	0.00	0.03	0.00	0.00
146	0.00	0.02	0.00	0.00	0.02	0.03	0.00	0.00	0.04	0.00	0.02	0.02	0.04	0.02	0.00	0.00	0.00
148	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.00	0.02	0.00	0.00	0.00	0.00
150	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00
154	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00
158	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.08	0.02	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00
160	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00
162	0.00	0.04	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.00
164	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.02	0.00
166	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.02	0.06	0.00	0.00	0.00	0.00
168	0.02	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.02	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00
192	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

LOCUS	SITES																
CM 5/23	*Sk2*	Sc2	Mo1	Mo3	Se1	Se3	Be2	Rh2	Go2	*Mi1*	Wo2	Tr2	We2	Pr1	Pr3	Pv2	*Fr2*
158	*0.00*	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	*0.00*	0.00	0.00	0.00	0.02	0.00	0.00	*0.00*
162	*0.00*	0.00	0.00	0.02	0.00	0.04	0.00	0.00	0.00	*0.00*	0.02	0.00	0.02	0.02	0.00	0.00	*0.00*
164	*0.00*	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.02	*0.00*	0.00	0.05	0.00	0.00	0.02	0.00	*0.00*
166	*0.00*	0.02	0.00	0.00	0.00	0.00	0.00	0.03	0.00	*0.00*	0.00	0.00	0.02	0.00	0.02	0.00	*0.00*
170	*0.00*	0.03	0.00	0.03	0.03	0.00	0.02	0.02	0.02	*0.02*	0.03	0.02	0.05	0.00	0.04	0.00	*0.02*
172	*0.00*	0.02	0.00	0.05	0.02	0.00	0.00	0.00	0.00	*0.03*	0.00	0.00	0.02	0.02	0.04	0.02	*0.02*
174	*0.02*	0.05	0.00	0.03	0.02	0.06	0.03	0.03	0.02	*0.05*	0.05	0.00	0.03	0.00	0.04	0.04	*0.05*
176	*0.50*	0.52	0.61	0.42	0.61	0.48	0.55	0.50	0.63	*0.38*	0.55	0.52	0.43	0.50	0.54	0.54	*0.42*
178	*0.30*	0.23	0.22	0.21	0.16	0.23	0.22	0.31	0.18	*0.26*	0.19	0.18	0.24	0.27	0.14	0.25	*0.30*
180	*0.08*	0.08	0.11	0.15	0.11	0.17	0.15	0.09	0.13	*0.14*	0.10	0.03	0.10	0.03	0.09	0.14	*0.09*
182	*0.02*	0.00	0.00	0.03	0.03	0.02	0.02	0.00	0.02	*0.07*	0.05	0.07	0.05	0.08	0.02	0.00	*0.08*
184	*0.00*	0.06	0.03	0.03	0.00	0.00	0.00	0.00	0.00	*0.02*	0.02	0.03	0.03	0.05	0.04	0.02	*0.00*
186	*0.06*	0.00	0.00	0.00	0.02	0.00	0.00	0.02	0.00	*0.02*	0.00	0.08	0.00	0.02	0.00	0.00	*0.02*
188	*0.02*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.00*	0.00	0.00	0.00	0.00	0.02	0.00	*0.02*
190	*0.00*	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	*0.00*	0.00	0.00	0.00	0.00	0.00	0.00	*0.00*
192	*0.00*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.00*	0.00	0.00	0.00	0.02	0.00	0.00	*0.00*
194	*0.00*	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	*0.00*	0.00	0.02	0.00	0.00	0.00	0.00	*0.00*
202	*0.00*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.02*	0.00	0.00	0.00	0.00	0.00	0.00	*0.00*
210	*0.00*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.00*	0.00	0.00	0.00	0.00	0.02	0.00	*0.00*
216	*0.00*	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	*0.00*	0.00	0.00	0.00	0.00	0.00	0.00	*0.00*
218	*0.00*	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	*0.00*	0.00	0.00	0.00	0.00	0.00	0.00	*0.00*

Appendix 5.2 - Test for Hardy-Weinberg equilibrium (F_{IS}, Weir and Cockerham, 1984) for the 17 UK samples using Fisher's method, estimation of exact probability values (*P*-value) by Markov-chain randomization (10,000 dememorizations, 100 batches and 5,000 iterations per batch). Peripheral (*Sk2* and *Fr2*) and marginal (*Mi1*) populations are in italics. For abbreviations see Table 5.1.

LOCUS Cm2/15		
SITES	F_{IS} (W&C)	*P*-value
Sk2	*1.000*	*0.000*
Sc2	0.850	0.000
Mo1	1.000	0.016
Mo3	0.663	0.016
Se1		-
Se3	1.000	0.000
Be2		-
Rh2	-0.008	1.000
Go2	0.326	0.051
Mi1	*-0.008*	*1.000*
Wo2	0.789	0.004
Tr2	0.489	0.035
We2	0.489	0.033
Pr1	0.489	0.034
Pr3	0.659	0.051
Pv2	0.663	0.018
Fr2		-
LOCUS Cm 4/5		
SITES	F_{IS} (W&C)	*P*-value
Sk2	*0.567*	*0.000*
Sc2	0.632	0.000
Mo1	0.653	0.000
Mo3	0.822	0.000
Se1	0.692	0.000
Se3	0.747	0.000
Be2	0.554	0.000
Rh2	0.571	0.000
Go2	0.581	0.000
Mi1	*0.622*	*0.000*
Wo2	0.714	0.000
Tr2	0.458	0.000
We2	0.617	0.000
Pr1	0.653	0.000
Pr3	0.611	0.000
Pv2	0.417	0.000
Fr2	*0.540*	*0.000*
LOCUS Cm5/23		
SITES	F_{IS} (W&C)	*P*-value
Sk2	*0.157*	*0.143*
Sc2	-0.046	0.565
Mo1	0.408	0.003
Mo3	0.201	0.333
Se1	0.167	0.104
Se3	0.339	0.082

Be2	0.218	0.103
Rh2	0.138	0.606
Go2	0.379	0.003
Mi1	*-0.071*	*0.068*
Wo2	0.018	0.431
Tr2	0.089	0.303
We2	0.081	0.080
Pr1	0.175	0.495
Pr3	0.122	0.077
Pv2	-0.005	0.948
Fr2	*0.099*	*0.785*

ALL LOCI		
POPULATIONS	F_{IS} **(W&C)**	*P*-value
Sk2	*0.491*	*0.000*
Sc2	0.414	0.000
Mo1	0.576	0.000
Mo3	0.548	0.000
Se1	0.478	0.000
Se3	0.629	0.000
Be2	0.410	0.000
Rh2	0.379	0.000
Go2	0.493	0.000
Mi1	*0.298*	*0.000*
Wo2	0.458	0.000
Tr2	0.317	0.000
We2	0.390	0.000
Pr1	0.453	0.000
Pr3	0.419	0.000
Pv2	0.270	0.000
Fr2	*0.344*	*0.000*

Appendix 6.1 - Allele frequencies of the Atlantic and Mediterranean samples of *Chthamalus montagui* at six microsatellite loci. For site abbreviations see Table 6.1

