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TITOLO TESI

Population structure of aristeid shrimps (Decapoda, Aristeidae) in the Western Mediterranean Sea inferred by microsatellite loci

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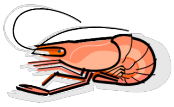

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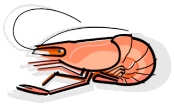


1 Introduction

The red and blue shrimp *Aristeus antennatus* (Risso, 1816) is a species widely distributed throughout the world. It occurs in the Mediterranean, with a higher frequency in the western central part of the basin (SYNDEM, 1999), and off eastern Atlantic coasts from Portugal to the Cape Verde islands. In the western Mediterranean, the species is of great economic interest and, together with *Aristaeomorpha foliacea* (Risso, 1827), it is the main target species of deep-trawling, which goes up to 800-1000m (Demestre and Martìn, 1993; Ragonese and Bianchini, 1996; Sardà and Cartés, 1994, Matarrese *et al*., 1997, Cau *et al*., 2002). The exploitation of the red and blue shrimp in the eastern Mediterranean has not been increased yet, since, in that area, trawling cannot exceed the limit of 400-500m of depth (Politou *et al*., 1998). For this reason, in the eastern part of the Mediterranean Sea, *Aristeus antennatus* is considered practically unexploited (Papaconstantinou and Kapiris, 2001; Kapiris, 2004).

Today, main informations on *A. antennatus* derive from scientific researches supported by the economic interest of the species and in fact, data are mainly from the central part of the Mediterranean basin, where it is widely fished. This knowledge focuses on the biology, ecology, and fishing exploitation of the species.

Due to the “deep” biological habits of this species, aspects such as maximum depth of distribution, factors that influence the larval dispersal and ecology, and the population structure are poorly known.

The peculiar biological traits, the limited ecological knowledge and the strong exploitation pose several questions about the sustainability of current rates of

exploitation, threatening the long-term survival of this important commercial resource.

In fact, as in the case of several other marine species, the studies on the ecology of the species are overcome by the simultaneous exploitation of the species, and may not allow us to perceive the real impact that fishing has on populations of *A. antennatus*.

Among possible approaches, genetic studies may allow to gather thorough information in order to study biological, physiological and ecological aspects of the species. In particular, genetic data are extremely useful for the identification of "stocks" and gene flow dynamics.

The latest techniques in molecular biology may reveal important information about the distribution in Mediterranean of *A. antennatus* allowing, for example, to highlight the existence of genetically isolated populations of the blue and red shrimp.

Since direct tracking of individuals is very difficult for marine species, and even more challenging for deep-water shrimps such as *A. antennatus*, the use of indirect methods for the measurement of connectivity among populations is important (Cowen et al., 2007). Among the most recently developed indirect methods to infer connectivity, genetic approaches have been particularly successful (Hedgecock *et al*., 2007).

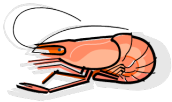
As mentioned before, information about the genetic structure and diversity of natural populations of *A. antennatus* throughout its natural range is very limited. Previous studies using either allozymes (Marchi *et al*., 1995; Pla *et al*., 1995; Sardà *et al*., 1998) or mtDNA markers (Maggio *et al*., 2009; Roldán *et al*., 2009; Sardà *et al*., 2010) have shown the absence of population differentiation. Genetic homogeneity of *A. antennatus* populations has been attributed to relatively recent separation in populations and/or ongoing gene flow, due to pelagic larvae dispersal, and adult migrations.

The use of more variable and polymorphic molecular markers, such as microsatellites (also known as simple sequence repeats, SSRs) could reveal the existence of genetically isolated populations, even if differentiation is very small, and populations are closely related (Wright and Bentzen, 1994).

In this study, the isolation and characterization of microsatellite loci for the red and blue shrimp are described for the first time and are applied in the study of genetic variation of *A. Antennatus* samples from nine different locations of the western Mediterranean area, in order to quantify the genetic diversity, analyze the genetic population structure and determine whether there are separate stocks of the species. Furthermore, due to different bathymetric distributions of the sexes and their possible differential dispersal capacity, we also tested the hypothesis of instantaneous sex-biased dispersal.

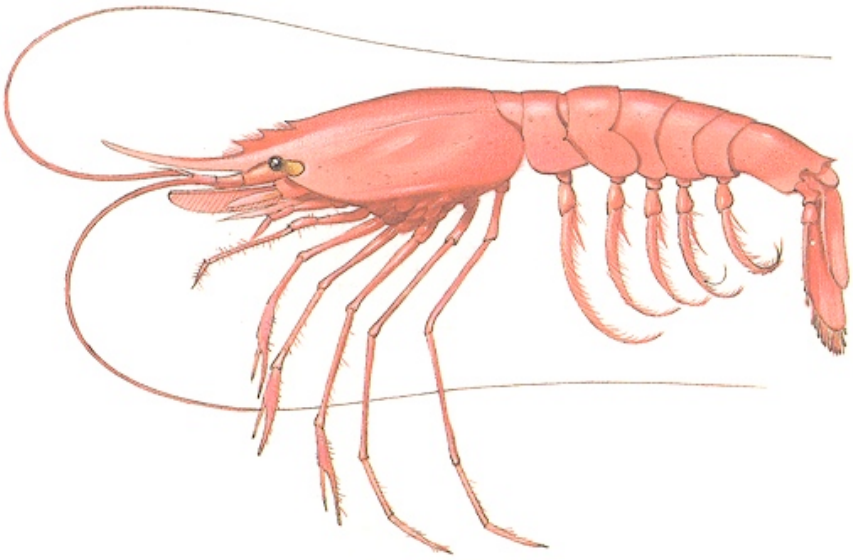
Finally, results are compared with those from a parallel PhD research project, developed with other fellows from the same institute, on *Aristaeomorpha foliacea*, analyzing differences and similarities between the two species.

Genetic information gathered in my study can provide important indications to be used on a scientific basis for regulating the exploitation of the resource, inaugurating a new course: Responsible and Aware Fisheries, that represent our primary goal. In fact, this PhD project was carried out at the Com.Bio.Ma., a centre of Competence on Marine Biodiversity, and funded by the "APQ RESEARCH – PROJECT P5 BIODIVERSITY" whose objectives are to realise laboratories on Marine Biodiversity, aimed to deepen knowledge on fishes and invertebrates from Sardinia.



2 Systematics, biology and management of the red and blue shrimp, Aristeus antennatus *(Risso, 1816)*

2.1. Classification and systematics



PHYLUM:	Arthropoda
CLASS:	Crustacea
SUBCLASS:	Malacostraca
ORDER:	Decapoda
FAMILY:	Aristeidae
GENDER:	*Aristeus*
SPECIES:	*Aristeus antennatus*

The red and blue shrimp, *Aristeus antennatus*, is a deep-water shrimp from the Aristeidae family and together with *Aritaeomorpha foliacea* they are the only demersal Mediterranean species.

2.2. Morphology

The red and blue shrimp, *Aristeus antennatus* (figure 2.1), is a big sized shrimp (maximum total length = 22cm), belonging to decapod crustaceans; adults present sexual dimorphism: males present a short rostrum, that overlaps the eye but not the distal end of the antennal scale (figure 2.2), while in females the rostrum is longer and sharp (figure 2.2). Nevertheless, sexually immature males present a rostrum as long in females (Mura and Cau, 1989). In both sexes, the rostrum presents three spines on the basal side. The carapace is not characterized by any hull neither empathic spine.

The red and blue shrimp main colour is pale red, often with bluish tones on the carapace.

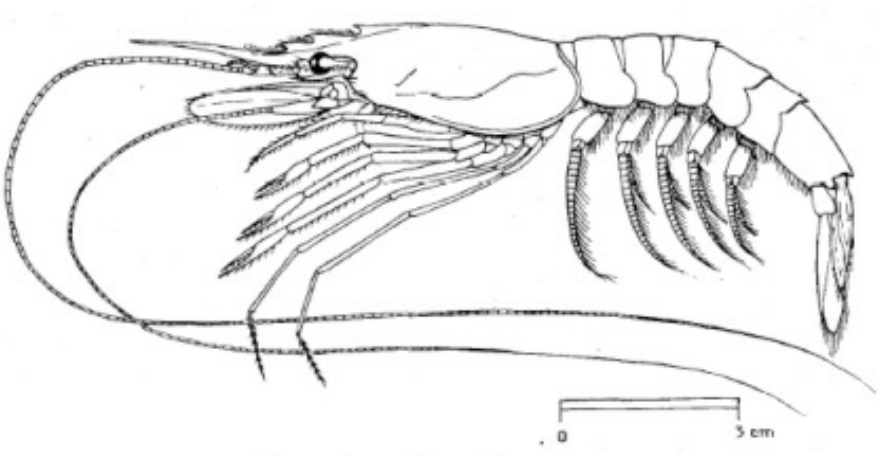


Fig.2.1 *A. antennatus* female with the typical long and sharp rostrum

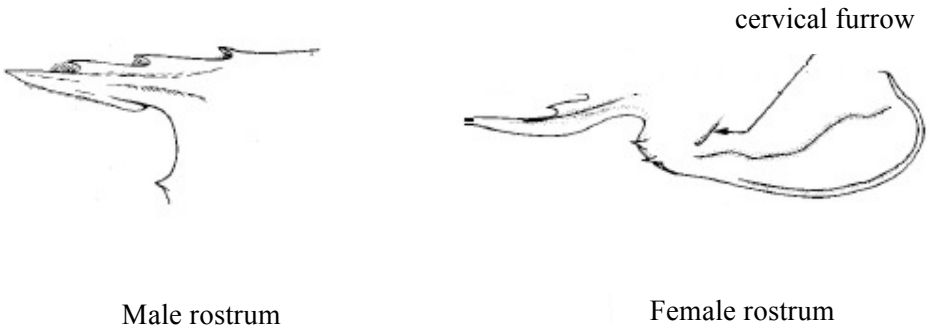


Fig.2.2 Sexual dimorphism of the rostrum in *A. Antennatus*

2.3 Geographical and bathymetric distribution

Aristeus antennatus is found along meso-bathyal muddy bottoms, and mostly in the central part of the western Mediterranean (SYNDEM, 1999).

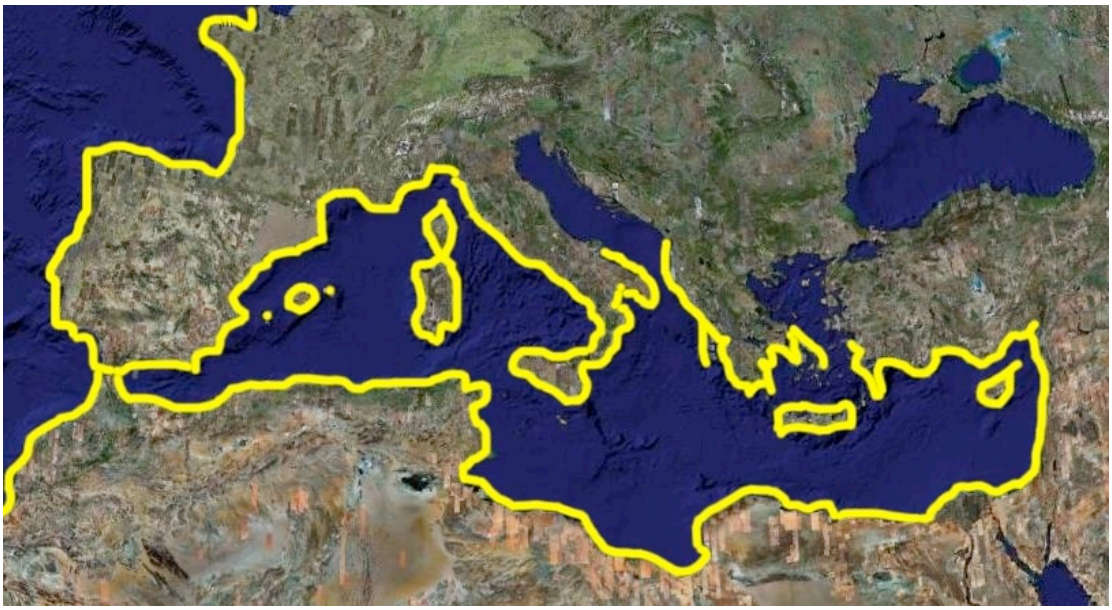


Fig.2.3 *Aristeus antennatus* geographical distribution in the Mediterranean Sea

In Italian seas the geographical distribution of the species is quite irregular. *A. antennatus* is completely absent in the northern and central Adriatic sea and very rare in the its southern basin (Vaccarella *et al.*, 1986). The blue and red shrimp is abundant in the Ionian Sea, Tyrrhenian Sea, but for its southern part (AA.VV., 1990), Ligurian Sea, in the Strait of Sicily and along the south-western coasts of Sardinia (SYNDEM, 1999).

A. antennatus is also found along the eastern side of Corsica, even if in lower concentration than the giant red shrimp *A. foliacea* (Campillo, 1994). Generally, the red and blue shrimp is found in deep waters, deeper than 400m , and it is the species with the widest depth distribution in the Mediterranean Sea. It occurs over muddy bottoms of the slope between 80 m (Nouar, 2001) and 3300 m (Sardà *et al.*, 2004). Different authors have observed vertical migrations to shallower waters during nighttime (Campillo, 1994; Matarrese *et al.*, 1995). Such events are mainly observable near to the edges and apices of underwater canyons. The depth distribution seems to influence also the sex-ratio of the species, which is biased towards females between 400 and 700m of depth as reported by different authors in different seas (Orsi Relini, 1980; Mura and Cau, 1989; Ragonese *et al.*, 1996; Matarrese *et al.*, 1995; Spedicato *et al.*, 1995). On the other side, males seem to be more abundant than females between 1000 and 2200m of depth (Sardà *et al.*, 1994).

2.4. Life cycle

The red and blue shrimp, directly disperse the eggs in the water column, where they hatch, starting a complex biological cycle (figure 2.4). Eggs free a very simple and little larvae: the nauplius, the first of 11 probable larval stages which include 5 nauplius stages, 3 protozoae stages and finally 3 mysis stages. *A. antennatus* larvae have been found only four times (Heldt, 1954; 1955; Seridji, 1971; Dos Santos, 1998; Carbonell *et al.*, 2010) and always in the upper water layers, far from the adult fishing grounds where they should have originated (Carbonell *et al.*, 2010). It is likely that newly hatched larvae perform a rapid and long vertical migration from the deeper spawning areas to the upper layers, where the subsequent stages develop, passively transported by the superficial currents (Carbonell *et al.*, 2010). During the early stages, larvae are transported near to the coast. Here, after the larval phase, the individual becomes a postlarva and settles on bottoms, changing its habits: from planktonic to benthic. This usually takes place 3 weeks after the egg has hatched. Another major ontogenetic migration takes place when the post-larva moves to deeper waters becoming young recruits and later adults. These can reproduce and

start a new biological cycle. Usually the “new comers” reach these reproductive grounds when they are about one year old (FAO, 1987).

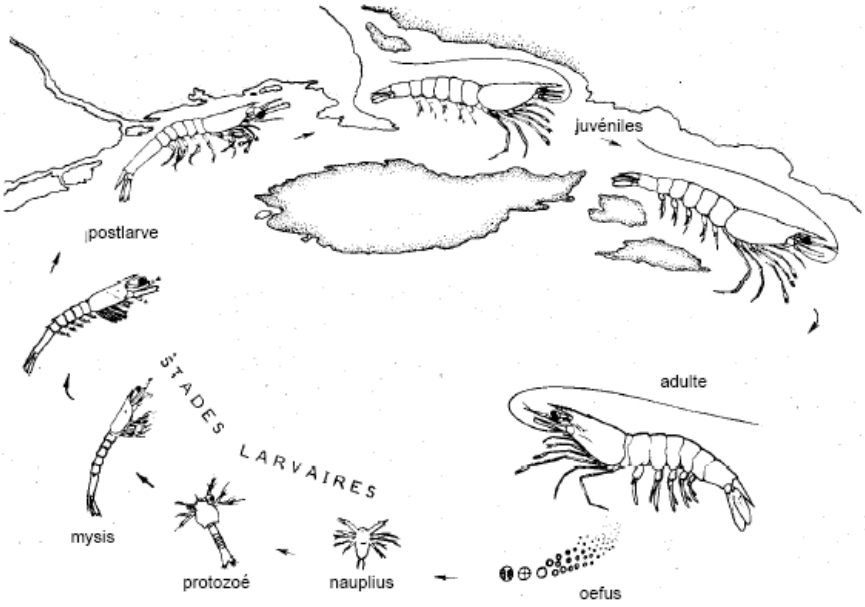


Fig.2.4 Biological life cycle of *A. antennatus*

2.5. Feeding habits

The red and blue shrimp shows, as described in Brian (1931) and Lagardère (1972), a euriphagous behaviour. Its diet includes organisms preyed on the bottom like worms, echinoderms, the decapod *Calocaris macandreae*, small bivalves, gastropods and crustaceans belonging to various groups, and also euribathyc micro-nekton organisms, in particular Eufausiacea and Decapoda (Relini and Orsi Relini, 1987). If we consider the total number of prey, the 50% of them are crustaceans (Cartés and Sardà, 1989). Nevertheless, considering not only the number but also the size of prey, pelagic decapods as Sergestidae, Pasiphaeidae e Oplophoridae assume a fundamental role in the diet (Orsi Relini *et al.*, 1995).

2.6. Size and growth

Size

The maximum carapace length (LC) observed for females and for males resulted 71mm and 40mm, respectively, in samples from the Ligurian Sea (Orsi Relini and Pestarino, 1981; Orsi Relini and Relini, 1996).

Growth

The estimated growth parameters show adaptation to two different scenarios, either of “slow” or “fast” growth (Ragonese and Bianchini, 1996). Actually, literature data (table 2.I) seem to agree in attributing slow growth and a longer life span to females (6-10 years; Orsi Relini and Relini, 1996; 1998).

Tab 2.I Growth parameters for *Aristeus antennatus* where, L∞, maximum theoretical length, K/year, slope; t0, age at size zero.

AUTHOR	AREA	SEX	L∞ (mm)	K/year	t0
Orsi Relini *et al*., 1996/98	Ligurian Sea	F	71.2 76.9	0.317 0.213	-0.047 -0.019
Spedicato *et al*., 1995	Central-southern Tyrrhenian Sea	F	66.81	0.558	-0.2337
Arculeo *et al.*, 1994	Southern Tyrrhenian Sea	F	69.4	0.337	
Cau *et al.*, 1994	Sardinian Seas	F	76.8	0.34	0.369
Colloca *et al.*, 1998	Central Tyrrhenian Sea	F	67.65	0.49	0
Ragonese and Bianchini, 1996	Strait of Sicily	F	69.1	0.532	0
Matarrese et al., 1997	Ionian Sea	F	77.18	0.35	-0.36

2.7. Sex and reproduction

A. antennatus is a gonochoric species; the breeding season begins in spring, in April, with peaks in summer, when most of the females reach sexual maturity, and ends in autumn, during the months of October-November (Demestre , 1995).

Females and males can be distinguished even in the juvenile stage due to the different location and morphology of the gonads and to the presence of secondary sexual characteristics. Females have an open *thelycum* structure. This small cavity, appointed to host the male spermatophores (figure 2.5) is located between the third pair of pereiopods.

Fig 2.5 Female specimen of *A. antennatus*, with a male's spermatophore.

In males, the basal part (endopodite) of the first pair of pleopods are transformed into a laminar structure (figure 2.6), the so called *petasm*, by which the spermatophores are transferred to the female thelycum. In young individuals this structure is split in two halves.

Fig 2.6 Male specimen of *A. antennatus*

In *A. antennatus*, *thelycum* and *petasma* seem to act in a synchronous manner during mating, carrying and supporting spermatophores before fertilization, which thus takes place in the absence of the male (Demestre and Fortuno, 1992). This strategy can be beneficial for species such as *A. antennatus* showing a gender-segregation and/or a depth range that allows little contact between males and females (Sardà *et al.*, 1997).

In species with this anatomical morphology, spermatophores are transferred during an inter-moult period. For the blue and red shrimp it has been suggested a possible a repetition of the sequence moult-mating-moult for several times during the same breeding season.

Four ovarian maturation stages have been described: they can be recognized using a macroscopic colour scale (Orsi Relini, 1980). Ovaries in immature females or in post-spawning are colourless or white (stage 1). With the progression of vitellogenesis and the inclusion of caroteneprotein, the ovaries first are stained pink (stage 2) and then lilac (stage 3, advanced maturation stage, oocytes up to 250 μm). At the final stage of development the gonad appears of a dark purple colour (stage 4, oocyte diameter about 300 μm).

In males, a two-stage spermatogenesis has been identified through macroscopic surveys. The young male (stage 1) has a white deferent vessel without spermatophores and emipetasms are separated. Mature males (stage 2) have spermatophores and emipetasms appear joined (Demestre and Fortuno, 1992).

Before sexual maturity, at the beginning of the breeding season, females are bigger in size. With the progress of the breeding season, the average size of first sexual maturity tends to decrease, but increases again at the end of the period (Mura et al, 1992; D'Onghia *et al.*, 1997).

In males, the reproductive phase seems to be more extended, in fact mature males with emispermatophores in the terminal portion of the sperm ducts have been

observed even in fall and winter (Orsi Relini and Pestarino, 1981; D'Onghia *et al.*, 1997).

2.8. Evaluation and exploitation

Indices of abundance for *A. antennatus*, (table 2.II) referred to the trawl-survey MEDITS-95, appear to be for the layer 200-800 m higher in the Ionian, Ligurian and Sardinian Seas.

Tab.2.II Indices of abundance for *A. antennatus*, where: kg/km2, biomass index, CV, variation coefficient.

INVESTIGATED AREA	LAYER 200-800 M	
	kg/km^2	**CV**
Ligurian Sea	4,46	44,05
Northern Tyrrhenian Sea	0,08	95,33
Central Tyrrhenian Sea	0,12	72,7
Southern Tyrrhenian Sea	0,78	61,31
Sardinian Seas	4,2	35,59
Strait of Sicily	0,46	53,43
Ionian Sea	4,94	30,07

Parameters of total and natural mortality, estimated for some Italian waters for female of *Aristeus antennatus*, are shown in table 2. III.

Tab.2.III Mortality rates for *Aristeus antennatus* where M, natural mortality rate, Z, total mortality rate.

AUTHOR	AREA	SEX	M	Z
Spedicato et al., 1995	Central-southern Tyrrhenian Sea	F	0.583-0.695	1.937-1.962
Cau *et al.*, 1994	Sardinian Seas	F	0.46	
Ragonese and Bianchini, 1996	Strait of Sicily	F	0.8	1.1
Matarrese et al., 1997	Ionian Sea	F		0.64

A. antennatus is an economically important resource: in the Ligurian Sea (Orsi Relini and Relini, 1985), in the Tyrrhenian Sea (Mailloux and Lembo, in AA.VV., 1990; Arculeo *et al*., 1994; Ardizzone *et al*., 1994), in the seas of Sardinia (Mura *et al*., 1992), in the Strait of Sicily (Ragonese and Bianchini, 1996) and in the Ionian Sea (Matarrese *et al*., 1992).

Despite the blue and red shrimp is subject to high fishing pressure, it is believed that this resource is currently submitted to a sustainable harvest rate (SYNDEM, 1999). The ability to maintain such levels of exploitation seems to be linked to the extremely broad distribution on bathyal bottoms of the species (Bianchini and Ragonese, 1994), and to the partial vulnerability of *A. antennatus* stocks, since only a fraction of the stock is accessible for commercial fishing fleets (Demestre and Lleonard, 1993). However, the application of "yield per recruit" models showed an overfishing condition (Mailloux *et al.*, 1995). Also in the Ligurian Sea, the trends of the commercial landings are in decline (Fiorentino *et al.*, 1995). Although it is possible that this resource is currently in a downturn in fluctuation cycle (Caddy, 1993), a

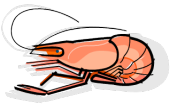
more conservative management may be advisable. The fishing yields of blue and red shrimp are nonetheless subject to seasonal variations and annual fluctuations.

In fact, catches of this shrimp are known to fluctuate considerably from year to year, and recent studies have demonstrated the existence of a relationship between environmental factors (e.g., marine currents, water masses, and climate variability) and abundance fluctuations of *A. antennatus* (Cartés *et al.*, 2008; Company *et al.*, 2008; Guijarro *et al.*, 2008; Massuti *et al.*, 2008; Maynou, 2008a; b; Cartés *et al.*, 2009). In particular, higher landings have been recorded 2-5 years after particularly cold winters and negative North Atlantic Oscillation index (NAO) periods. This pattern is likely due to the increased dense shelf water formation, with the cascading of superficial water masses that propagate along and across the continental slope. The transportation of particulate organic matter to the deep basin determines higher macrobenthos production, higher prey availability, and consequently higher fecundity. The enhancement of recruitment of *A. antennatus* could be also due to higher larval survival resulting from increased food availability and to the decrease of predation pressure due to the turbidity anomalies generated in the deep layers after cascading events (Company *et al.*, 2008).

2.9. State of resource and recommendations

An increase in the size of recruits, by adopting a more selective mesh, will lead to future increases in productivity, thus leading to a less serious condition of exploitation without economic repercussions in the short term (Ragonese and Bianchini, 1996) .

Moreover, for Ionian Sea a period of fishing closure during the late summer, is recommended to reduce the fishing pressure on the recruits, particularly vulnerable during this period (D'Onghia *et al.*, 1997).



3 Molecular Genetic Markers

The main instruments used to study the genetic variability within or between populations and species are genetic markers. A genetic marker is any morphological, biochemical or molecular character that is inherited through generations, so that they can be monitored over time (Brooke, 1999).

Genetic markers are used in a multitude of applications in the study of ecology and evolution (mating models, population structure and gene flow, fitness, etc.).

In recent years, scientists using a 'molecular approach' in population-based studies may apply a wide range of methodologies of analysis: this is because the continuous discovery and development of investigative techniques increasingly sophisticated does never completely replace the older ones. Further, many markers for the direct study of the DNA (RAPD, RFLP, AFLP, minisatellites and microsatellites) and markers for the study of its products (Allozyme) differ in the kind and degree of variability which they identify, in the ease of employment, and last but not least, costs of development and application (Ouborg *et al.*, 1999).

Analysis conducted with allozymatic markers, though, are nowadays less and less used, as the allozymes (enzymes encoded by different alleles of a single locus, with different electrophoretic mobility due to alterations in the overall ionic charge of the protein) can be considered as indirect indicators of genetic variability. They are not able to detect modifications in the DNA that do not involve changes in the ionic charge and thus the overall protein mobility in an electric field.

A molecular marker has to be selectively neutral and basically must follow the Mendelian principles to be used as a tool to reveal the demographic pattern.

Among the many features that differentiate one molecular marker from the other, three aspects in particular should be highlighted:

- *Dominance / Codominance*

Dominant markers (e.g. RAPD, AFLP) are those in which one allele is dominant on the other and the heterozygote profile is essentially indistinguishable from dominant homozygote. For this reason it can recognize only two phenotypes, dominant or recessive.

Codominant markers (RFLP, allozyme, microsatellites), instead, are those whose homozygous profiles can be distinguished from the heterozygous profile allowing precise estimation of allele frequencies in a population.

- *Inheritance criterion*

Markers differ in their criterion of inheritance: the nuclear DNA is inherited from both parents, while the DNA of organelles (mitochondrion and chloroplast) has more often a uniparental inheritance.

- *Genetic variability*

Finally, in a gonochoric situation, owing to amphimixis, the nuclear genome of the new individual takes origin from the contribution of two parents allowing genetic variability, while the DNA of organelles in general doesn't, as it is uniparentally inherited.

The degree of variation that a marker must have depends on the specific question to which you want to answer and also on the type of populations and organisms you want to study.

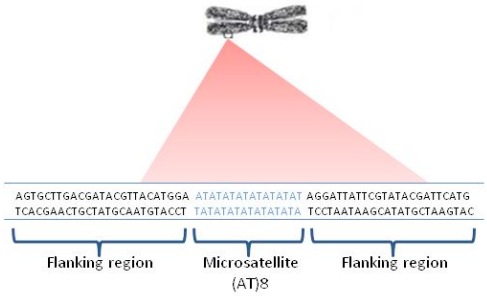
In this particular study I decided to conduct an analysis of the genetic variability of *Aristeus antennatus* at the nuclear level using microsatellites which are codominant and biparental inherited markers.

3.1 Microsatellites

Animals nuclear DNA is a diploid genome transmitted according to Mendelian segregation and it is subject to recombination. Its more polymorphic regions, microsatellite loci, originally studied in relation to genetic diseases, are highly informative in population structure and dynamics studies (De-Xing Zhang and Godfrey M. Hewitt, 2003). Their high variability is due to the fact that these portions are not coding, and therefore not subject to selection.

3.1.1 What are microsatellites?

Microsatellites are high-frequency repeats of 1-6 nucleotides modules found in the nuclear genome of many taxa. They are also known as simple sequence repeats (SSR), variable number of tandem repeats (VNTR) and short tandem repeats (STR).



Type	Sequence example
Perfect	CACACACACACACACACACA
Compound	CACACAGAGAGAGAGAGAGA
Interrupted	CACATTCACACATTCATTCACA

Fig.3.1 Schematic representation of a microsatellite with reported aside a few sequence examples.

Our understanding on the behaviour microsatellites in terms of mutation, function, evolution and distribution in the genome and across taxa is rapidly increasing (Li *et al.*, 2002; Ellegren, 2004).

A microsatellite locus typically ranges in length from 5 to 40 repetitions, but longer repeats are possible. Typical microsatellites widely chosen for molecular genetic studies are those made of dinucleotides, trinucleotides and tetranucleotide. It should be noted that the trinucleotides (and also esanucleotides) can occur in coding

sequences since they do not cause the frameshift of the sequence (shift of the sequence that totally changes the reading of the triplets by the ribosome).

Sequences of DNA around the microsatellite locus are known as “flanking regions”. Since these flanking regions are conserved in different individuals of the same species, and sometimes even between related species, a microsatellite locus can be identified by its flanking regions on which primers can be designed to allow the specific amplification of the microsatellite locus.

Many microsatellites have a very high mutation rate, between 10^{-2} and 10^{-6} mutations per locus per generation and an average of 5×10^{-4}, which creates a high level of allelic diversity necessary for genetic studies (Schlotterer, 2000).

Microsatellite repeat sequences frequently change due to errors during DNA replication, primarily by changing the number of repetitions of the sequence (Eisen, 1999). Because the alleles differ in length, they can be distinguished by high resolution electrophoresis gel, allowing rapid genotyping of many individuals for several loci.

3.1.2 Why choose microsatellites?

Many issues in molecular ecological studies are discussed with more than one marker. Microsatellites are of particular interest because they allow researchers to deepen into complex ecological questions.

Ease of sample preparation.

An ideal marker requires small tissue samples that can be easily stored for future use. In contrast with methods based on allozymes, DNA-based techniques, just as microsatellite, use PCR to amplify markers from very small portions of sample. The stability of DNA is another big advantage over enzymes, as this allows the use of simple substances and reagents for the tissue preservation. Moreover, microsatellite sequences are usually short (100-300bp) and can almost always be amplified, despite the possible degradation of DNA (Taberlet *et al*, 1999). This feature of

microsatellites allows easy and economic DNA extraction even from ancient DNA, from hair and faecal samples used in non-invasive sampling (Taberlet *et al.*, 1999). Finally, microsatellites, being species-specific, present no risk of cross contamination from other non-target organisms, which, can occur with other techniques employing universal primers (e.g. AFLP).

Highly informative content.

Each genomic locus can be considered as a representative sample for its genome, but, given that the different evolutionary events (recombination, mutation, genetic drift, selection) does not affect all loci equally, an investigation cannot be based on a single locus which could lead to high error rates in assessments. So taking advantage of many loci, as in the case of studies with microsatellites, allows us to perform multiple sampling of the genome with the possibility of crossing the results and get a broader picture of the situation. Although techniques such as AFLP, allozyme and RAPDs are considered multi locus, these do not have the same resolving power of microsatellites (Sunnucks, 2000). F.e., Gerber *et al*. (2000) demonstrated that 159 AFLP loci revealed even a lower resolution than six polymorphic microsatellite loci in determining paternity.

Microsatellites, due to their high mutation rate, are useful in studies involving the actual demography, the connection structures, determination of changes in the recent past (10-100 generations), studies of population structure and migration (Kalinowsky, 2002, Wilson and Rannala, 2003).

3.1.3 Drawbacks with microsatellites

Despite many advantages, microsatellites exhibit also many difficulties and problems that can complicate the data analysis, and even restrict their use or make the results very cryptic.

Isolation of species-specific markers

Primers amplifying regions common to many species (such as 12S, D-loop and others) and used in most molecular studies, are usually highly conserved sequences within the species and sometimes across taxonomic groups. This high conservation in the sequence involves many difficulties in optimizing the use of these primers when widening the analysis to new species.