LOCUS SITES

CM 2/15	Sk2	Mo1	Mo3	Se1	Se3	Pr1	Pr3	Fr2	Vigo	Alme	Baia	Si1	Si3	Gr1	Gr3	Po1	Po3	Moly
N	23	32	32	32	29	30	30	32	24	23	23	31	32	29	29	30	28	22
138	0.000	0.000	0.000	0.000	0.000	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
142	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.017	0.000	0.000	0.000	0.000
148	0.000	0.000	0.000	0.000	0.000	0.033	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
150	0.000	0.000	0.031	0.000	0.035	0.000	0.000	0.000	0.000	0.000	0.044	0.016	0.063	0.000	0.017	0.083	0.036	0.046
152	0.826	0.969	0.953	1.000	0.862	0.933	0.950	1.000	0.938	0.848	0.283	0.323	0.375	0.431	0.345	0.317	0.375	0.318
154	0.174	0.031	0.000	0.000	0.103	0.000	0.000	0.000	0.063	0.109	0.348	0.194	0.203	0.155	0.328	0.250	0.375	0.296
156	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.087	0.210	0.219	0.138	0.138	0.100	0.143	0.091
158	0.000	0.000	0.000	0.000	0.000	0.017	0.000	0.000	0.000	0.000	0.130	0.065	0.094	0.069	0.069	0.100	0.018	0.068
160	0.000	0.000	0.000	0.000	0.000	0.000	0.050	0.000	0.000	0.000	0.044	0.048	0.000	0.052	0.000	0.000	0.000	0.091
162	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.032	0.031	0.017	0.000	0.067	0.036	0.068
164	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.052	0.069	0.000	0.000	0.000
166	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.035	0.000	0.017	0.000	0.000
168	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.044	0.000	0.000	0.000	0.000	0.000	0.050	0.018	0.000
170	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.022	0.065	0.000	0.000	0.000	0.000	0.000	0.000
172	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.022	0.032	0.016	0.035	0.000	0.000	0.000	0.000
176	0.000	0.000	0.016	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
180	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.017	0.000	0.023
198	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.022	0.016	0.000	0.000	0.035	0.000	0.000	0.000

LOCUS SITES

CM 4/5	Sk2	Mo1	Mo3	Se1	Se3	Pr1	Pr3	Fr2	Vigo	Alme	Baia	Si1	Si3	Gr1	Gr3	Po1	Po3	Moly
N	23	25	30	26	30	28	30	31	22	23	24	27	28	27	29	28	29	23
74	0.000	0.000	0.000	0.000	0.000	0.018	0.000	0.000	0.000	0.000	0.250	0.315	0.286	0.259	0.328	0.321	0.259	0.587
76	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.042	0.000	0.000	0.000	0.000	0.000	0.000	0.000
78	0.000	0.000	0.017	0.000	0.033	0.054	0.033	0.000	0.000	0.065	0.021	0.000	0.000	0.019	0.000	0.000	0.000	0.000
80	0.000	0.140	0.167	0.000	0.100	0.036	0.083	0.081	0.114	0.044	0.000	0.019	0.071	0.000	0.000	0.054	0.069	0.000
82	0.000	0.000	0.000	0.000	0.017	0.000	0.017	0.000	0.000	0.065	0.063	0.056	0.000	0.000	0.000	0.000	0.035	0.000
94	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.071	0.000	0.035	0.000	0.000	0.000

Appendix 6.1 - continued

98	0.044	0.100	0.100	0.289	0.067	0.036	0.000	0.016	0.114	0.065	0.125	0.037	0.161	0.111	0.103	0.054	0.138	0.044
100	0.044	0.140	0.133	0.135	0.083	0.232	0.133	0.145	0.091	0.130	0.000	0.074	0.000	0.056	0.069	0.036	0.000	0.174
102	0.283	0.060	0.117	0.154	0.217	0.143	0.167	0.081	0.091	0.152	0.000	0.056	0.018	0.000	0.000	0.036	0.000	0.087
104	0.087	0.200	0.017	0.135	0.067	0.036	0.067	0.194	0.136	0.065	0.188	0.074	0.000	0.037	0.069	0.125	0.086	0.065
106	0.022	0.000	0.033	0.039	0.133	0.054	0.017	0.081	0.000	0.022	0.083	0.111	0.214	0.148	0.138	0.036	0.103	0.022
108	0.044	0.020	0.033	0.039	0.000	0.054	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.052	0.000
110	0.022	0.000	0.067	0.000	0.067	0.071	0.033	0.032	0.046	0.022	0.000	0.056	0.000	0.000	0.000	0.054	0.000	0.022
112	0.087	0.020	0.033	0.000	0.050	0.036	0.050	0.016	0.091	0.044	0.125	0.019	0.000	0.093	0.052	0.036	0.035	0.000
114	0.130	0.080	0.083	0.058	0.000	0.036	0.083	0.161	0.046	0.022	0.000	0.019	0.036	0.111	0.017	0.000	0.035	0.000
116	0.000	0.000	0.067	0.039	0.000	0.000	0.017	0.000	0.000	0.000	0.000	0.000	0.036	0.000	0.000	0.071	0.017	0.000
118	0.044	0.020	0.000	0.000	0.000	0.036	0.067	0.000	0.114	0.022	0.021	0.000	0.000	0.037	0.052	0.036	0.000	0.000
120	0.044	0.000	0.033	0.000	0.033	0.036	0.033	0.032	0.000	0.044	0.000	0.037	0.036	0.074	0.000	0.000	0.000	0.000
122	0.000	0.040	0.000	0.000	0.017	0.000	0.050	0.000	0.000	0.065	0.042	0.037	0.000	0.019	0.052	0.000	0.086	0.000
124	0.000	0.020	0.000	0.039	0.000	0.036	0.000	0.016	0.000	0.000	0.000	0.000	0.036	0.000	0.000	0.071	0.035	0.000
126	0.022	0.000	0.000	0.000	0.000	0.000	0.000	0.048	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.017	0.000
128	0.044	0.000	0.000	0.000	0.000	0.000	0.033	0.000	0.046	0.000	0.000	0.000	0.000	0.019	0.000	0.000	0.000	0.000
130	0.000	0.000	0.000	0.000	0.033	0.000	0.000	0.000	0.000	0.000	0.000	0.074	0.000	0.000	0.035	0.000	0.000	0.000
132	0.000	0.040	0.017	0.000	0.000	0.018	0.000	0.016	0.000	0.044	0.000	0.019	0.000	0.000	0.000	0.000	0.035	0.000
134	0.000	0.040	0.000	0.019	0.033	0.036	0.017	0.032	0.000	0.022	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
136	0.000	0.000	0.000	0.000	0.017	0.000	0.000	0.016	0.000	0.065	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
138	0.000	0.000	0.033	0.000	0.000	0.000	0.000	0.000	0.023	0.000	0.000	0.000	0.000	0.019	0.000	0.018	0.000	0.000
140	0.022	0.020	0.017	0.039	0.000	0.018	0.017	0.032	0.046	0.044	0.000	0.000	0.000	0.000	0.017	0.000	0.000	0.000
144	0.022	0.000	0.000	0.000	0.000	0.000	0.033	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
146	0.000	0.000	0.000	0.019	0.033	0.018	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
148	0.000	0.040	0.000	0.000	0.000	0.000	0.000	0.000	0.023	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
150	0.000	0.020	0.000	0.000	0.000	0.000	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
154	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.042	0.000	0.000	0.000	0.000	0.000	0.000	0.000
158	0.022	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.023	0.000	0.000	0.000	0.000	0.000	0.035	0.036	0.000	0.000
160	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.036	0.000	0.000	0.000	0.000	0.000
162	0.000	0.000	0.000	0.000	0.000	0.000	0.033	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
168	0.022	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
176	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.018	0.000	0.000
192	0.000	0.000	0.033	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

LOCUS SITES

CM 5/23	Sk2	Mo1	Mo3	Se1	Se3	Pr1	Pr3	Fr2	Vigo	Alme	Baia	Si1	Si3	Gr1	Gr3	Po1	Po3	Moly
N	25	32	31	32	26	32	28	32	24	22	24	27	30	27	28	29	29	24
158	0.000	0.016	0.000	0.000	0.000	0.016	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
162	0.000	0.000	0.016	0.000	0.039	0.016	0.000	0.000	0.000	0.023	0.000	0.000	0.000	0.000	0.000	0.000	0.017	0.021
164	0.000	0.000	0.016	0.000	0.000	0.000	0.018	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
166	0.000	0.000	0.000	0.000	0.000	0.000	0.018	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
170	0.000	0.000	0.032	0.031	0.000	0.000	0.036	0.016	0.000	0.023	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
172	0.000	0.000	0.048	0.016	0.000	0.016	0.036	0.016	0.000	0.000	0.000	0.019	0.000	0.037	0.000	0.000	0.000	0.000
174	0.020	0.000	0.032	0.016	0.058	0.000	0.036	0.047	0.083	0.046	0.479	0.130	0.000	0.000	0.000	0.069	0.017	0.021
176	0.500	0.609	0.419	0.609	0.481	0.500	0.536	0.422	0.396	0.591	0.167	0.019	0.000	0.000	0.000	0.000	0.000	0.021
178	0.300	0.219	0.210	0.156	0.231	0.266	0.143	0.297	0.396	0.023	0.000	0.000	0.000	0.056	0.000	0.000	0.000	0.021
180	0.080	0.109	0.145	0.109	0.173	0.031	0.089	0.094	0.021	0.136	0.000	0.000	0.017	0.000	0.018	0.052	0.069	0.000
182	0.020	0.000	0.032	0.031	0.019	0.078	0.018	0.078	0.042	0.046	0.000	0.000	0.000	0.019	0.000	0.017	0.000	0.000
184	0.000	0.031	0.032	0.000	0.000	0.047	0.036	0.000	0.042	0.023	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.021
186	0.060	0.000	0.000	0.016	0.000	0.016	0.000	0.016	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
188	0.020	0.000	0.000	0.000	0.000	0.000	0.018	0.016	0.000	0.000	0.021	0.037	0.000	0.019	0.018	0.000	0.000	0.000
190	0.000	0.000	0.016	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.052	0.017	0.000
192	0.000	0.000	0.000	0.000	0.000	0.016	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.035	0.000	0.063
194	0.000	0.000	0.000	0.016	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.019	0.036	0.000	0.069	0.042
196	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.042	0.019	0.017	0.019	0.000	0.000	0.052	0.000
198	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.033	0.056	0.000	0.000	0.052	0.000
200	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.017	0.000	0.036	0.017	0.000	0.021
202	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.021	0.000	0.017	0.019	0.000	0.035	0.017	0.021
204	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.033	0.037	0.018	0.000	0.000	0.000
206	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.021	0.000	0.000	0.019	0.017	0.037	0.000	0.000	0.017	0.000
208	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.023	0.000	0.000	0.000	0.019	0.018	0.017	0.017	0.042
210	0.000	0.000	0.000	0.000	0.000	0.000	0.018	0.000	0.000	0.000	0.000	0.037	0.067	0.000	0.036	0.052	0.035	0.042
212	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.063	0.056	0.017	0.000	0.018	0.000	0.000	0.042
214	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.021	0.000	0.033	0.056	0.000	0.017	0.000	0.042
216	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.021	0.000	0.017	0.000	0.036	0.035	0.000	0.021
218	0.000	0.016	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.021	0.037	0.017	0.000	0.036	0.000	0.000	0.000
220	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.056	0.050	0.000	0.000	0.000	0.000	0.042
222	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.042	0.000	0.017	0.093	0.036	0.035	0.017	0.083
224	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.056	0.033	0.019	0.018	0.017	0.000	0.000

	Sk2	Mo1	Mo3	Se1	Se3	Pr1	Pr3	Fr2	Vigo	Alme	Baia	Si1	Si3	Gr1	Gr3	Po1	Po3	Moly
226	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.037	0.083	0.037	0.000	0.000	0.069	0.042
228	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.037	0.000	0.000	0.071	0.035	0.138	0.063
230	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.019	0.050	0.037	0.000	0.103	0.017	0.000
232	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.042	0.074	0.033	0.019	0.018	0.017	0.000	0.021
234	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.021	0.037	0.050	0.037	0.036	0.000	0.052	0.000
236	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.056	0.017	0.019	0.071	0.035	0.017	0.000
238	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.017	0.000	0.000	0.000	0.000	0.000
240	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.037	0.033	0.037	0.054	0.000	0.035	0.063
242	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.021	0.000	0.083	0.037	0.089	0.035	0.035	0.000
244	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.037	0.000	0.000	0.036	0.017	0.000	0.000
246	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.019	0.033	0.000	0.054	0.017	0.069	0.021
248	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.019	0.017	0.019	0.036	0.000	0.000	0.000
250	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.037	0.000	0.000	0.035	0.021
252	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.019	0.000	0.037	0.018	0.052	0.000	0.042
254	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.023	0.000	0.019	0.083	0.037	0.000	0.000	0.000	0.000
256	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.019	0.000	0.019	0.000	0.035	0.069	0.000
258	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.019	0.000	0.056	0.000	0.035	0.017	0.000
262	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.046	0.000	0.037	0.000	0.000	0.000	0.035	0.000	0.000
266	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.019	0.000	0.037	0.089	0.069	0.017	0.042
268	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.017	0.000	0.036	0.000	0.000	0.021
272	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.033	0.019	0.000	0.017	0.000	0.021
274	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.021	0.019	0.017	0.019	0.018	0.000	0.035	0.042
278	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.021
282	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.033	0.000	0.036	0.000	0.000	0.000
284	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.018	0.000	0.000	0.000
288	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.052	0.000	0.021

LOCUS SITES

CM 4/3	Sk2	Mo1	Mo3	Se1	Se3	Pr1	Pr3	Fr2	Vigo	Alme	Baia	Si1	Si3	Gr1	Gr3	Po1	Po3	Moly
N	16	21	15	13	21	21	21	21	15	22	25	18	19	25	24	22	25	24
74	0.000	0.000	0.000	0.000	0.000	0.048	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
80	0.000	0.000	0.000	0.000	0.000	0.048	0.048	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
82	0.000	0.071	0.000	0.000	0.048	0.000	0.095	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
96	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.046	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
100	0.000	0.167	0.000	0.000	0.000	0.048	0.024	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
102	0.000	0.024	0.000	0.000	0.048	0.238	0.167	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