In contrast, a couple of primers for a microsatellite locus rarely works in different taxonomic groups, and so primers have to be designed ex-novo for each new species that is studied (Glenn and Schabl, 2005). Today, isolation of new microsatellite markers is cheap and fast, although it has still a significant failure rate, especially in certain marine invertebrates (e.g. Cruz *et al.*, 2005), Lepidoptera (Meglecz *et al.*, 2004) and birds (Primmer *et al.*, 1997).

Unclear mutational mechanisms

Microsatellite mutation model appears to be complex and, fortunately for most ecological applications is does not seem important to know the exact mutation mechanism at each locus, because this kind of analyses is insensitive to these mechanisms (Neigel, 1997) even if we can find methods based on the estimation of allele frequency using an explicit mutation model.

To date, four different models have been proposed to explain the evolution of microsatellite loci, i.e. the origin of new alleles: IAM (Infinite Alleles Model; Kimura and Crown, 1964), SMM (Stepwise Mutation Model; Kimura and Ohta, 1978); KAM (K-Allele Model; Crown and Kimura, 1970) and TPM (Two-Phase Model; Di Rienzo *et al*., 1994). Between these, It is to be noted that the first two models are more extreme, while the others represent variants of the firsts.

Traditionally, in population genetic analysis, we use the infinite allele model, IAM, (Di Rienzo *et al*., 1994). According to his model, mutations occurring in microsatellites involve the loss or the gain of one or more repeats simultaneously and lead to the formation of a new allele never met before in the population. *F* statistic is

based on this model which is also still the most popular one because it appears to be the simplest and generic model.

The other principal specific model for microsatellites, the stepwise mutation model, SMM, (Di Rienzo *et al.*, 1994), assumes that mutations lead to loose or acquire only one repeat at a time at a constant rate, mimicking the replication errors in the polymerase that generate mutations, creating a Gaussian type of allele (Ellegren, 2004). Under these conditions, the more two alleles differ in size, the more they can be considered divergent from a common ancestor. *R* statistic is based on this model.

But we must also remember that non-staged mutations occur, including point mutations or recombinant events such as gene conversion and unequal crossovers (Richard and Paques, 2000). Gradualism seems to be predominant in the microsatellite mutational events, but a more complex model that would include both gradual and non-staged events could better explain the mutation dynamics of such loci.

The allelic identification based on sizes in the microsatellite genotyping appears to be a good shortcut compared to the sequencing of each individual; however, it assumes that all alleles differ only in length and the ones that exhibit sequence differences but same length, will then be considered equal. This phenomenon is called "homoplasy", which can be revealed if it is subsequently highlighted by sequencing the alleles, or undetectable when two alleles have the same sequence but different genealogical histories. This non-identity is found in the gradual mutation behavior when there is a reverse mutation or the allele is different from its counterpart and then returns to original form. The homoplasy can finally concern the flanking regions, because the mutation can occur in this stretch, leaving unchanged the size of microsatellite allele.

Amplification issues

Finding a good locus marker for DNA implies to find a region of the genome that has a mutation rate high enough to produce multiple versions of the locus, alleles, in the

same population, flanked by highly conserved regions on the other side, over which the PCR primers anneal in almost all individuals of the species (about 99-100%). In fact, if there are mutations in the flanking regions, some individuals will have only one allele amplified or even none.

It was found that some taxa appear to be more afflicted by amplification problems than others, as in the case of molluscs, corals and other invertebrates.

Despite the problems listed above, the versatility of microsatellites overcomes these drawbacks by far. Also, fortunately, many of these problems can be avoided through careful selection of loci during the process of isolation.

4 Materials and Methods

4.1 Field collections

Individuals of *A. antennatus* analyzed in this study come from nine sampling areas of the western Mediterranean basin: Algeria, San Remo, Cala Gonone (Sardinia EC), Sant'Antioco (Sardinia SW), Gulf of Cagliari (Sardinia S), Vapore (S Sardinia), Terrasini (NW Sicily) and a site called PSP, which covers a south-east area of Sardinian sea (table 4.I and figure 4.1). Individuals were collected in 2006–2008 using both commercial bottom trawling (≤ 800 m depth) and experimental deep bottom trawling (from 800 to 1600 m). In the latter instance, individuals named PSP come from two different experimental deep fishing trawls, conducted by the Department of Animal Biology and Ecology, University of Cagliari. PSP South and PSP North are the only localities for which two different temporal replicates (2006 and 2007) were collected (table 4.I, figure 4.1).

Tab.4.I Location, year of sampling, depth of capture, number, and sex of individuals of each sample analyzed. Individuals from commercial hauls (*) were presumably caught at 800 m depth or shallower. The sex of the individuals from Algeria, San Remo, and Terrasini is unknown (ND = not determined) because only pereiopods were available for the genetic analyses. PSP (Pesca-Profonda-Sperimentale) indicates the experimental hauls.

Sampling locations	***Year of sampling***	***Depth in m mean (max)***	***N of individuals (males, females)***
Algeria	2006	<800 *	20 (NA)
San Remo	2007	<800 *	29 (NA)
Cala Gonone	2007	<800 *	20 (0,20)
Terrasini	2007	<800 *	20 (NA)
PSP North	2006,	1507 (1621)	12 (4,8)
PSP North	2007	1421 (1422)	12 (1/11)
Sant'Antioco	2007	<800 *	26 (6,20)
Cagliari	2006	<800 *	17 (0,17)
PSP South	2006	1117 (1227)	29 (22,7)
PSP South	2007	1110 (1173)	26 (21,5)
Vapore	2008	<800 *	16 (16,0)
Total			*227*

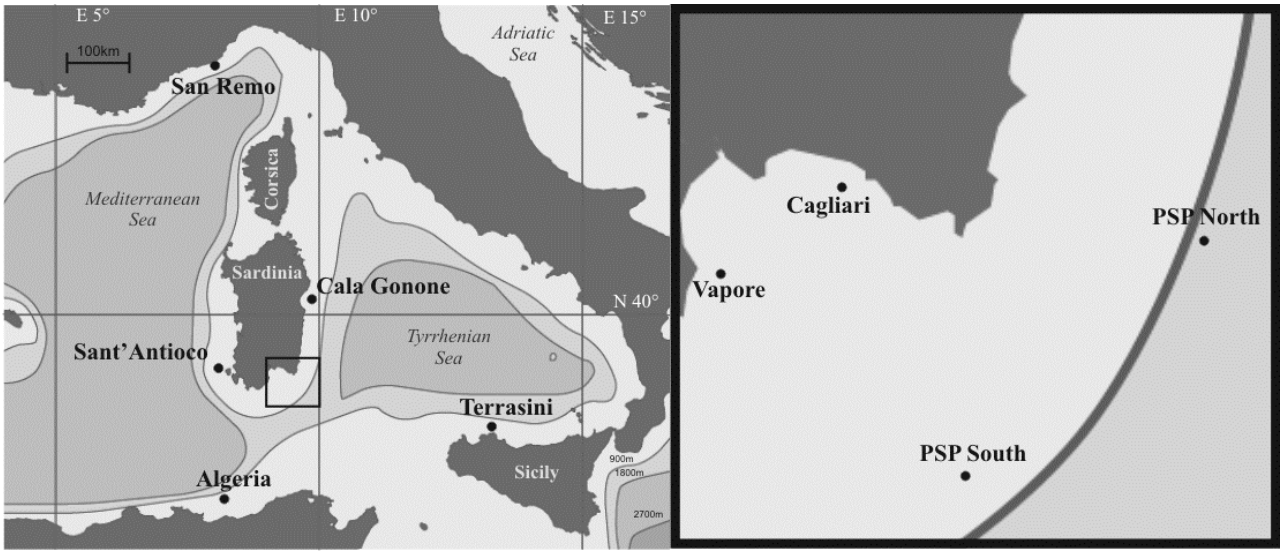


Fig. 4.1 Map of the sampling sites. The right square represents an enlargement of the Gulf of Cagliari.

4.2 Tissue sampling and DNA Extraction

Muscle tissue was sampled from pereiopods or tails and preserved in 70–100% ethanol at 4 °C. Total DNA was extracted from tissue (5-10 mg), following "Salting Out" extraction protocol (Miller *et al*., 1988),with minor modifications. This protocol provides protein precipitation and their removal from aqueous solution containing the DNA, according to the principle of variation of ionic strength due to the addition of a salt, in this case NaCl> 6M: added salt ions compete with the ions in the solution facilitating protein aggregation.

The extracted DNA was eluted in 50 µl of sterile ddH_2O. Quality and quantity of isolated DNA were verified by electrophoresis in agarose gel 1%, in TAE 1X Buffer (*BIO-RAD*), at 70V for 15 minutes (figure 4.2).

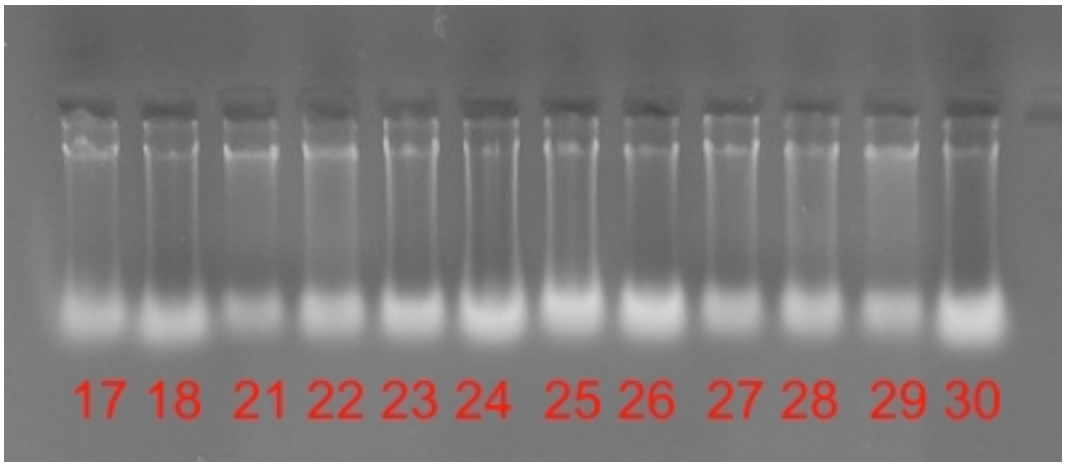


Fig.4.2 Control of DNA extracted from various samples on 1% agarose gel.

4.3 Microsatellite loci

4.3.1 Isolation of microsatellite nuclear markers

Isolation of microsatellite markers has been done using the FIASCO protocol - *Fast Isolation by AFLP of Sequences COntaining repeats* - (Zane *et al*., 2002) with minor modifications. This allowed the construction of a partial genomic *library* enriched for *AC* repeat.

The protocol is part of recent isolation protocols through selective hybridization using biotinylated probes. It can be schematically divided into three phases (figure 4.3):

1. PREPARATION FOR ENRICHMENT;
2. ENRICHMENT;
3. CLONING.

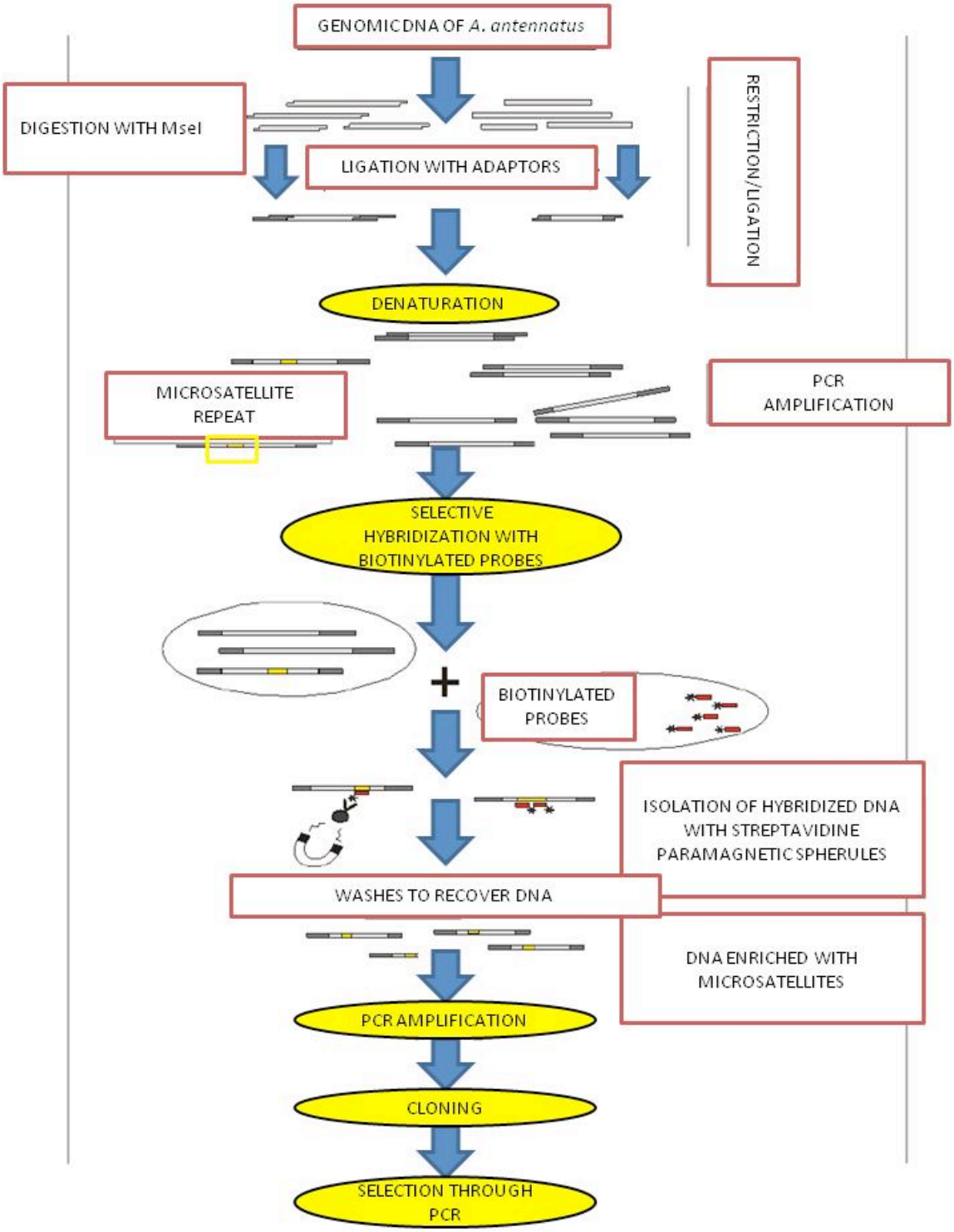


Fig.4.3 Schematic representation of FIASCO protocol

4.3.1.1 DNA preparation for enrichment

A genomic DNA mix from 5 different individuals of *Aristeus antennatus* from the sample PSP South was digested with restriction *MseI (Invitrogen)* and contemporaneously ligated with *MseI* adaptors used for the *AFLP* procedure (Vos *et al*., 1995), as follows:

- Genomic DNA 25-250 ng;

- Buffer OnePhorAll 1X (*Pharmacia*);

- DTT 5mM

- BSA 50 μg/ml

- Adaptors 1 μM

- ATP 200 μM

- *MseI* 2,5 unità

- T4 DNA ligase 1 unit

Total volume of reaction: 25 μl

The reaction mix has been incubated for 3 hours at 37°C.

The DNA fragments ligated to adapters were amplified using PCR (figure 4.4) .
DNA from the ligation reaction for was not diluted (1:10) following protocol, but the restricted-ligated DNA was used as template, along with four *AFLP primers* specific for the adaptors. The primers differ in the terminal base at the 3 'end (A or C or G or T).
The amplification mixture was as follows:
- non diluted restriction-ligation reaction 5 μl
- Buffer 1X

- $MgCl^2$ 1,5 mM
- primer 1,5 ng/µl each
- dNTP 200 µM
- Taq DNA polymerase 0,4 unit

_____

Total volume of reaction: 20 µl

The amplification reaction was performed in an *Invitrogen* thermocycler, with the following temperature profile:

Initial denaturation	94°C	2'	
Denaturation	94°C	30"	22 cycles
Annealing	53°C	1'	
Extension	72°C	1'	
Final extension	72°C	5'	

Fragments obtained with PCR conditions adopted above, are typically sized between 200 and 600 bp and visible on agarose gel as a smear (figure 4.5).

The PCR reaction was replicated twenty times in order to have several hundreds nanograms of amplified product, enough for the following enrichment step.