	Sk2	Mo1	Mo3	Se1	Se3	Pr1	Pr3	Fr2	Vigo	Alme	Baia	Si1	Si3	Gr1	Gr3	Po1	Po3	Moly
104	0.000	0.214	0.000	0.000	0.000	0.238	0.167	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.023	0.000	0.000
106	0.000	0.095	0.000	0.000	0.000	0.000	0.048	0.000	0.067	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
112	0.000	0.000	0.000	0.000	0.000	0.048	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
116	0.000	0.024	0.000	0.000	0.000	0.048	0.024	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
120	0.000	0.000	0.000	0.000	0.000	0.000	0.048	0.048	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
124	0.000	0.048	0.000	0.000	0.000	0.048	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
130	0.000	0.000	0.000	0.000	0.048	0.000	0.048	0.000	0.000	0.046	0.000	0.000	0.000	0.000	0.000	0.068	0.000	0.000
140	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.042
174	0.250	0.071	0.300	0.231	0.143	0.191	0.167	0.095	0.067	0.568	0.320	0.250	0.211	0.380	0.271	0.136	0.420	0.188
176	0.344	0.143	0.633	0.577	0.262	0.000	0.048	0.452	0.600	0.136	0.020	0.028	0.132	0.000	0.000	0.000	0.020	0.000
178	0.063	0.048	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.026	0.000	0.000	0.000	0.000	0.000
188	0.188	0.048	0.000	0.000	0.000	0.000	0.000	0.024	0.067	0.023	0.140	0.194	0.053	0.040	0.146	0.114	0.040	0.250
190	0.000	0.000	0.000	0.000	0.071	0.000	0.048	0.095	0.000	0.136	0.060	0.028	0.026	0.020	0.083	0.046	0.020	0.063
192	0.000	0.024	0.067	0.077	0.191	0.048	0.048	0.000	0.067	0.000	0.180	0.278	0.158	0.060	0.063	0.227	0.160	0.167
194	0.063	0.000	0.000	0.077	0.000	0.000	0.024	0.048	0.067	0.000	0.080	0.083	0.105	0.140	0.021	0.159	0.160	0.188
196	0.000	0.000	0.000	0.000	0.095	0.000	0.000	0.000	0.000	0.000	0.160	0.028	0.026	0.100	0.021	0.023	0.120	0.063
198	0.063	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.111	0.211	0.220	0.250	0.159	0.040	0.021
202	0.000	0.024	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.053	0.040	0.104	0.000	0.000	0.021
204	0.000	0.000	0.000	0.039	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
206	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.020	0.000	0.000	0.000	0.000	0.000	0.000	0.000
208	0.000	0.000	0.000	0.000	0.048	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.020	0.000
212	0.000	0.000	0.000	0.000	0.048	0.000	0.000	0.000	0.067	0.000	0.000	0.000	0.000	0.000	0.042	0.046	0.000	0.000
224	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.020	0.000	0.000	0.000	0.000	0.000	0.000	0.000
228	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.048	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
234	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.046	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
236	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.048	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
244	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.048	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
246	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.095	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
254	0.031	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

LOCUS SITES

CM 5/18	Sk2	Mo1	Mo3	Se1	Se3	Pr1	Pr3	Fr2	Vigo	Alme	Baia	Si1	Si3	Gr1	Gr3	Po1	Po3	Moly
N	25	25	24	26	23	29	25	31	24	25	25	20	21	25	25	23	19	24
70	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.020	0.000	0.000	0.000	0.000
76	0.020	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.021	0.000	0.040	0.000	0.000	0.000	0.020	0.000	0.000	0.021
92	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.021	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

98	0.000	0.020	0.000	0.039	0.065	0.035	0.000	0.000	0.042	0.000	0.000	0.000	0.000	0.040	0.000	0.000	0.000	0.000
100	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.032	0.042	0.000	0.060	0.025	0.024	0.000	0.020	0.000	0.000	0.042
102	0.000	0.020	0.000	0.039	0.022	0.000	0.000	0.016	0.042	0.000	0.000	0.000	0.000	0.020	0.000	0.000	0.000	0.000
104	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.021	0.000	0.040	0.100	0.048	0.080	0.040	0.065	0.184	0.229
106	0.000	0.000	0.021	0.019	0.000	0.035	0.000	0.032	0.000	0.080	0.000	0.050	0.000	0.020	0.060	0.022	0.000	0.021
108	0.000	0.000	0.042	0.039	0.000	0.000	0.000	0.065	0.021	0.040	0.020	0.025	0.000	0.000	0.000	0.000	0.053	0.000
110	0.000	0.020	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.020	0.025	0.024	0.020	0.000	0.000	0.000	0.042
112	0.000	0.000	0.021	0.039	0.000	0.000	0.000	0.000	0.000	0.020	0.020	0.000	0.024	0.000	0.000	0.000	0.026	0.000
114	0.000	0.000	0.000	0.000	0.000	0.017	0.040	0.000	0.000	0.000	0.000	0.050	0.000	0.000	0.000	0.000	0.079	0.000
116	0.000	0.000	0.000	0.000	0.000	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
118	0.020	0.000	0.021	0.000	0.000	0.000	0.000	0.048	0.000	0.000	0.000	0.000	0.048	0.000	0.000	0.000	0.000	0.000
120	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.020	0.000	0.000	0.000	0.000	0.000	0.000
126	0.080	0.040	0.042	0.000	0.000	0.000	0.020	0.000	0.000	0.100	0.100	0.100	0.095	0.100	0.120	0.109	0.000	0.000
128	0.620	0.300	0.563	0.596	0.565	0.224	0.380	0.597	0.625	0.480	0.680	0.500	0.619	0.620	0.740	0.674	0.658	0.583
130	0.000	0.000	0.042	0.077	0.044	0.000	0.000	0.000	0.000	0.020	0.000	0.075	0.048	0.000	0.000	0.022	0.000	0.000
132	0.020	0.020	0.000	0.000	0.000	0.017	0.000	0.000	0.000	0.000	0.000	0.025	0.000	0.040	0.000	0.000	0.000	0.000
134	0.020	0.000	0.021	0.000	0.065	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.024	0.000	0.000	0.000	0.000	0.000
136	0.000	0.020	0.000	0.000	0.000	0.000	0.000	0.016	0.042	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
138	0.000	0.000	0.000	0.000	0.000	0.017	0.000	0.016	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
144	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.020	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
154	0.000	0.560	0.000	0.000	0.000	0.638	0.500	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
160	0.000	0.000	0.000	0.000	0.000	0.000	0.020	0.016	0.000	0.020	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
174	0.000	0.000	0.000	0.000	0.044	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.044	0.000	0.000
180	0.120	0.000	0.167	0.154	0.174	0.000	0.000	0.129	0.125	0.220	0.000	0.000	0.024	0.020	0.000	0.000	0.000	0.021
182	0.100	0.000	0.042	0.000	0.000	0.000	0.000	0.032	0.000	0.000	0.000	0.000	0.000	0.020	0.000	0.044	0.000	0.021
206	0.000	0.000	0.000	0.000	0.000	0.000	0.040	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.022	0.000	0.021
208	0.000	0.000	0.000	0.000	0.022	0.000	0.000	0.000	0.000	0.000	0.000	0.025	0.024	0.000	0.000	0.000	0.000	0.000
250	0.000	0.000	0.021	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

LOCUS	SITES																	
CM 9/11	Sk2	Mo1	Mo3	Se1	Se3	Pr1	Pr3	Fr2	Vigo	Alme	Baia	Si1	Si3	Gr1	Gr3	Po1	Po3	Moly
N	25	25	22	22	22	27	25	31	22	22	24	20	21	25	25	22	25	25
88	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.021	0.000	0.000	0.000	0.140	0.000	0.000	0.000
94	0.040	0.000	0.000	0.046	0.046	0.019	0.000	0.016	0.000	0.046	0.167	0.300	0.405	0.440	0.300	0.500	0.400	0.100
96	0.000	0.000	0.000	0.000	0.000	0.037	0.040	0.000	0.000	0.000	0.250	0.075	0.071	0.100	0.140	0.136	0.100	0.000
98	0.100	0.000	0.000	0.046	0.000	0.000	0.040	0.048	0.000	0.023	0.042	0.050	0.024	0.060	0.060	0.000	0.020	0.040

Appendix 6.1 - continued

100	0.000	0.040	0.000	0.000	0.023	0.000	0.000	0.000	0.068	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
102	0.080	0.000	0.046	0.046	0.023	0.056	0.040	0.065	0.091	0.114	0.063	0.025	0.000	0.020	0.000	0.023	0.020	0.060
104	0.020	0.040	0.046	0.068	0.046	0.000	0.000	0.032	0.046	0.023	0.021	0.000	0.000	0.000	0.020	0.000	0.000	0.040
106	0.040	0.000	0.023	0.091	0.136	0.093	0.080	0.129	0.046	0.023	0.000	0.000	0.000	0.000	0.040	0.000	0.040	0.100
108	0.120	0.000	0.114	0.046	0.114	0.019	0.040	0.145	0.046	0.136	0.042	0.000	0.000	0.000	0.000	0.000	0.020	0.000
110	0.020	0.060	0.046	0.046	0.114	0.056	0.000	0.065	0.068	0.068	0.000	0.050	0.000	0.000	0.040	0.000	0.040	0.020
112	0.140	0.020	0.159	0.023	0.046	0.037	0.020	0.113	0.046	0.091	0.000	0.050	0.024	0.000	0.000	0.023	0.000	0.020
114	0.060	0.040	0.046	0.091	0.023	0.056	0.040	0.097	0.205	0.136	0.000	0.000	0.024	0.000	0.040	0.000	0.000	0.020
116	0.060	0.080	0.091	0.136	0.068	0.074	0.020	0.065	0.046	0.068	0.021	0.000	0.000	0.000	0.020	0.000	0.000	0.040
118	0.080	0.000	0.091	0.068	0.114	0.019	0.020	0.097	0.000	0.068	0.021	0.025	0.024	0.000	0.000	0.023	0.020	0.020
120	0.080	0.060	0.091	0.046	0.046	0.000	0.060	0.016	0.091	0.023	0.021	0.025	0.048	0.000	0.000	0.000	0.000	0.000
122	0.000	0.020	0.000	0.114	0.000	0.000	0.020	0.016	0.023	0.000	0.021	0.000	0.000	0.000	0.020	0.000	0.000	0.080
124	0.060	0.000	0.000	0.068	0.046	0.037	0.000	0.000	0.046	0.091	0.042	0.000	0.048	0.020	0.000	0.023	0.040	0.100
126	0.000	0.000	0.114	0.023	0.068	0.019	0.000	0.016	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.023	0.040	0.020
128	0.000	0.000	0.046	0.000	0.000	0.000	0.000	0.016	0.000	0.000	0.000	0.100	0.024	0.000	0.000	0.000	0.000	0.000
130	0.020	0.000	0.000	0.000	0.000	0.000	0.020	0.032	0.046	0.000	0.000	0.100	0.048	0.020	0.020	0.023	0.020	0.020
132	0.040	0.000	0.000	0.000	0.000	0.019	0.000	0.000	0.000	0.023	0.042	0.000	0.071	0.120	0.020	0.114	0.020	0.060
134	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.063	0.025	0.048	0.040	0.000	0.046	0.020	0.040
136	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.046	0.000	0.000	0.025	0.000	0.060	0.060	0.000	0.000	0.020
140	0.000	0.000	0.000	0.046	0.000	0.000	0.000	0.000	0.023	0.023	0.083	0.000	0.000	0.020	0.000	0.046	0.060	0.060
142	0.040	0.000	0.023	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.042	0.000	0.000	0.000	0.020	0.023	0.080	0.020
148	0.000	0.000	0.023	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.042	0.125	0.024	0.080	0.020	0.000	0.000	0.060
152	0.000	0.000	0.000	0.000	0.046	0.000	0.000	0.000	0.023	0.000	0.000	0.000	0.000	0.000	0.020	0.000	0.020	0.000
154	0.000	0.000	0.023	0.000	0.000	0.000	0.000	0.000	0.046	0.000	0.000	0.025	0.000	0.020	0.020	0.000	0.000	0.000
156	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.020
158	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.040	0.000
166	0.000	0.000	0.023	0.000	0.000	0.000	0.020	0.000	0.000	0.000	0.000	0.000	0.071	0.000	0.000	0.000	0.000	0.000
174	0.000	0.000	0.000	0.000	0.046	0.019	0.080	0.000	0.000	0.000	0.000	0.000	0.048	0.000	0.000	0.000	0.000	0.000
178	0.000	0.420	0.000	0.000	0.000	0.333	0.360	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.020
180	0.000	0.160	0.000	0.000	0.000	0.056	0.100	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.020
182	0.000	0.060	0.000	0.000	0.000	0.056	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
224	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.046	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
246	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.032	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Appendix 6.2 - Test for Hardy-Weinberg equilibrium (F_{IS}, Weir and Cockerham, 1984) using Fisher's method, and estimation of exact probability values (P-value) by Markov-chain randomization (10,000 dememorizations, 100 batches and 5,000 iterations per batch). For abbreviations see Table 6.1.

LOCUS CM 2/15			
SITES	F_{IS} (W&C)	P-value	S.E.
Sk2	1.000	0.000	-
Mo1	1.000	0.016	-
Mo3	0.663	0.016	-
Se1		-	
Se3	1.000	0.000	-
Pr1	0.489	0.017	-
Pr3	0.659	0.051	-
Fr2		-	
Vigo	0.657	0.064	-
Alme	0.844	0.000	-
Baia	0.508	0.000	0.000
Si1	0.330	0.001	0.000
Si3	0.312	0.008	0.001
Gr1	0.198	0.026	0.003
Gr3	0.275	0.000	0.000
Po1	0.514	0.000	0.000
Po3	0.399	0.000	0.000
Moly	0.267	0.039	0.003
LOCUS CM 4/5			
SITES	F_{IS} (W&C)	P-value	S.E.
Sk2	0.567	0.000	0.000
Mo1	0.653	0.000	0.000
Mo3	0.822	0.000	0.000
Se1	0.692	0.000	0.000
Se3	0.747	0.000	0.000
Pr1	0.653	0.000	0.000
Pr3	0.611	0.000	0.000
Fr2	0.540	0.000	0.000
Vigo	0.663	0.000	0.000
Alme	0.591	0.000	0.000
Baia	0.765	0.000	0.000
Si1	0.749	0.000	0.000
Si3	0.750	0.000	0.000
Gr1	0.626	0.000	0.000
Gr3	0.722	0.000	0.000
Po1	0.716	0.000	0.000
Po3	0.732	0.000	0.000
Moly	0.448	0.000	0.000
LOCUS CM 5/23			
SITES	F_{IS} (W&C)	P-value	S.E.
Sk2	0.157	0.217	0.007
Mo1	0.408	0.008	0.001
Mo3	0.201	0.006	0.002
Se1	0.167	0.000	0.000
Se3	0.339	0.027	0.002
Pr1	0.175	0.067	0.007