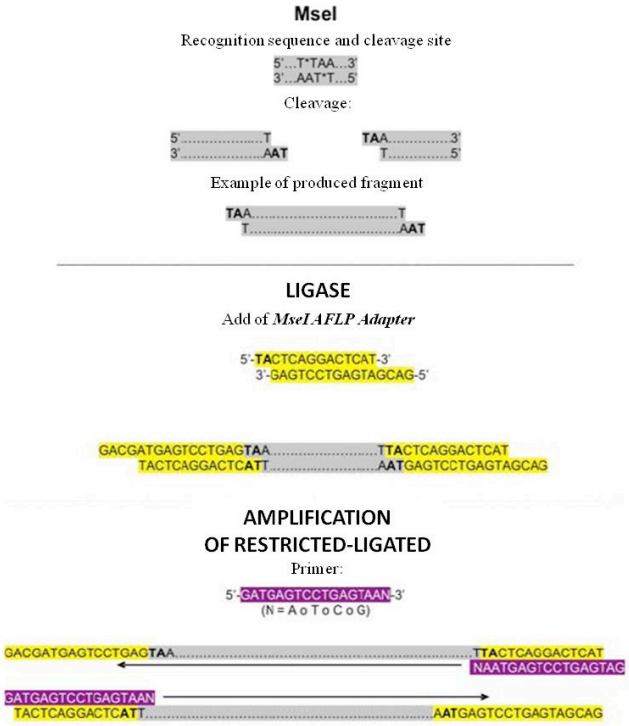


Fig.4.4 Preparation for enrichment: restriction-ligation and amplification of restricted-ligated DNA.

Fig.4.5 Control of restricted-ligated amplification on a 1,8% agarose gel

4.3.1.2 Enrichment

Hybridization

DNA hybridization was carried out with a 5' end biotinylated $(AC)_{17}$ probe. Hybridization was performed in 100 µl of 6X SSC / 0.07% SDS, mixing 200-300 ng of purified restricted-ligated DNA with 50-80 pmol of the probe, heating at 95 ° C for 3' and letting slowly cool to room temperature.

The solution was diluted with 300 µl of TEN100 (10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 7.5).

Selection of hybridized DNA using streptavidin paramagnetic spherules

Probe hybridized DNA was selected with the use of streptavidin paramagnetic spherules (Streptavidin Magnetic Particles, *Roche*; 1 mg per reaction). The streptavidin binds irreversibly to the biotin and, being associated with paramagnetic spherules, it can be removed from the solution by applying a magnetic field generated in this case by a special eppendorf tubes magnetic rack (Magnetic Rack, *Invitrogen*). The magnetic spherules were prepared by treating them with 2-3 TEN100 washes, each 5' long, at room temperature. After mixing 100 µl of hybridized DNA with 300 µl of TEN100, 5 µl of tRNA (10 mg / ml) and 50 µl of previously prepared spherules, everything was incubated at 50°C for 30'.

Definitive removal of non hybridized DNA

In order to remove the DNA not linked to the probe, three not stringent washes (400 µl of TEN100) and three stringent washes(400 µl of 0.2 X SSC buffer and 0.1% SDS) were performed. After each wash, 5' long at room temperature, shaking gently the solution, the hybridized DNA was removed using the magnetic field and the washing solution was discarded.

Separation of the microsatellite-enriched DNA from the probe-beads complex

To separate the enriched DNA from the biotinylated probe-magnetic spherules complex, the mixture has been subjected to two denaturing steps:

- 1st Denaturation: 95°C for 5' with 50µl of TE 10:1 (Tris 10 mM, EDTA 1 mM), pH 8 (the supernatant containing the DNA of interest was quickly removed and stored);
- 2nd Denaturation: the pellet from the first denaturation was incubated 5' at room temperature with 20µl of TE 10:1, 8µl of 0.15 M NaOH/acetic acid 0.166 M pH 7.

The DNA from the 2 denaturation steps was stored for the next step: the volume of the eluate was measured and the DNA was precipitated with an equal volume of isopropanol and sodium acetate (final concentration 0.15 M).

Finally, I carried out the control of the two previous denaturation steps and of the three washes on an 1,8% agarose gel (figure 4.6).

Amplification of enriched DNA

The enriched DNA obtained from the first and the second denaturation steps was precipitated with 85% ethanol and resuspended in 50µl of sterile water; 2µl were amplified for 30 cycles using AFLP primers specific for the adapter under conditions described in “DNA preparation for enrichment”.

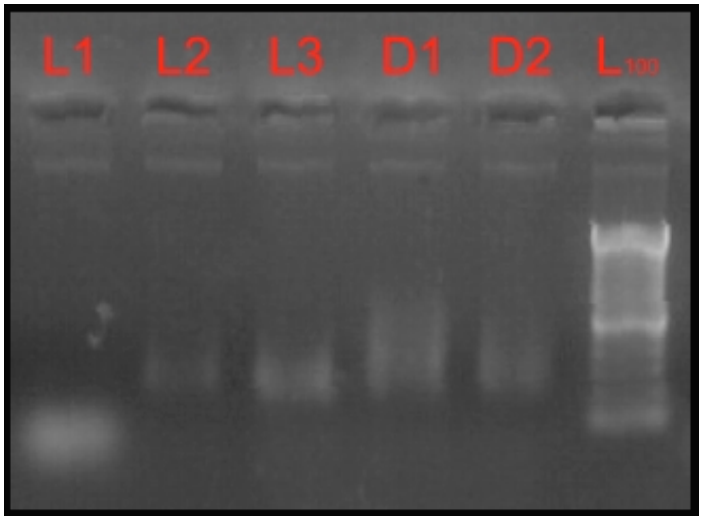


Fig.4.6 Control of denaturations and washes of enriched DNA

L1 = contains tRNA, which works as a carrier for RNA, taking it away with it. Smear hasto be focused under 300bp.

L2 = Represents DNA not ligated to the probe.

L3 = Represents DNA not ligated to the probe.

D1(4) = Microsatellite-enriched DNA separated from the probe.

D2 (5) = Microsatellite-enriched DNA separated from the probe.

DNA concentration is lower, but its quality higher.

L100 = Ladder 100 bp

4.3.1.3 Cloning

The microsatellite-enriched DNA has been cloned (figure4.7) with the TOPO TA CLONING® kit (*Invitrogen*) containing *Escherichia coli* cells and the plasmid vector *pCR®II-TOPO®.*

This vector is characterized by a dominant selective marker for resistance to ampicillin and it is linearizedwith3'desossitimidineresidues to which Topo isomerise I is covalently linked. This (with the addition of the non-specific Taq polymerase with terminal transferase activity) allows the microsatellite- enriched DNA - PCR produced - to bind efficiently the carrier thanks to the presence of a 3'desossiadenine (figure 4.8).

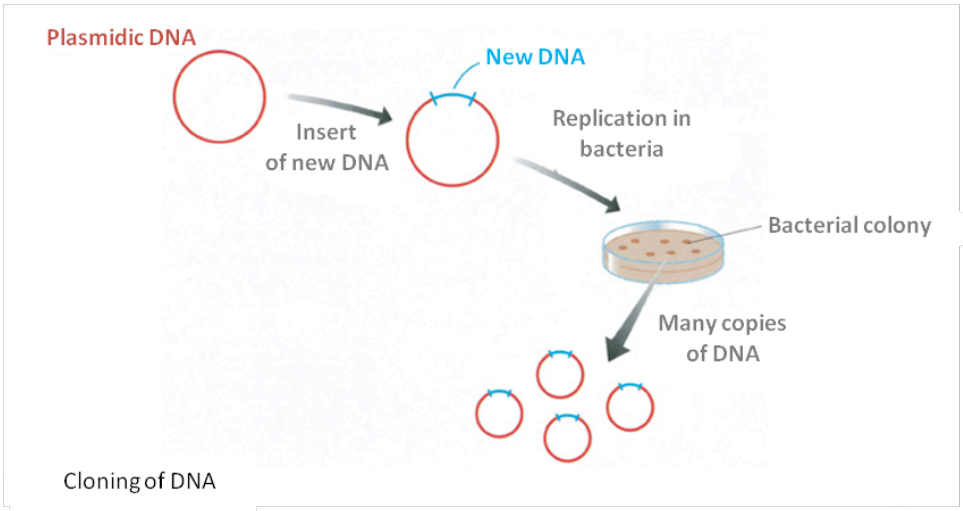


Fig. 4.7 Cloning of DNA

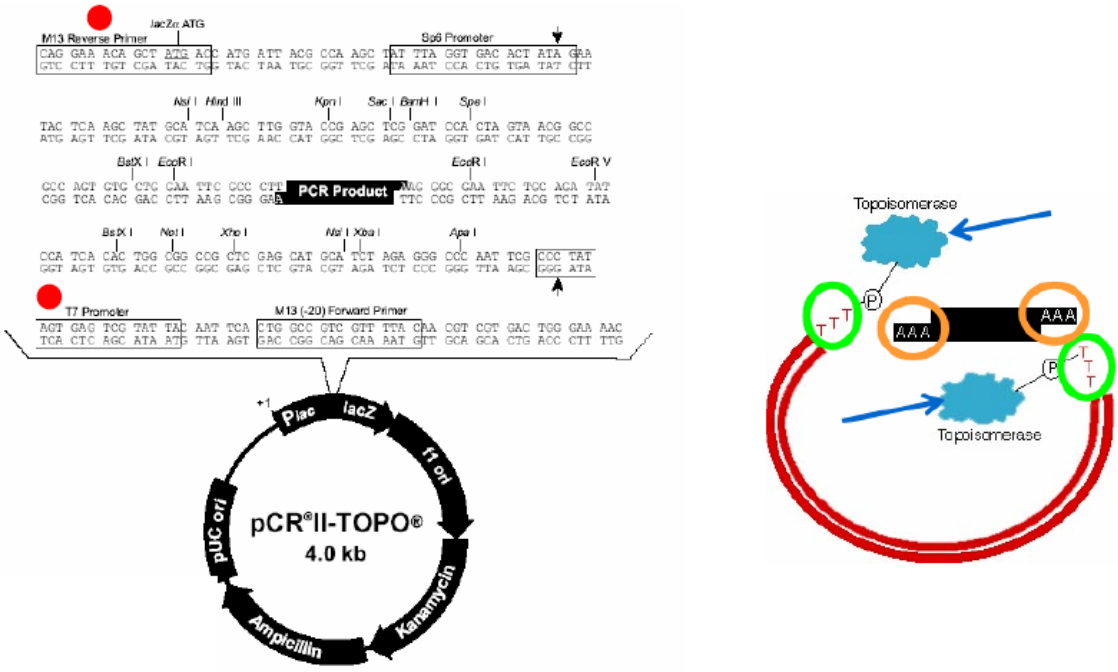


Fig.4.8 pCR®II-TOPO® vector of the cloning kit *TOPO TA CLONING® (Invitrogen).*

Ligation reaction

In a total volume of 6µl, 1µl of DNA (PCR product) was mixed with 1µl of saline solution (1.2 M NaCl and 0.06 M $MgCl^2$) and 1µl of plasmid pCR®II-TOPO®vector.

The solution was mixed gently and incubated for 5'at room temperature, then placed on ice.

Transformation

Two µl of the previous reaction (TOPO cloning reaction) were added into a tube containing cells of *Escherichia coli*. To allow cell transformation the mix was incubated on ice for 30' then subjected to heat shock at 42°C for 30" and placed back on ice. We proceeded with the addition of 250µl of SOC cells, and gently mixed the tube at an oblique position for 1h at 37°C. In the meantime, culture plates were prepared with Luria-Bertani solid medium (1% Tryptone, 0.5% yeast extract, 1% NaCl, pH 7, 15 g/L agar), 50g/ml ampicillin and 40µl of X-Gal 40mg/ml (beta-galactosidase substrate).

Fig.4.9 Plating of cells

Cell seeding and cell growth

Transformed cells were plated at different volumes (100, 75, 50 and 25µl) to obtain, at least for one plate, spread of colonies that would facilitate the withdrawal (figure 4.9). The plates were incubated overnight at 37°C. After cells of *E. coli* raised to colonies on the plates with solid medium, we proceeded to the selection of those containing the insert, on a colorimetric basis thanks to the X-Gal reaction (figure 4.10). Colonies with recombinant plasmids, unable to metabolize X-Gal (a galactose analogue), appear white, while the few colonies without insert appear blue. In fact, the plasmid vector has the reading code for ß-galactosidase, which, if interrupted by our insert, cannot produce working ß-galactosidase.

4.3.2 Colony screening and insert amplification

128 colonies putatively containing the AC repeat were removed or 'peaked' from the plates. Each colony was transferred into a volume of 12µl of sterile H_2O and then lysed at 96°C for 6'. We proceeded with the amplification of the inserts, using a pair

of universal primers M13FOR (GTCATAGCTGTTTCCTG-5'-3') and M13REV (GTAAAACGACGGCCAG-5'-3'), designed on the sequence of the plasmid.

Amplification conditions were:

- Buffer (Promega) 1X
- MgCl2 2,5 mM
- primer (M13FOR-M13REV) 1 µM each
- dNTP 0,2 mM
- Taq DNA polymerase (Promega) 0,5 unit

Total volume of reaction: 20µl

Lisate volume used: 5µl

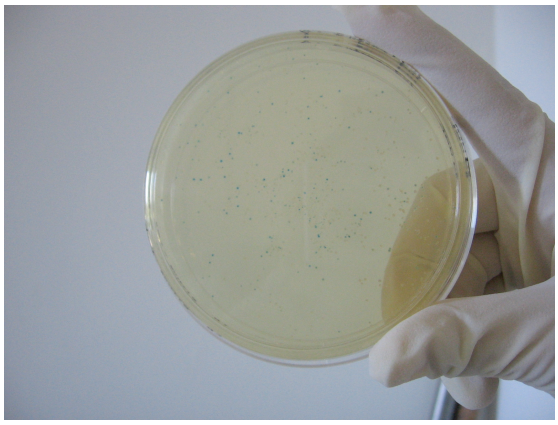


Fig. 4.10 Plate with white (with insert) and blue (without insert) colonies

Thermic profile for amplification was:

Initial Denaturation	95°C	2’	
Denaturation	95°C	30”	
Annealing	56°C	1’	33cycles
Extension	72°C	1’30”	
Final extension	72°C	5’	

PCR products were run on agarose gel to determine which colonies contained fragments and their sizes by means of a ladder (figure 4.11). The fragment must have a length of at least 600bp to have a better chance of finding a microsatellite sequence and clean flanking regions where to build primers. Of these, 121 out of 128 amplicons were of the optimal size; they were purified (PCR Clean Up Charge Switch® Kit, *Invitrogen*) and sent for sequencing at the sequencing center BMR GENOMICS, University of Padua.

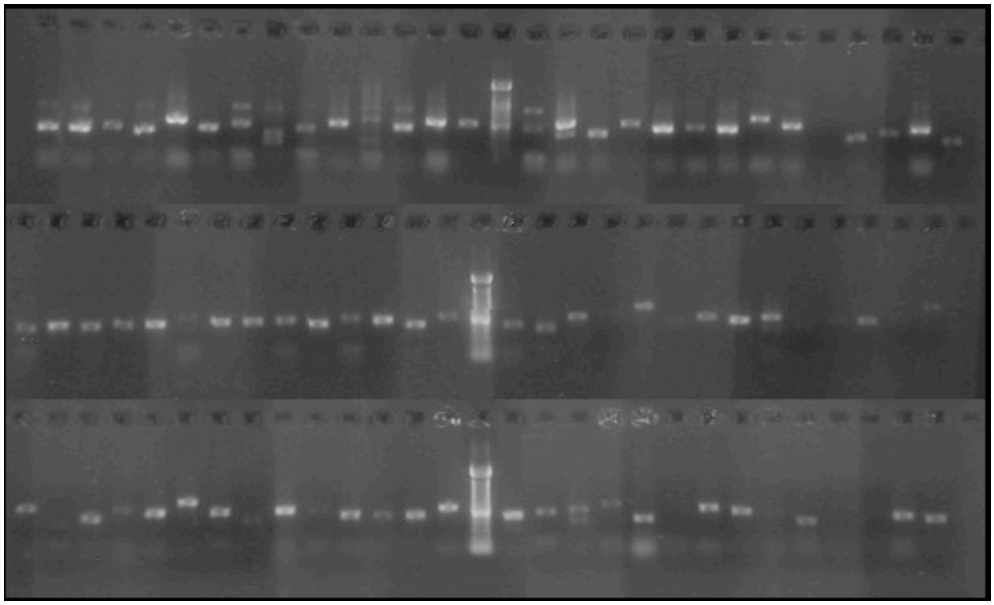


Fig. 4.11 Control of amplified colonies on agarose gel. Fragment length can be deduced from the ladder in the center well for each row.

Sequences are produced as chromatograms with peaks resulting from the sequencing with colours corresponding to the nucleotide base at that position in the sequence. This colourful sequence of peaks can then be transformed into a sequence of letters corresponding to the nucleotide bases (figure 4.12).

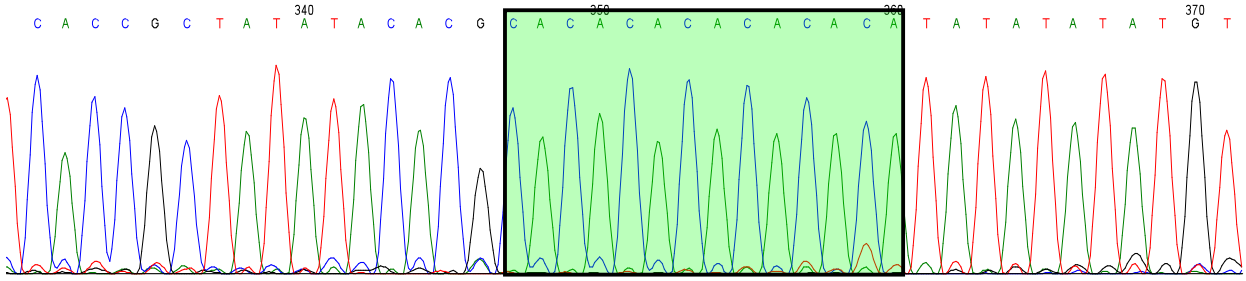


Fig. 4.12 Example of a chromatogram and its translation in the nucleotide sequence above. We note a possible CA module repeated seven times.

4.3.3 Primers design and relative conditions

When a microsatellite with at least six sequential repetitions of the same form (eg, AC_6) and with flanking regions of adequate length was identified, primers were designed on the flanking regions through the programs *OLIGO ANALYZER 1.1.2* and *OLIGO EXPLORER 1.2* following a set of rules for optimal functioning of these primers:

-	Primers must be at least **18-20 nucleotide** long (max 25).
-	Primers with **long traits made of a single base** should be avoided. It is particularly important to avoid 3 or more G or C in a row.
-	Primers should have a **melting temperature around 50°C**.
-	Primers should have a **content of G/C between 40% and 60%**.
-	Primers should not contain **complementary regions**; that means that there shouldn't be the possibility to create hairpins. In case, the primer would bend on itself resulting with reduced effectiveness.
-	Avoid primers that produce **dimers** between them. Discard primers that have 4 or more consecutive links or 8 in total; discard particularly those that form dimmers in the 3' region..
-	If possible launch a **blast research** of primers in order to assess if the DNA sequence is unique, especially the last 8-10 bases in 3'.
-	Don't design **degenerated primers**. Don't use **Inosine** in primers. They would give low specific amplicons.
-	Primers have to be **resuspended in ddH_2O**.
-	The **sequenced region** on which the primers are designed have to be reliable and the first 20-30 bases of the sequence have to be discarded as they're not correctly readable.

On these principles, 30 pairs of primers were designed and tested for amplification success and to find optimal condition.

Optimal conditions for amplification were found as follows:

- Buffer (Promega) 1X
- $MgCl^{++}$ 25 mM
- primer 0,3 µM each
- dNTP 0,2 mM
- Taq DNA polymerase (Promega) 1 unit

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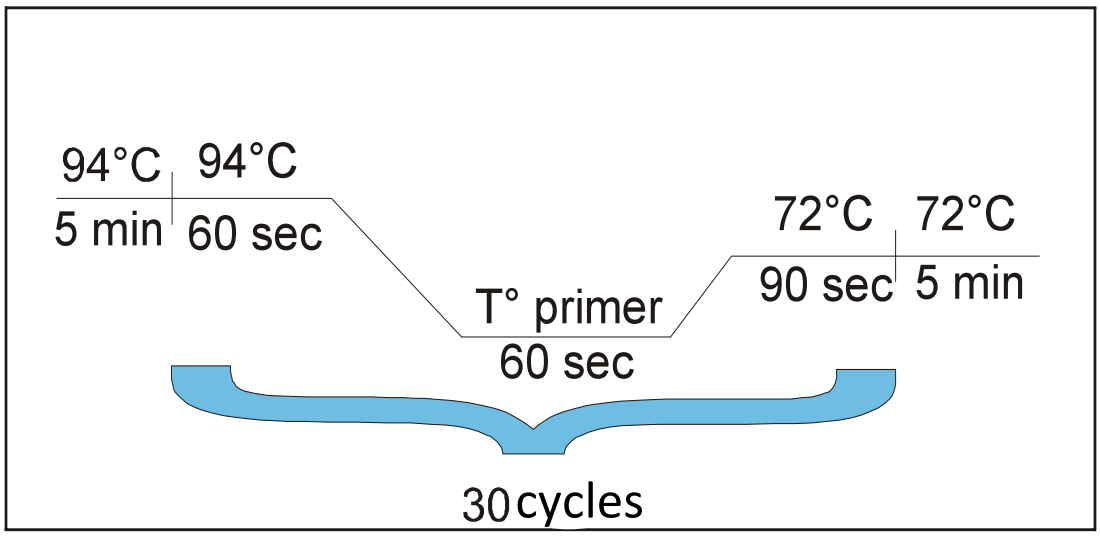
Total volume of amplification: 25µl

Template volume: 5µl

Amplification conditions vary in annealing temperature for every primer pair, but all followed the following cycle type:



The various PCR tests have been all checked on a 1.5% agarose gel to control that there was the amplified indeed.

4.3.4 Amplification with cold and labelled primers

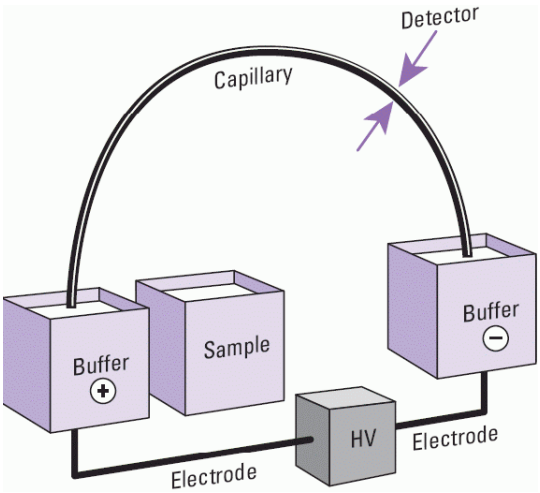
Primers were first tested in “cold” conditions (unlabelled), in order to understand which work optimally.

Among the tested primers, I decided to use the following pairs that successfully amplified:

Locus	*Primer Sequence 5'-3'*	*Fluorophore*	*annealing T°*
Ant 99	*F: TGTCATAGCGGCTTCCAT*	*HEX*	*52°*
	R: CGAGGGTCGTAACAAGATAT		
Ant 6	*F: AGCAATAATTAGATGATGCC*	*HEX*	*52°*
	R: GTTTATGGTGGAAGATGAAT		
Ant 51	*F: CCTTCCGTTTCTTCTACAGT*	*TAMRA*	*52°*
	R: AAAACCCACTTACGCTACTC		
Ant 66	*F: TACTGCCTTGAGATCGTT*	*HEX*	*52°*
	R: CCTTCCGTTTCTTCTACAGT		
Ant 93	*F: TGCTGATACAGAAGGTAGGC*	*FAM*	*51°*
	R: TTGGTACTGTTTCCCCATGC		
Ant 16	*F: TGAGACCCTCAGACTCAC*	*FAM*	*51°*
	R: TCTTTCTTTCTACTTCCCCTC		
Ant 94	*F: GAGGTGTAGGCAGAGTGA*	*TAMRA*	*56°*
	R: GCCTCTTTTACGTTACGCTG		
Ant 9	*F: CGCCTACACCGATGGTTCCT*	*HEX*	*61°*
	R: GCCTCCCACTGCCAACATGA		
Ant 20	*F: TAGTGTTCCATAGACTTATA*	*TAMRA*	*45°*
	R: ACTAGACAAATCTAAATGCT		
Ant 37	*F: TGTACGGGGCGACAGTCTAC*	*FAM*	*61°*
	R: GGGGAGACGGCGAAGCAAAC		
Ant 34	*F: AACGTGCCAATCAAAGTGAT*	*TAMRA*	*51°*
	R: TGAGGTAGAGACAAAGACTG		
Ant 46	*F: AATCAGAGGATCACGACACT*	*FAM*	*52°*
	R: TACTGTGTCTTTGGCAACTG		
Ant 82	*F: TGTTACGATTCCTGGTAAGG*	*HEX*	*52°*
	R: ATGAAGTGGTTGAGTAGTCC		
Ant 194	*F: ATGGATTCAATAATTTCGGC*	*HEX*	*47°*
	R: ATATTCTGGCATTTTGTAGG		
Ant 60	*F: TGCGAAGTAATCACGACTAG*	*FAM*	*59°*
	R: CTAATCCAACCGTAAAGCAC		
Ant 54	*F: ATTACTGGAGCCTTACTACC*	*TAMRA*	*55°*
	R: CATGTACTGAGGGAGTGTGC		
Ant 2	*F: CGCAGACGATGAAGGCGAGG*	*FAM*	*60°*
	R: AGCACCTTACGTCGGGATGA		
Ant 104	*F: ATATCTAGGGAATCTCCCCA*	*HEX*	*52°*
	R: GCTCTTCAGGGCATATACTA		

For these 18 pairs, the forward primers were resynthesized and labeled with a fluorophore. Amplification conditions were unchanged but the labelled amplicon could be now easily dimensioned by a capillary electrophoresis. In fact, the

fluorophore responds with an impulse when struck by a pulse of light in the capillary during electrophoresis.



4.3.5 Fragment analysis, allele sizing and genotype assignment

The length polymorphisms between the different alleles of a microsatellite locus corresponds to fragments of different lengths that differ in the number of repeated modules (*repeats*). The difference in size of these fragments are not visible as two distinct bands by agarose gel electrophoresis. For this reason capillary electrophoresis is indispensable.

The term electrophoresis indicates the migration of different electrically charged molecules in an electric field, and we know that many pharmaceutical and biological molecules have ionizable groups so that they can exist in solution as anions or as cations.

In the case of capillary electrophoresis, the apparatus comprises a fused silica capillary of very small diameter, containing an appropriate buffer, which is immersed in two separate tanks containing two electrodes, which are responsible for generating the electric field.

When the sample migrates it can be analyzed by an instrument connected to the capillary called "detector". This sends a pulse of light by a laser, that affects the DNA fragment at the time of its passage. The amplicon due to its labelling by fluorophore, will send a response to the instrument. Individual alleles can be sized by comparison with an internal *standard*: for each capillary, together with the amplified portion, a standard of known molecular weight is loaded, formed by a set of fragments of known size (between 50 and 500 bp) labeled with a fluorophore.

Specifically, the analysis of our samples was carried out using a genotyping service on automatic sequencer (ABI PRISM 3700 DNA Analyzer or ABI PRISM 3100 DNA Analyzer; internal *standard* GS 400 HD Rox) by BMR Genomics at the CRIBI centre, Padua.

Results processing went first through *Genographer 1.6.0* (Benham, 2001) software which displays the size of the fragments, reconstructing the graphic image of the electrophoretic run from file formats *ABI3100 - ABI3700*. Then microsatellite profiles were analyzed through *GeneMarker® v.1.75* (*Softgenetics*): this software allows to examine the profiles of microsatellite marker for each fluorophore and each profile can be identified on the assumption that every microsatellite locus amplicon is displayed in a graph having as abscissa the time of migration on gel and the intensity of the amplified product ordered.

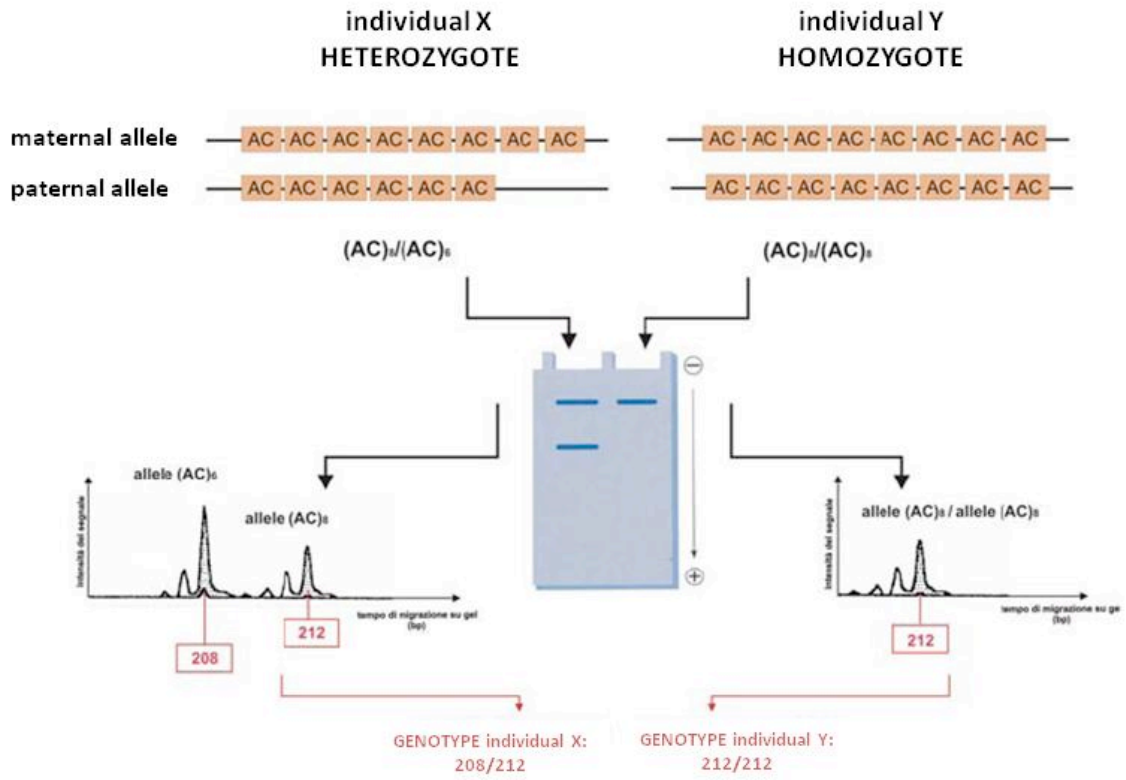
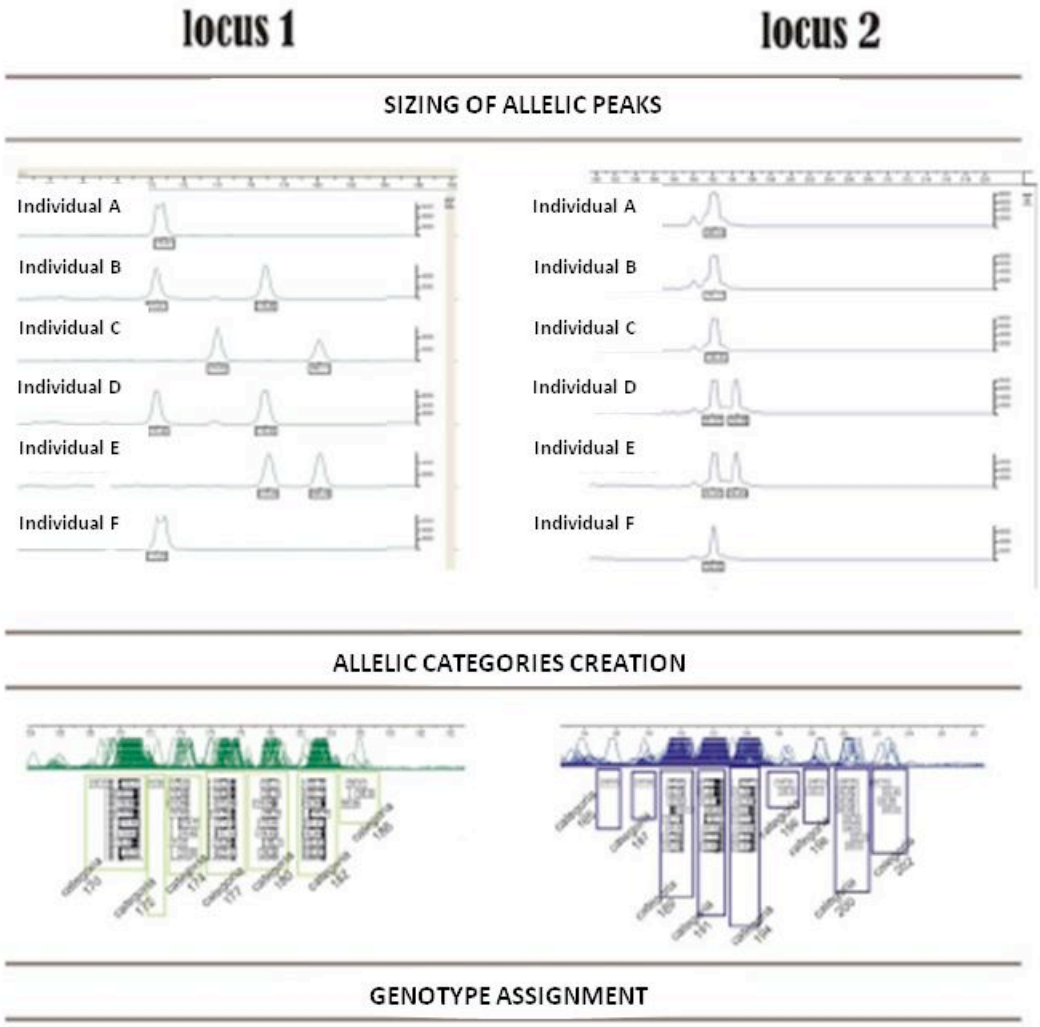


Fig.4.13 Representation of sizing and genotype assignment for different individuals. From peak sizing allele categories were derived and genotype identified.