Pr3	0.122	0.441	0.023
Fr2	0.099	0.384	0.015
Vigo	0.218	0.054	0.004
Alme	0.435	0.000	0.000
Baia	-0.063	0.003	0.002
Si1	0.468	0.000	0.000
Si3	0.385	0.000	0.000
Gr1	0.436	0.000	0.000
Gr3	0.451	0.000	0.000
Po1	0.399	0.000	0.000
Po3	0.464	0.000	0.000
Moly	0.494	0.000	0.000
LOCUS CM 4/3			
SITES	F_{IS} **(W&C)**	***P*-value**	**S.E.**
Sk2	0.924	0.000	0.000
Mo1	0.529	0.000	0.000
Mo3	0.876	0.000	-
Se1	0.881	0.000	0.000
Se3	0.839	0.000	0.000
Pr1	0.836	0.000	0.000
Pr3	0.640	0.000	0.000
Fr2	0.940	0.000	0.000
Vigo	1.000	0.000	0.000
Alme	0.793	0.000	0.000
Baia	0.472	0.000	0.000
Si1	0.602	0.000	0.000
Si3	0.282	0.012	0.002
Gr1	0.649	0.000	0.000
Gr3	0.606	0.000	0.000
Po1	0.694	0.000	0.000
Po3	0.381	0.003	0.001
Moly	0.660	0.000	0.000
LOCUS CM 5/18			
SITES	F_{IS} **(W&C)**	***P*-value**	**S.E.**
Sk2	0.467	0.001	0.000
Mo1	-0.469	1.000	0.000
Mo3	0.183	0.000	0.000
Se1	0.447	0.000	0.000
Se3	0.337	0.000	0.000
Pr1	0.313	0.004	0.001
Pr3	-0.177	0.039	0.002
Fr2	0.231	0.004	0.001
Vigo	0.377	0.000	0.000
Alme	0.446	0.000	0.000
Baia	0.172	0.072	0.006
Si1	0.048	0.301	0.016
Si3	0.305	0.000	0.000
Gr1	0.411	0.000	0.000
Gr3	0.186	0.032	0.002
Po1	0.275	0.002	0.001
Po3	0.809	0.000	0.000
Moly	0.463	0.000	0.000

LOCUS CM 9/11			
SITES	F_{IS} (W&C)	P-value	S.E.
Sk2	0.748	0.000	0.000
Mo1	0.143	0.005	0.002
Mo3	0.518	0.000	0.000
Se1	0.572	0.000	0.000
Se3	0.618	0.000	0.000
Pr1	0.021	0.039	0.010
Pr3	0.156	0.000	0.000
Fr2	0.656	0.000	0.000
Vigo	0.616	0.000	0.000
Alme	0.616	0.000	0.000
Baia	0.029	0.521	0.029
Si1	0.323	0.000	0.000
Si3	0.429	0.000	0.000
Gr1	0.646	0.000	0.000
Gr3	-0.055	0.393	0.028
Po1	0.191	0.069	0.009
Po3	0.326	0.000	0.000
Moly	0.083	0.000	0.000
ALL LOCI			
SITES	F_{IS} (W&C)	P-value	S.E.
Sk2	0.629	0.000	0.000
Mo1	0.314	0.000	0.000
Mo3	0.524	0.000	0.000
Se1	0.568	0.000	0.000
Se3	0.626	0.000	0.000
Pr1	0.424	0.000	0.000
Pr3	0.326	0.000	0.000
Fr2	0.517	0.000	0.000
Vigo	0.586	0.000	0.000
Alme	0.596	0.000	0.000
Baia	0.330	0.000	0.000
Si1	0.431	0.000	0.000
Si3	0.416	0.000	0.000
Gr1	0.499	0.000	0.000
Gr3	0.383	0.000	0.000
Po1	0.485	0.000	0.000
Po3	0.507	0.000	0.000
Moly	0.395	0.000	0.000

Appendix 6.3 – Tests for linkage disequilibrium in each location and for each locus pair, estimation of exact probability values (*P*-value) and standard error (S.E.) determined by Markov-chain randomization (10,000 dememorizations, 1,00 batches and 5,000 iterations per batch). Significant *P*-values in bold.

SITE	LOCUS 1	LOCUS 2	*P*-values	S.E.
Sk2	Cm2/15	Cm 4/5	0.4692	0.0050
Sk2	Cm2/15	Cm5/23	0.0893	0.0017
Sk2	Cm5/23	Cm 4/5	**0.0110**	0.0033
Sk2	Cm2/15	Cm 4/3	0.7530	0.0025
Sk2	Cm 4/5	Cm 4/3	0.2124	0.0143
Sk2	Cm5/23	Cm 4/3	0.3300	0.0103
Sk2	Cm2/15	Cm5/18	0.0670	0.0022
Sk2	Cm 4/5	Cm5/18	0.0110	0.0040
Sk2	Cm5/23	Cm5/18	0.4785	0.0159
Sk2	Cm 4/3	Cm5/18	0.5157	0.0095
Sk2	Cm2/15	Cm9/11	0.8981	0.0025
Sk2	Cm 4/5	Cm9/11	1.0000	0.0000
Sk2	Cm5/23	Cm9/11	0.7398	0.0179
Sk2	Cm 4/3	Cm9/11	0.5095	0.0157
Sk2	Cm5/18	Cm9/11	0.6974	0.0223
Mo1	Cm2/15	Cm 4/5	1.0000	0.0000
Mo1	Cm2/15	Cm5/23	1.0000	0.0000
Mo1	Cm5/23	Cm 4/5	0.9973	0.0015
Mo1	Cm2/15	Cm 4/3	0.6175	0.0053
Mo1	Cm 4/5	Cm 4/3	1.0000	0.0000
Mo1	Cm5/23	Cm 4/3	0.5827	0.0166
Mo1	Cm2/15	Cm5/18	0.3954	0.0051
Mo1	Cm 4/5	Cm5/18	0.8544	0.0115
Mo1	Cm5/23	Cm5/18	0.3468	0.0153
Mo1	Cm 4/3	Cm5/18	0.1918	0.0155
Mo1	Cm2/15	Cm9/11	0.7265	0.0050
Mo1	Cm 4/5	Cm9/11	1.0000	0.0000
Mo1	Cm5/23	Cm9/11	0.8753	0.0097
Mo1	Cm 4/3	Cm9/11	1.0000	0.0000
Mo1	Cm5/18	Cm9/11	0.9413	0.0070
Mo3	Cm2/15	Cm 4/5	0.4422	0.0095
Mo3	Cm2/15	Cm5/23	0.3647	0.0084
Mo3	Cm5/23	Cm 4/5	0.7436	0.0261
Mo3	Cm2/15	Cm 4/3	Not calculable	
Mo3	Cm 4/5	Cm 4/3	0.8642	0.0045
Mo3	Cm5/23	Cm 4/3	1.0000	0.0000
Mo3	Cm2/15	Cm5/18	0.4200	0.0053
Mo3	Cm 4/5	Cm5/18	**0.0469**	0.0089
Mo3	Cm5/23	Cm5/18	0.2518	0.0204
Mo3	Cm 4/3	Cm5/18	0.5697	0.0073
Mo3	Cm2/15	Cm9/11	1.0000	0.0000
Mo3	Cm 4/5	Cm9/11	1.0000	0.0000
Mo3	Cm5/23	Cm9/11	1.0000	0.0000
Mo3	Cm 4/3	Cm9/11	Not calculable	
Mo3	Cm5/18	Cm9/11	1.0000	0.0000
Se1	Cm2/15	Cm 4/5	Not calculable	