For each sample it was possible to separate the amplified fragments of the fluorophore by colour and size alleles at every microsatellite locus (figure 4.13). The

analysis of each peak and its allelic size in base pairs has allowed the construction of the genotype for each individual (figure 4.14).



	locus 1			locus 2	
individual A	170	170	individual A	191	191
individual B	170	177	individual B	191	191
individual C	174	180	individual C	191	191
individual D	170	177	individual D	191	194
individual E	177	180	individual E	191	194
individual F	170	170	individual F	191	191

Fig.4.14 Genotype assignment from allele categories resulting from peak sizing.

4.4.6 Polymorphism and genetic indices of isolated loci

Population structure analysis followed polymorphism evaluation for each locus. This first step was necessary to identify the usefulness of each individual locus, resulting in the exclusion of those not enough variable for population structure analyses. The level of polymorphism and information of individual loci was derived by analyzing 20 individuals from the sample PSP North 2006.

Allele frequencies

Allele frequencies were evaluated for each microsatellite locus. Allele frequency corresponds to the ratio between the number of times an allele is present in a population and the total number of alleles. The P_i frequency of the i-th allele of any gene is given by:

$$\mathbf{P_i = (2n_{il} + n_{i2})/2N}$$

where:

n_{il} = number homozygotes for the allele i-th

n_{i2} = number heterozygotes for allele i-th

N = number of individuals sampled

Polymorphism of a given locus

In population genetics, two criteria are generally used to determine whether a locus is polymorphic: the 1% and the 5% level. According to these limits, a locus is polymorphic if the most common allele has a frequency $\leq$ 0.99 or $\leq$ 0.95, respectively.

Hardy-Weinberg equilibrium

The Hardy-Weinberg principle is the basis of population genetics and states that in an infinitely large population with random mating and no mutations, migrations or selective pressure, allele frequencies and genotypes are constant from one generation to the next generation and there is a simple relationship between allelic and genotypic frequencies (Guo and Thompson, 1992). For an autosomal locus with alleles $A_1,A_2,...,A_n$ the genotypic frequencies inferred by the Hardy-Weinberg law are:

$$\mathbf{\Sigma p_i^2 A_i A_j + \Sigma 2 p_i p_j A_i A_j}$$

Where:

p_i = frequency of allele $A_i = (2n_1+n_2)/2N$, where n_1 is the number of homozygotes for the allele i-th, n_2 the number of heterozygotes for the i-th allele, N is the number of individuals.

To test for Hardy-Weinberg equilibrium, the exact test was used (Guo and Thompson, 1992). This test is preferred when the sample sizes are small and/or there are loci with rare alleles. For the test, a significance level α (α = 0.05) is given, consisting of the maximum probability of error that you are willing to accept in rejecting the zero hypothesis (Beghi, 1992). In the case of multiple comparisons, the threshold was adjusted according to the standard Bonferroni procedure, as described below.

The test proceeds by evaluating all possible genotype frequencies for the particular set of observed allele frequencies and rejecting the hypothesis of Hardy-Weinberg equilibrium (*HWE*) whether the observed genotype frequencies under *HWE* are unlikely.

Linkage equilibrium

Under random mating conditions, alleles at any locus reach rapidly the random association of genotypes. A situation of gamete random association between alleles of different genes is called *linkage equilibrium* (balance of associated genes).

However, the alleles of a gene may not be in random association with alleles of the other gene, and this situation states that genes are in *linkage disequilibrium*.

For the study of microsatellite loci, contingency tables were performed for all pairs of loci in population. Assumption zero is that the genotypes of a locus are independent from genotypes of another locus. Each pair of comparison was associated with a probability value, that corresponds to the probability of getting the value of linkage as from the original dataset by chance. The global significance level of the test is α=0.05, adjusted using the sequential Bonferroni correction, where the threshold for individual comparisons is corrected for the number of tests performed.

The calculation of genetic indexes mentioned above were performed using the softwares *Genalex v.6.2* (Peakall and Smouse, 2006), *Genepop v.4.0* (Rousset, 2008) and *Fstat v.2.9.3* (Goudet *et al*., 2002).

4.3.7 Population genetic structure and dynamics analyses

4.3.7.1 Population structure

Once determined the most suitable microsatellite loci for studying the genetic structure of *A. antennatus* populations, we repeated the *allele frequencies*, *polymorphism at a given locus*, *Hardy-Weinberg equilibrium* and *Linkage equilibrium* analyses including all individuals sampled.

Genetic variability

Genetic variability for each sampling site was quantified using standard descriptive statistics. Number of alleles (*Na*), observed heterozygosity (*Ho*), unbiased expected heterozygosity (*UHe*), and *Fis* inbreeding coefficient (Weir and Cockerham, 1984) were calculated using the software *GenAlEx v.6.2* (Peakall and Smouse, 2006) and *Genepop v.4.0* (Rousset, 2008). Allelic richness (*Ra*), which was calculated with *FSTAT 2.9.3* using the rarefaction method (Goudet *et al*., 2002), was corrected to the sampling location with the fewest individuals (Vapore) to increase the power of detecting differences in *Ra* (Leberg, 2002), and results were averaged across loci.

Polymorphism information content (*PIC*) for all loci over all samples was calculated using the Excel *Microsatellite Toolkit v. 3.1.1* (Park, 2001).

ANOVA

Differences among sampling locations and temporal samples for the mean *Ho* and *UHe* were assessed using the ANOVA F statistic, whereas *Na* and *Ra* differences were tested using the Mann-Whitney U test.

Neutrality test

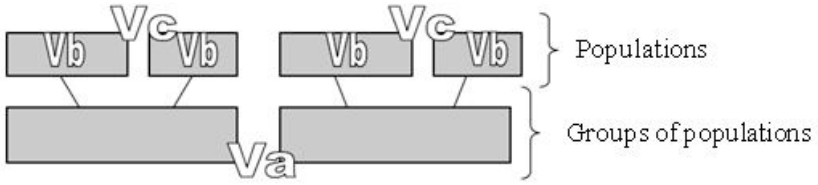
Microsatellite data were used to perform the Ewens-Watterson neutrality test for all loci using the algorithm given in Manly (1985) and implemented in the software *PopGene v 1.32* (Yeh *et al*., 1998). The observed sum of the squared allele frequencies (observed F) was compared with the 95% confidence intervals (calculated using 1000 simulated samples) for the expected sum of the squared allele frequencies (expected F). This test is useful for detecting recent or current deviations from mutation-drift equilibrium due to selection or demographic changes.

To determine whether the departure from panmixia was due to the presence of null alleles, the *FreeNa package* (Chapuis and Estoup, 2007) was used, and a maximum-likelihood estimate was calculated for the frequency of null alleles according to Dempster *et al.* (1977). The same software was used to compute a genotype data set corrected for null alleles following the Including Null Alleles (INA) method described in Chapuis and Estoup (2007). This new data set was used to recalculate the mean expected heterozygosity (*He*; Nei, 1987) as a measure of genetic variability within geographical locales, because the presence of null alleles has only a limited effect on this statistic (Chapuis and Estoup, 2007).

AMOVA

The Analysis of MOlecular VAriance (AMOVA, Excoffier et al., 1992), typically used to test genetic structures and to combine more populations, was used on the global dataset. The AMOVA, in fact, estimate show much of the total variance of

allele frequencies is due to differences between population groups (Va), how much can be attributed to differences between individuals within groups (Vb) and , finally, differences between populations within groups (Vc).



The software *Arlequinv.3.1.1* (Excoffier, Laval and Schneider, 2005) estimates the"*F*" fixation indices from Wright (Wright, 1951, 1965), based on the variance components which measure the loss of heterozygosity compared to HWE or the increase of homozygosity that inevitably leads to 'fix' one or another allele, thus allowing an integrated view of genetic variation and population structure at different hierarchical levels.

The significance level of fixation indices was tested randomly permuting 1,000 times, genotypes, individuals, or populations, between individuals, between populations, or among groups of populations (Excoffier *et al*., 1992). After each permutation, the *F* indices are recomputed and compared with the observed one, so it is possible to estimate the frequency with which values equal or exceed the ones in the observed distribution. Then the associated probability that values would be observed by chance is calculated. If these values are significantly greater than zero, i.e., a probability of being observed by chance less than 0,05, they indicate the presence of differentiation.

Bayesian analysis in Structure

AMOVA was joined with a further analys is also aimed at verifying the possible presence of underlying genetic structure in the sample. This analysis is based on the method from Pritchard *et al.* (2000), implemented in the software *Structure v.2.3.3*, that applies a Bayesian approach to define clusters of individuals based on allele frequencies of multiple loci, without considering population groups defined a priori.

Structure, allows you to create a series of simulations designed to estimate the value of natural logarithm of probability 'LnP(D)' that individuals are subdivided into a number of populations *K* (all in HW equilibrium and loci in linkage equilibrium).

Each simulation is based on a high number of iterations. The maximum value of 'LnP(D)' obtained is association with the most likely situation that the population is divided into a number (*K*) of populations. The probability (*Q*) that every group and every individual has to belong to each *K* cluster (population) as defined by the program is also calculated.

For each possible *K* value, the simulation was repeated four times.

4.3.7.2 Test for possible reductions or expansions in population size

Using the whole dataset from isolated microsatellite loci, it was possible to test the possibility that analyzed populations have undergone recent demographic fluctuations, for example by reducing its effective size (*Ne*), as in a "bottleneck" event. The software *Bottleneck v.1.2.02* (Cornuet and Luikart, 1996; Piry *et al*., 1999) was used for this purpose in order to identify any signs of recent population reduction on the basis of allele frequencies. In fact, populations which show a reduction in the number of alleles (*Na*) also reduce their "observed heterozygosity" (*Ho*) per polymorphic locus. In particular, *Na* is reduced much faster than *Ho*, and for this reason, the observed heterozygosity will be higher than expected heterozigosity, which is calculated precisely from *Na*. By taking three of the four models proposed for the evolution of microsatellites, SMM, IAM and TPM, the program tests the excess of heterozygosity in all loci and for all populations through three different statistical tests. In this study it was decided to use the intermediate model TPM - Two-Phase Model with a 7% multi-step changes (Di Rienzo *et al*., 1994, Luikart *et al.*, 1998) and the "Wilcoxon sign-rank test" (Luikart *et al*., 1998), better suited when you have access to less than twenty microsatellite loci.

The same software was used to look at a graphical descriptor of the shape of the allele frequency distribution: a population that has not suffered a recent bottleneck event will have an L-shape distribution (as expected in a stable population in mutation-gene drift equilibrium).

Possible signs of expansion were tested using two different approaches: imbalance index β (Kimmel *et al*., 1998, King *et al*., 2000) and the interlocus g test (Reich and Goldstein, 1998; Reich *et al*., 1999).

The imbalance index compares the distribution of length differences among microsatellite alleles; in particular, the lnβ metric was chosen because of its ability and power to detect historic signals of population expansion (King *et al*., 2000). First, the variance estimator (θ_V) and the homozygosity estimator (θ_H) were calculated from equations (1) and (3) in Kimmel *et al.* (1998). Next, the imbalance index (lnβ) was calculated as the difference in the natural logarithm of these two estimators averaged over all microsatellite loci. The statistic g test, calculated using the Kgtests Excel macro (Bilgin, 2007), is interpreted as an indication of an expansion when it has an unusually low value (Reich *et al*., 1999); in our study, g values lower than 0.14–0.16 are indicative of population expansion. Significance levels for the interlocus g test were obtained in Table 1 in Reich *et al*. (1999).

4.3.8 Sex-biased Dispersal

Due to the different bathymetric distribution of males and females, reported to be associated with different water masses and hence with possible differential dispersal capacity between sexes, the hypothesis of sex-biased dispersal was tested.

The following software were used to test for sex-biased instantaneous dispersal: *FSTAT* v.2.9.3, which allows analysis of genotypes for which missing data are present but not populations exclusively composed of individuals of the same sex (as in the case of Cala Gonone); *GENECLASS v.2.0* (Piry *et al*., 2004) and *GenAlEx*

v.6.2, which exclude individuals bearing missing data but allow populations composed by individuals of only one sex. F_{IS}, F_{ST}, mean and variance of corrected Assignment Index (*mAIc* and *vAIc*, respectively) were calculated with *FSTAT v.2.9.3*; statistical significance for these indices was determined using 10,000 randomizations.

The Assignment Index reflects the probability that a particular genotype of an individual was originated from the sampled population.

Assignment values for each individual in each sample were transformed to corrected Assignment Indices (*AIc*, Favre *et al*., 1997; Goudet *et al*., 2002).

The *AIc* is proportional to population size, estimated for each individual using the following formula:

***AIc* = log px – log P'**

The mean population *AIc* (*mAIc*) is zero, and the lower *AIc* value an individual has, the higher the probability that the individual is a disperser from another population (Bekkevold *et al*., 2004). Because of the accuracy and higher discriminant power of the Bayesian assignment method of individual-based assignment to measure dispersal was recently assessed (Berry *et al*., 2004; Lawson Handley and Perrin,2007), *mAIc* assignment indices were also computed with *GENECLASS v.2.0* (Piry *et al*., 2004) using the Bayesian assignment approach developed by Rannala and Mountain (1997). The Mann–Whitney U test was applied to determine if *AIc* values for males and females were significantly different.

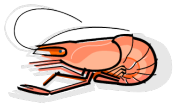
The mean relatedness index (*r*) between individuals of each sex was calculated using the method of Queller and Goodnight (1989) implemented in *GenAlEx v.6.2*. It is worth noting that the biparental markers utilized in this study (i.e., microsatellites) convey information on short-term dispersal (i.e., after dispersal, before mating). Therefore, the sex-biased migration signal will disappear after one generation of random mating due to Mendelian segregation if the dispersal is no longer sex-biased (Goudet *et al*., 2002; Prugnolle and de Meeus, 2002). However, a significant

proportion of the migrants may disperse but not reproduce in the new population. This would maintain allele frequency differences between the populations and allow the detection of immigrants within every generation.

4.3.9 Correction for multiple tests

Sequential Bonferroni correction

The sequential Bonferroni method performs more than one hypothesis test simultaneously. All multiple p-values performed in this thesis were corrected using this method implemented in the software*SGoF+* (Carvajal-Rodriguez *et al*., 2009).



5 Results

5.1 Isolation of nuclear microsatellite markers

From the partial genomic library enriched for AC repeat, 121 colonies putatively containing the microsatellite were selected and sequenced. Forty-four sequences were found to include repeats and 30 of these showed suitable flanking regions (in quality and length) to primers design. Among these 30 primer pairs, 18 have been tested.

The 18 pairs of primers were initially tested on a restricted sample of individuals to verify the effectiveness of amplification. Once verified the functionality in amplification, the 18 loci were amplified in 20 individuals from the sample PSP North 2006.

5.2 Polymorphism and genetic indices of isolated loci

Loci polymorphism

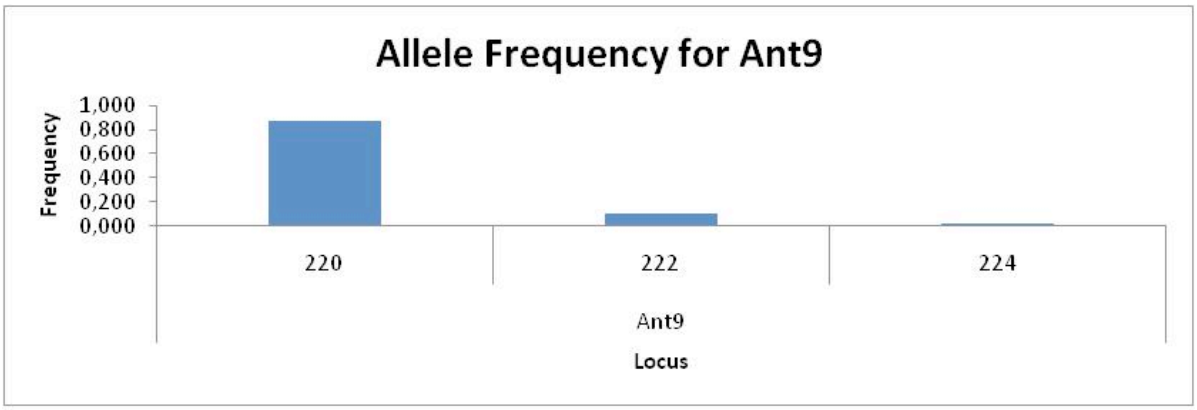
Of the 18 loci, 14 were found to be polymorphic, even after correction for null alleles, and therefore suitable for population analysis. This result has produced a publication on international scientific journal (Appendix 1).

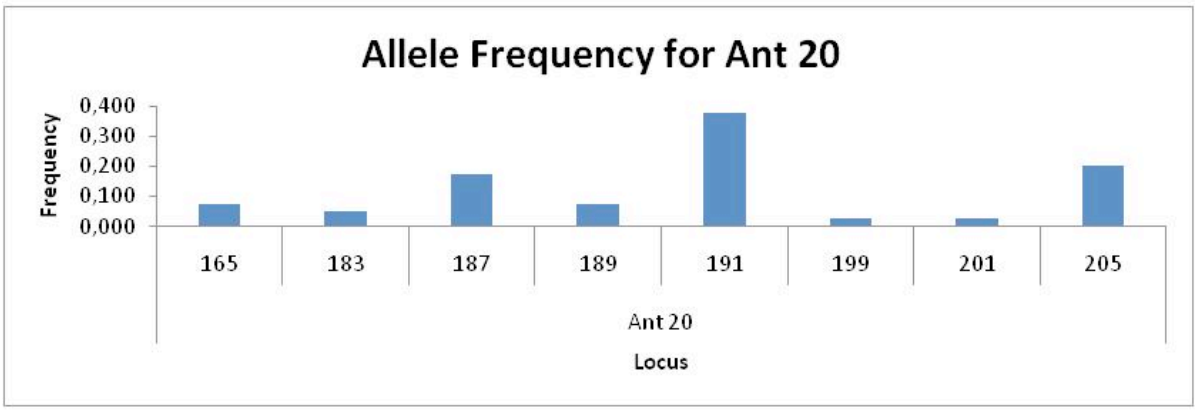
In table 5.I details for each isolated locus are presented. For loci Ant60, Ant54, Ant2 and Ant104 the heterozygosity is 0 because loci are to be considered monomorphic with the 5% criterion.

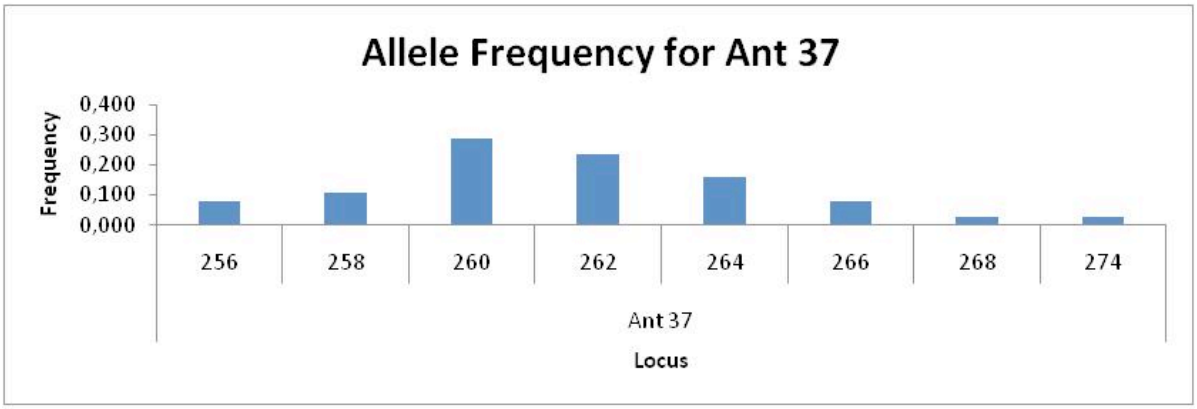
Table 5.I Characteristics of 14 microsatellite loci isolated from *Aristeus antennatus* (n = 20 individuals), with primer sequences, fluorochrome dye of forward primer, touchdown (TD) annealing temperatures (T°) in °C, repeat motif derived from a sequenced clone, number of alleles observed (NA), observed allele size ranges, number of individuals assayed (N), observed (HO) and expected (HE) heterozygosities (boldface numbers indicate significant deviation from Hardy–Weinberg equilibrium with *P < 0.05, **P < 0.01)

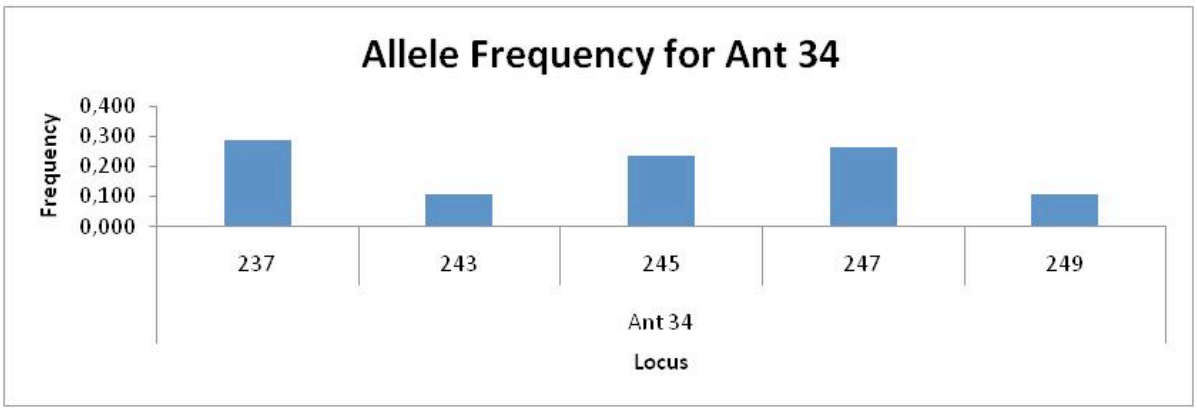
Locus	*Primer sequence 5'-3'*	*Dye*	*Repeat sequence 5'-3'*	*Na*	*Ho*	*He*
Ant 99	*F: TGTCATAGCGGCTTCCAT*	*HEX*	*(TG)4CT(TG)5CG(TG)3*	*7*	*0,700*	*0,687*
	R: CGAGGGTCGTAACAAGATAT					
Ant 6	*F: AGCAATAATTAGATGATGCC*	*HEX*	*(GT)5AT(GT)12*	*3*	*0,350*	*0,304*
	R: GTTTATGGTGGAAGATGAAT					
Ant 51	*F: CCTTCCGTTTCTTCTACAGT*	*TAMRA*	*(TG)10*	*4*	*0,550*	*0,645*
	R: AAAACCCACTTACGCTACTC					
Ant 66	*F: TACTGCCTTGAGATCGTT*	*HEX*	*(CA)10*	*4*	*0,500*	*0,619*
	R: CCTTCCGTTTCTTCTACAGT					
Ant 93	*F: TGCTGATACAGAAGGTAGGC*	*FAM*	*(AC)10*	*5*	***0,350****	***0,745***
	R: TTGGTACTGTTTCCCCATGC					
Ant 16	*F: TGAGACCCTCAGACTCAC*	*FAM*	*(GT)9*	*8*	***0,200****	***0,870***
	R: TCTTTCTTTCTACTTCCCCTC					
Ant 94	*F: GAGGTGTAGGCAGAGTGA*	*TAMRA*	*(TG)12*	*10*	***0,600****	***0,882***
	R: GCCTCTTTTACGTTACGCTG					
Ant 9	*F: CGCCTACACCGATGGTTCCT*	*HEX*	*(GT)5G(GT)3*	*3*	*0,200*	*0,230*
	R: GCCTCCCACTGCCAACATGA					
Ant 20	*F: TAGTGTTCCATAGACTTATA*	*TAMRA*	*(CA)7*	*8*	***0,550****	***0,793***
	R: ACTAGACAAATCTAAATGCT					
Ant 37	*F: TGTACGGGGCGACAGTCTAC*	*FAM*	*(TG)11*	*8*	*0,850*	*0,823*
	R: GGGGAGACGGCGAAGCAAAC					
Ant 34	*F: AACGTGCCAATCAAAGTGAT*	*TAMRA*	*(AC)9*	*6*	*0,650*	*0,838*
	R: TGAGGTAGAGACAAAGACTG					
Ant 46	*F: AATCAGAGGATCACGACACT*	*FAM*	*(AC)6AG(AC)6*	*13*	***0,600****	***0,890***
	R: TACTGTGTCTTTGGCAACTG					
Ant 82	*F: TGTTACGATTCCTGGTAAGG*	*HEX*	*(AC)10*	*9*	*0,750*	*0,882*
	R: ATGAAGTGGTTGAGTAGTCC					
Ant 194	*F: ATGGATTCAATAATTTCGGC*	*HEX*	*(CA)8*	*4*	*0,500*	*0,705*
	R: ATATTCTGGCATTTTGTAGG					
Ant 60	*F: TGCGAAGTAATCACGACTAG*	*FAM*	*(AC)5AT(AC)5*	*1*	*0*	*0*
	R: CTAATCCAACCGTAAAGCAC					
Ant 54	*F: ATTACTGGAGCCTTACTACC*	*TAMRA*	*(TG)2TA(TG)9*	*1*	*0*	*0*
	R: CATGTACTGAGGGAGTGTGC					
Ant 2	*F: CGCAGACGATGAAGGCGAGG*	*FAM*	*(GT)8*	*1*	*0*	*0*
	R: AGCACCTTACGTCGGGATGA					
Ant 104	*F: ATATCTAGGGAATCTCCCCA*	*HEX*	*(CT)6*	*1*	*0*	*0*
	R: GCTCTTCAGGGCATATACTA					

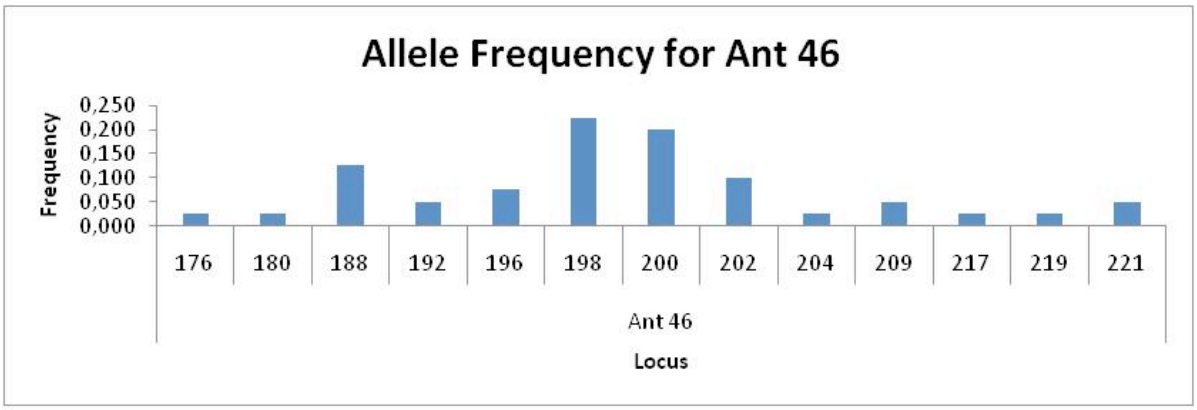
Histograms reported in figure 5.1 represent the allele frequencies per locus for the group of individuals. The graphical representation highlights the different levels of polymorphism within loci.