Se1	Cm2/15	Cm5/23	Not calculable	
Se1	Cm5/23	Cm 4/5	0.9776	0.0054
Se1	Cm2/15	Cm 4/3	Not calculable	
Se1	Cm 4/5	Cm 4/3	1.0000	0.0000
Se1	Cm5/23	Cm 4/3	0.3053	0.0059
Se1	Cm2/15	Cm5/18	Not calculable	
Se1	Cm 4/5	Cm5/18	0.6158	0.0199
Se1	Cm5/23	Cm5/18	0.4785	0.0198
Se1	Cm 4/3	Cm5/18	0.0715	0.0043
Se1	Cm2/15	Cm9/11	Not calculable	
Se1	Cm 4/5	Cm9/11	1.0000	0.0000
Se1	Cm5/23	Cm9/11	1.0000	0.0000
Se1	Cm 4/3	Cm9/11	Not calculable	
Se1	Cm5/18	Cm9/11	1.0000	0.0000
Se3	Cm2/15	Cm 4/5	0.5920	0.0057
Se3	Cm2/15	Cm5/23	0.0715	0.0030
Se3	Cm5/23	Cm 4/5	0.3013	0.0201
Se3	Cm2/15	Cm 4/3	1.0000	0.0000
Se3	Cm 4/5	Cm 4/3	0.4420	0.0220
Se3	Cm5/23	Cm 4/3	0.6964	0.0145
Se3	Cm2/15	Cm5/18	0.5554	0.0090
Se3	Cm 4/5	Cm5/18	0.3442	0.0235
Se3	Cm5/23	Cm5/18	0.0298	0.0052
Se3	Cm 4/3	Cm5/18	0.9875	0.0027
Se3	Cm2/15	Cm9/11	0.7768	0.0076
Se3	Cm 4/5	Cm9/11	1.0000	0.0000
Se3	Cm5/23	Cm9/11	1.0000	0.0000
Se3	Cm 4/3	Cm9/11	1.0000	0.0000
Se3	Cm5/18	Cm9/11	0.4976	0.0229
Pr1	Cm2/15	Cm 4/5	0.7355	0.0129
Pr1	Cm2/15	Cm5/23	0.0865	0.0064
Pr1	Cm5/23	Cm 4/5	0.6523	0.0305
Pr1	Cm2/15	Cm 4/3	0.3036	0.0090
Pr1	Cm 4/5	Cm 4/3	0.0001	0.0001
Pr1	Cm5/23	Cm 4/3	0.8699	0.0140
Pr1	Cm2/15	Cm5/18	0.1391	0.0068
Pr1	Cm 4/5	Cm5/18	0.9426	0.0103
Pr1	Cm5/23	Cm5/18	0.5250	0.0235
Pr1	Cm 4/3	Cm5/18	0.1181	0.0065
Pr1	Cm2/15	Cm9/11	0.5931	0.0134
Pr1	Cm 4/5	Cm9/11	0.1700	0.0308
Pr1	Cm5/23	Cm9/11	0.1563	0.0192
Pr1	Cm 4/3	Cm9/11	1.0000	0.0000
Pr1	Cm5/18	Cm9/11	0.5026	0.0212
Pr3	Cm2/15	Cm 4/5	0.5963	0.0113
Pr3	Cm2/15	Cm5/23	0.1257	0.0071
Pr3	Cm5/23	Cm 4/5	0.8331	0.0221
Pr3	Cm2/15	Cm 4/3	0.7221	0.0051
Pr3	Cm 4/5	Cm 4/3	0.1436	0.0239
Pr3	Cm5/23	Cm 4/3	0.1363	0.0218
Pr3	Cm2/15	Cm5/18	0.0587	0.0027
Pr3	Cm 4/5	Cm5/18	0.1122	0.0138
Pr3	Cm5/23	Cm5/18	0.1108	0.0148

Pr3	Cm 4/3	Cm5/18	0.4444	0.0141
Pr3	Cm2/15	Cm9/11	0.3001	0.0088
Pr3	Cm 4/5	Cm9/11	1.0000	0.0000
Pr3	Cm5/23	Cm9/11	**0.0147**	0.0085
Pr3	Cm 4/3	Cm9/11	0.2534	0.0274
Pr3	Cm5/18	Cm9/11	0.9188	0.0103
Fr2	Cm2/15	Cm 4/5	Not calculable	
Fr2	Cm2/15	Cm5/23	Not calculable	
Fr2	Cm5/23	Cm 4/5	0.8668	0.0171
Fr2	Cm2/15	Cm 4/3	Not calculable	
Fr2	Cm 4/5	Cm 4/3	0.7283	0.0213
Fr2	Cm5/23	Cm 4/3	0.5938	0.0130
Fr2	Cm2/15	Cm5/18	Not calculable	
Fr2	Cm 4/5	Cm5/18	0.3787	0.0308
Fr2	Cm5/23	Cm5/18	0.7171	0.0192
Fr2	Cm 4/3	Cm5/18	0.1784	0.0148
Fr2	Cm2/15	Cm9/11	Not calculable	
Fr2	Cm 4/5	Cm9/11	1.0000	0.0000
Fr2	Cm5/23	Cm9/11	0.3907	0.0287
Fr2	Cm 4/3	Cm9/11	0.2670	0.0201
Fr2	Cm5/18	Cm9/11	0.0958	0.0153
Vigo	Cm2/15	Cm 4/5	1.0000	0.0000
Vigo	Cm2/15	Cm5/23	0.1254	0.0035
Vigo	Cm5/23	Cm 4/5	1.0000	0.0000
Vigo	Cm2/15	Cm 4/3	0.6978	0.0055
Vigo	Cm 4/5	Cm 4/3	0.1645	0.0094
Vigo	Cm5/23	Cm 4/3	0.9441	0.0036
Vigo	Cm2/15	Cm5/18	0.1133	0.0063
Vigo	Cm 4/5	Cm5/18	0.5487	0.0223
Vigo	Cm5/23	Cm5/18	0.0809	0.0075
Vigo	Cm 4/3	Cm5/18	0.9676	0.0043
Vigo	Cm2/15	Cm9/11	0.3902	0.0087
Vigo	Cm 4/5	Cm9/11	1.0000	0.0000
Vigo	Cm5/23	Cm9/11	0.6715	0.0190
Vigo	Cm 4/3	Cm9/11	0.0524	0.0060
Vigo	Cm5/18	Cm9/11	0.8823	0.0137
Alme	Cm2/15	Cm 4/5	0.3356	0.0138
Alme	Cm2/15	Cm5/23	0.1842	0.0082
Alme	Cm5/23	Cm 4/5	1.0000	0.0000
Alme	Cm2/15	Cm 4/3	0.2498	0.0075
Alme	Cm 4/5	Cm 4/3	0.5086	0.0208
Alme	Cm5/23	Cm 4/3	0.9592	0.0044
Alme	Cm2/15	Cm5/18	0.3274	0.0093
Alme	Cm 4/5	Cm5/18	0.6171	0.0243
Alme	Cm5/23	Cm5/18	0.5289	0.0181
Alme	Cm 4/3	Cm5/18	0.1538	0.0096
Alme	Cm2/15	Cm9/11	0.8541	0.0061
Alme	Cm 4/5	Cm9/11	0.0710	0.0171
Alme	Cm5/23	Cm9/11	1.0000	0.0000
Alme	Cm 4/3	Cm9/11	0.8689	0.0102
Alme	Cm5/18	Cm9/11	1.0000	0.0000
Baia	Cm2/15	Cm 4/5	0.0988	0.0119
Baia	Cm2/15	Cm5/23	0.0941	0.0138

Baia	Cm5/23	Cm 4/5	0.2155	0.0192
Baia	Cm2/15	Cm 4/3	1.0000	0.0000
Baia	Cm 4/5	Cm 4/3	1.0000	0.0000
Baia	Cm5/23	Cm 4/3	0.2381	0.0195
Baia	Cm2/15	Cm5/18	0.4058	0.0221
Baia	Cm 4/5	Cm5/18	0.6170	0.0183
Baia	Cm5/23	Cm5/18	0.1607	0.0203
Baia	Cm 4/3	Cm5/18	0.2293	0.0188
Baia	Cm2/15	Cm9/11	1.0000	0.0000
Baia	Cm 4/5	Cm9/11	1.0000	0.0000
Baia	Cm5/23	Cm9/11	1.0000	0.0000
Baia	Cm 4/3	Cm9/11	1.0000	0.0000
Baia	Cm5/18	Cm9/11	1.0000	0.0000
Si1	Cm2/15	Cm 4/5	0.7554	0.0222
Si1	Cm2/15	Cm5/23	1.0000	0.0000
Si1	Cm5/23	Cm 4/5	1.0000	0.0000
Si1	Cm2/15	Cm 4/3	0.4620	0.0192
Si1	Cm 4/5	Cm 4/3	1.0000	0.0000
Si1	Cm5/23	Cm 4/3	Not calculable	
Si1	Cm2/15	Cm5/18	0.5275	0.0222
Si1	Cm 4/5	Cm5/18	0.4508	0.0275
Si1	Cm5/23	Cm5/18	0.0540	0.0141
Si1	Cm 4/3	Cm5/18	0.5606	0.0218
Si1	Cm2/15	Cm9/11	0.3759	0.0232
Si1	Cm 4/5	Cm9/11	1.0000	0.0000
Si1	Cm5/23	Cm9/11	1.0000	0.0000
Si1	Cm 4/3	Cm9/11	0.0000	0.0000
Si1	Cm5/18	Cm9/11	1.0000	0.0000
Si3	Cm2/15	Cm 4/5	0.8340	0.0165
Si3	Cm2/15	Cm5/23	1.0000	0.0000
Si3	Cm5/23	Cm 4/5	Not calculable	
Si3	Cm2/15	Cm 4/3	0.1576	0.0202
Si3	Cm 4/5	Cm 4/3	1.0000	0.0000
Si3	Cm5/23	Cm 4/3	Not calculable	
Si3	Cm2/15	Cm5/18	0.9389	0.0102
Si3	Cm 4/5	Cm5/18	0.3838	0.0227
Si3	Cm5/23	Cm5/18	Not calculable	
Si3	Cm 4/3	Cm5/18	1.0000	0.0000
Si3	Cm2/15	Cm9/11	1.0000	0.0000
Si3	Cm 4/5	Cm9/11	0.6638	0.0251
Si3	Cm5/23	Cm9/11	Not calculable	
Si3	Cm 4/3	Cm9/11	1.0000	0.0000
Si3	Cm5/18	Cm9/11	0.8734	0.0155
Gr1	Cm2/15	Cm 4/5	1.0000	0.0000
Gr1	Cm2/15	Cm5/23	1.0000	0.0000
Gr1	Cm5/23	Cm 4/5	0.0554	0.0180
Gr1	Cm2/15	Cm 4/3	0.6931	0.0200
Gr1	Cm 4/5	Cm 4/3	0.9425	0.0069
Gr1	Cm5/23	Cm 4/3	1.0000	0.0000
Gr1	Cm2/15	Cm5/18	0.3475	0.0285
Gr1	Cm 4/5	Cm5/18	0.9965	0.0017
Gr1	Cm5/23	Cm5/18	1.0000	0.0000
Gr1	Cm 4/3	Cm5/18	0.1160	0.0155

Gr1	Cm2/15	Cm9/11	0.1218	0.0178
Gr1	Cm 4/5	Cm9/11	0.8316	0.0192
Gr1	Cm5/23	Cm9/11	1.0000	0.0000
Gr1	Cm 4/3	Cm9/11	0.5238	0.0258
Gr1	Cm5/18	Cm9/11	0.7581	0.0258
Gr3	Cm2/15	Cm 4/5	0.8657	0.0184
Gr3	Cm2/15	Cm5/23	1.0000	0.0000
Gr3	Cm5/23	Cm 4/5	1.0000	0.0000
Gr3	Cm2/15	Cm 4/3	1.0000	0.0000
Gr3	Cm 4/5	Cm 4/3	0.7539	0.0224
Gr3	Cm5/23	Cm 4/3	1.0000	0.0000
Gr3	Cm2/15	Cm5/18	0.6276	0.0134
Gr3	Cm 4/5	Cm5/18	0.6781	0.0211
Gr3	Cm5/23	Cm5/18	1.0000	0.0000
Gr3	Cm 4/3	Cm5/18	0.9531	0.0044
Gr3	Cm2/15	Cm9/11	1.0000	0.0000
Gr3	Cm 4/5	Cm9/11	0.4583	0.0351
Gr3	Cm5/23	Cm9/11	1.0000	0.0000
Gr3	Cm 4/3	Cm9/11	0.4397	0.0267
Gr3	Cm5/18	Cm9/11	0.9146	0.0104
Po1	Cm2/15	Cm 4/5	0.8241	0.0199
Po1	Cm2/15	Cm5/23	1.0000	0.0000
Po1	Cm5/23	Cm 4/5	1.0000	0.0000
Po1	Cm2/15	Cm 4/3	0.6669	0.0233
Po1	Cm 4/5	Cm 4/3	1.0000	0.0000
Po1	Cm5/23	Cm 4/3	1.0000	0.0000
Po1	Cm2/15	Cm5/18	0.1267	0.0146
Po1	Cm 4/5	Cm5/18	0.1033	0.0135
Po1	Cm5/23	Cm5/18	1.0000	0.0000
Po1	Cm 4/3	Cm5/18	0.2911	0.0162
Po1	Cm2/15	Cm9/11	0.4374	0.0277
Po1	Cm 4/5	Cm9/11	0.0176	0.0071
Po1	Cm5/23	Cm9/11	1.0000	0.0000
Po1	Cm 4/3	Cm9/11	1.0000	0.0000
Po1	Cm5/18	Cm9/11	0.0750	0.0114
Po3	Cm2/15	Cm 4/5	0.7896	0.0147
Po3	Cm2/15	Cm5/23	0.6710	0.0192
Po3	Cm5/23	Cm 4/5	0.4015	0.0360
Po3	Cm2/15	Cm 4/3	0.7521	0.0113
Po3	Cm 4/5	Cm 4/3	0.8397	0.0200
Po3	Cm5/23	Cm 4/3	0.4943	0.0289
Po3	Cm2/15	Cm5/18	0.1643	0.0065
Po3	Cm 4/5	Cm5/18	0.0712	0.0082
Po3	Cm5/23	Cm5/18	0.6390	0.0167
Po3	Cm 4/3	Cm5/18	0.9535	0.0046
Po3	Cm2/15	Cm9/11	0.4738	0.0199
Po3	Cm 4/5	Cm9/11	0.7090	0.0296
Po3	Cm5/23	Cm9/11	1.0000	0.0000
Po3	Cm 4/3	Cm9/11	1.0000	0.0000
Po3	Cm5/18	Cm9/11	0.4500	0.0174
Moly	Cm2/15	Cm 4/5	0.3756	0.0164
Moly	Cm2/15	Cm5/23	Not calculable	
Moly	Cm5/23	Cm 4/5	Not calculable	

Appendix 6.3 - continued

Moly	Cm2/15	Cm 4/3	0.7536	0.0180
Moly	Cm 4/5	Cm 4/3	0.4310	0.0148
Moly	Cm5/23	Cm 4/3	Not calculable	
Moly	Cm2/15	Cm5/18	0.9740	0.0047
Moly	Cm 4/5	Cm5/18	0.8634	0.0093
Moly	Cm5/23	Cm5/18	Not calculable	
Moly	Cm 4/3	Cm5/18	0.5016	0.0232
Moly	Cm2/15	Cm9/11	1.0000	0.0000
Moly	Cm 4/5	Cm9/11	0.2179	0.0185
Moly	Cm5/23	Cm9/11	Not calculable	
Moly	Cm 4/3	Cm9/11	1.0000	0.0000
Moly	Cm5/18	Cm9/11	1.0000	0.0000

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