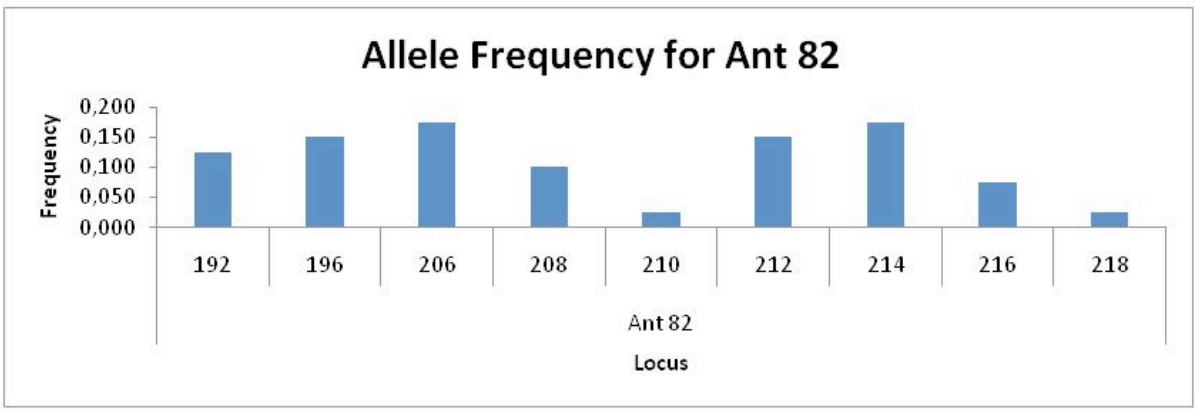


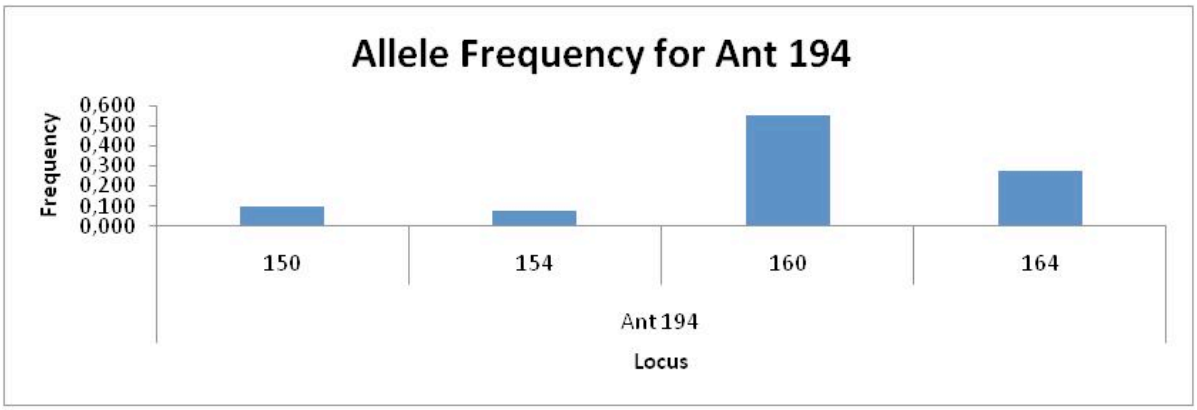


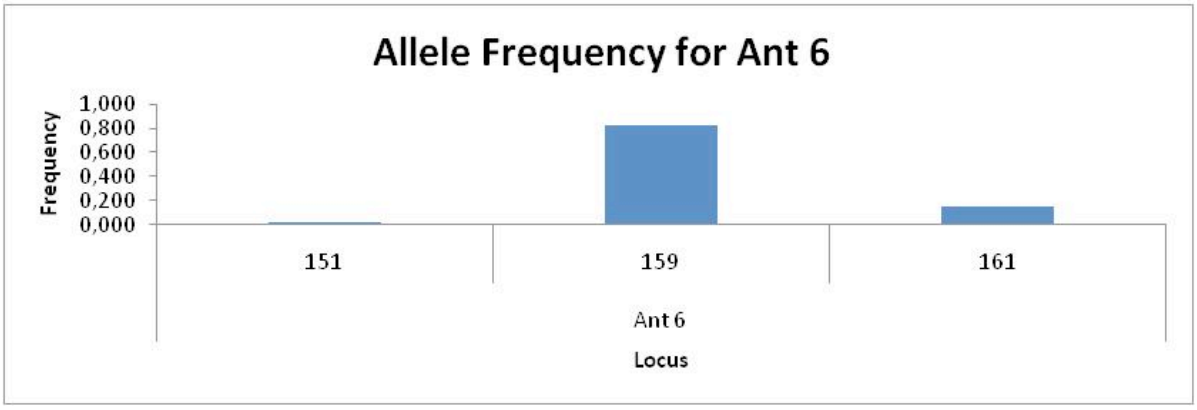


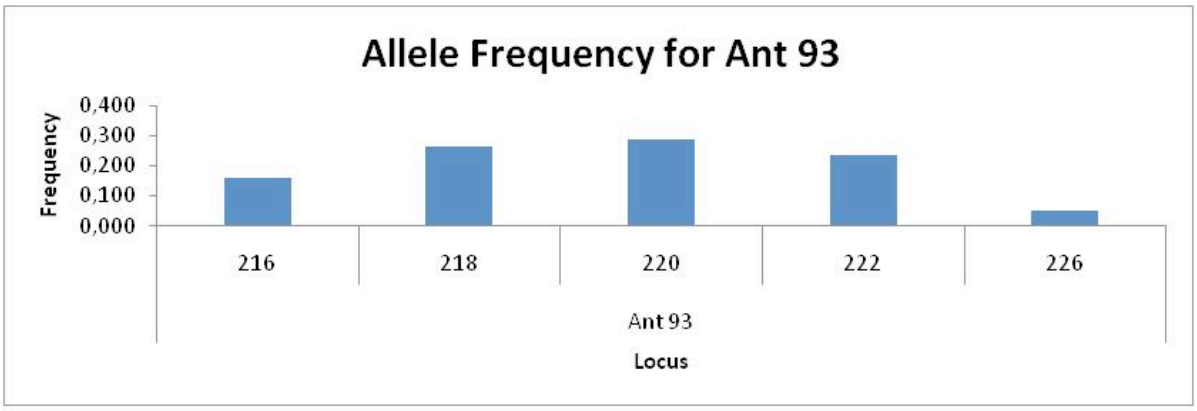


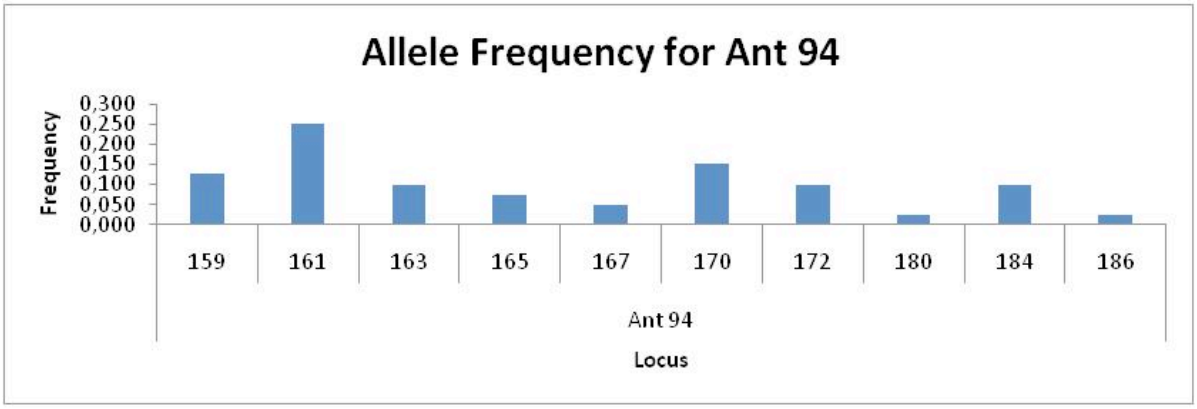

Allele Frequency for Ant 46
Frequency
0,250
0,200
0,150
0,100
0,050
0,000
176
180
188
192
196
198
200
202
204
209
217
219
221
Ant 46
Locus

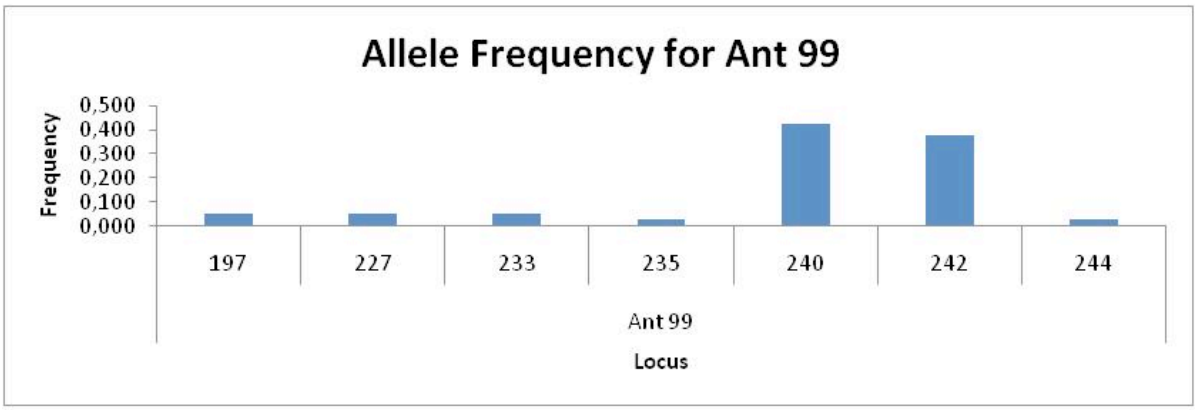

Allele Frequency for Ant 82
Frequency
0,200
0,150
0,100
0,050
0,000
192
196
206
208
210
212
214
216
218
Ant 82
Locus

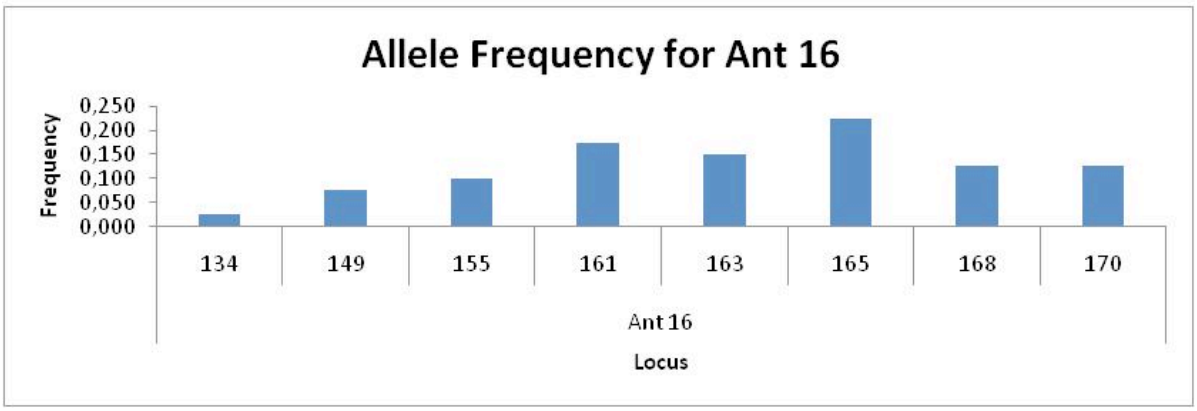

Allele Frequency for Ant 194
Frequency
0,600
0,500
0,400
0,300
0,200
0,100
0,000
150
154
160
164
Ant 194
Locus


Allele Frequency for Ant 6
Frequency
1,000
0,800
0,600
0,400
0,200
0,000
151
159
161
Ant 6
Locus


Allele Frequency for Ant 93
Frequency
0,400
0,300
0,200
0,100
0,000
216
218
220
222
226
Ant 93
Locus


Allele Frequency for Ant 94
Frequency
0,300
0,250
0,200
0,150
0,100
0,050
0,000
159
161
163
165
167
170
172
180
184
186
Ant 94
Locus


Allele Frequency for Ant 99
Frequency
0,500
0,400
0,300
0,200
0,100
0,000
197
227
233
235
240
242
244
Ant 99
Locus


Allele Frequency for Ant 16
Frequency
0,250
0,200
0,150
0,100
0,050
0,000
134
149
155
161
163
165
168
170
Ant 16
Locus

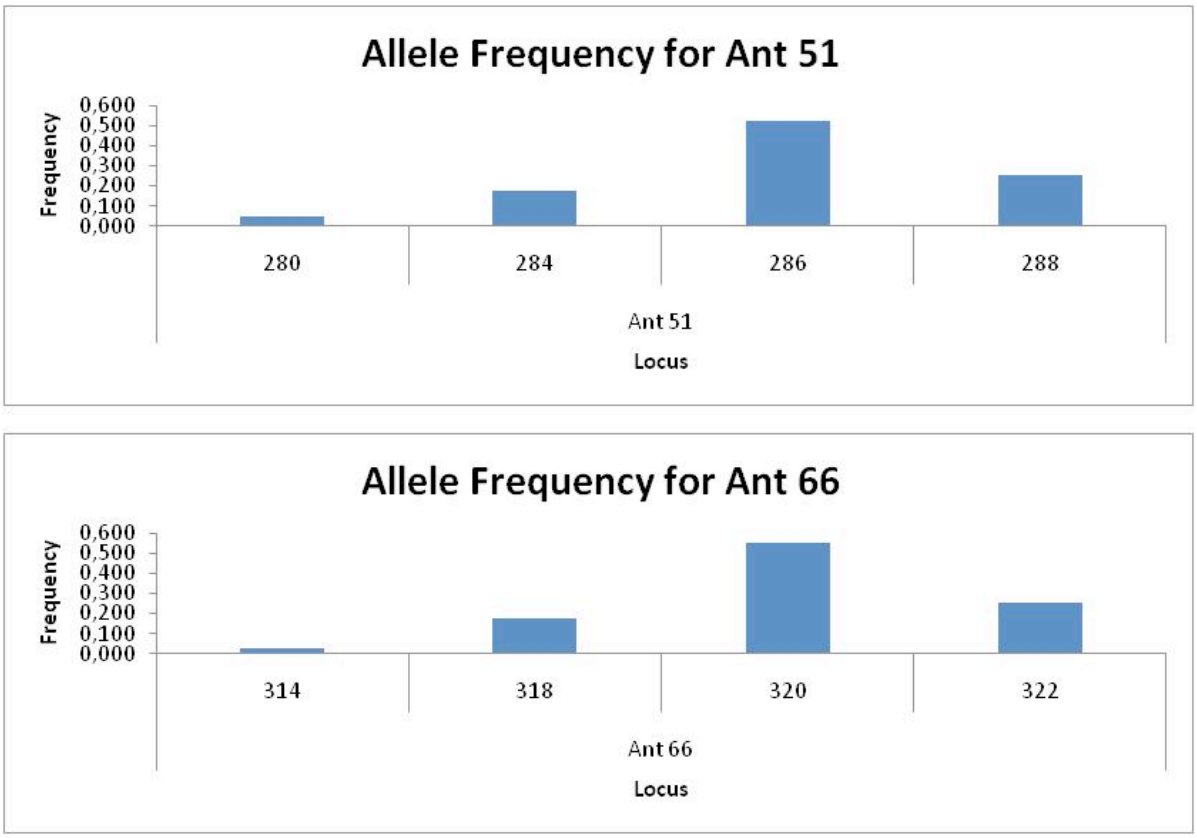


Fig. 5.1 Allele frequencies histograms at each locus. Loci Ant 60, Ant 54, Ant 2 and Ant 104 are not represented as they resulted monomorphic.

Linkage equilibrium

Genotypic *linkage equilibrium* analysis between all pairs of loci showed *linkage disequilibrium* for one pair, even after Bonferroni correction for multiple comparisons. linked loci (in *linkage disequilibrium*) are Ant 51 and Ant 66.

Hardy-Weinberg equilibrium

Of the 14 polymorphic loci, five showed significant deviations from Hardy-Weinberg equilibrium: Ant 16, Ant 20, Ant 46, Ant 93 and Ant 94. In particular, these five loci showed a deficit in heterozygotes.

5.3 Population genetic structure and dynamics

From the above reported preliminary loci only some of the available markers were selected in order to optimize resources and time available. From the starting 18 available loci, 8 markers were selected for further analyses.

As a first principle of selection, the index of polymorphism was analyzed. In this way 4 monomorphic loci (Ant 60, Ant 54, Ant 2, Ant 104) were excluded. In addition, the loci Ant 6 and Ant 9, showed only 3 alleles with a frequency of an allele over 90%. These loci have been found so poorly informative that were excluded from the population structure analysis, bringing to 12 the number of possible markers.

In a second step, we considered the *linkage disequilibrium* between loci: the locus Ant 51 resulted linked to Ant 66 and consequently excluded, reducing the number of markers to 11. Finally, the loci Ant 16, Ant 20 and Ant 46 were excluded from the population structure analysis due to technical difficulties related to the amplification of the loci and the allelic peak reading.

In conclusion, the population structure analysis was performed using the following 8 microsatellite loci: Ant 93, Ant 94, Ant 99, Ant 66, Ant 37, Ant 194, Ant 34, Ant 82.

5.3.1 Population genetic structure

5.3.1.1 Descriptive statistics

Allele frequencies and polymorphism

A total of 227 individuals were successfully genotyped; none of the individuals shared the same diploid genotype. All eight microsatellite loci were polymorphic across all populations and characterized by high PIC values (mean, 0.77; range, 0.65–0.86, see table 5.II).

Genetic variability

Estimators of genetic diversity varied among loci and populations, with variable levels of polymorphism (table 5.II). *Na* ranged from a minimum of 9 (locus Ant 99) to a maximum of 21 (locus Ant 94), with a mean of 13.5 alleles per locus. *Ho* for the eight loci ranged from 0.38 to 0.79 (mean, 0.65), *UHe* varied from 0.69 to 0.87 (mean, 0.8), and *Ra* varied from 5.28 to 10.06 (mean, 7.57). No significant differences in mean *Na*, *Ho*, *Uhe*, or *Ra* were observed among sampling sites and temporal samples (for all tests, P-value > 0.05); thus, in all of the following analyses the samples collected in different years from the same locality were pooled.

Hardy-Weinberg equilibrium

Genepop v.4.0 was used to obtain results for exact tests of Hardy-Weinberg equilibrium performed on populations. Initially, probabilities associated with each population to verify the null hypothesis of a deficit of heterozygotes, with a significance level of $\alpha = 0.05$ were calculated, followed by a sequential Bonferroni correction (Table 5.II).

Some samples deviated significantly from *HWE* at three microsatellite loci (table 5.II). After sequential Bonferroni corrections, a significant heterozygote deficit was found in 14 out of 72 comparisons (eight loci x nine samples). If the nine samples were considered as a whole, six out of eight loci in multi-sample tests deviated from *HWE*. The program *FreeNA* indicated that the departures from *HWE* probably were due to the presence of null alleles; all loci exhibited null allele frequencies > 0, but

only locus Ant 93 was in excess of 20% and three other loci were in excess of 5% (table 5.II).

Significant positive *Fis* estimates, which are indicative of heterozygote deficiencies, were observed at locus Ant 93 in all samples (except for Vapore) and at other two loci (Ant 34 and Ant 82) in a few samples (table 5.II).

Neutrality test

The overall Ewens-Watterson test for neutrality (pooled across samples) showed that none of the values for the eight loci fell outside of the lower and upper boundaries of the 95% confidence region.

Tab. 5.II Parameters of genetic diversity in *A. antennatus* analyzed for eight microsatellite loci. Number of individuals (*N*), total alleles (*Na*), allelic richness (*Ra*), observed heterozygosity (*Ho*), and unbiased expected heterozygosity (*UHe*) for each sample, each locus, and average values for all loci/all samples are reported. Significant departures from Hardy–Weinberg (*HWE*) after sequential Bonferroni correction are indicated next to the *Fis* values: *** $P < 0.001$, * $P < 0.05$. Frequencies of null alleles (*Nu*) estimated by *FreeNa* are shown for each locus. For all loci, Polymorphism Information Content (*PIC*) is indicated.

Sampling locale		*Ant93*	*Ant94*	*Ant99*	*Ant66*	*Ant37*	*Ant194*	*Ant34*	*Ant82*	*All loci*
Algeria	**N**	20	20	20	20	20	20	20	20	20
	Na	5	9	6	8	11	12	11	6	8.5
	Ra	4.7	8.56	5.51	7.01	9.46	9.81	10.1	5.89	8.08
	Ho	0.35	0.6	0.55	0.7	0.9	0.95	0.7	0.5	0.66
	UHe	0.72	0.87	0.68	0.78	0.82	0.84	0.89	0.8	0.8
	Fis	0.52***	0.32	0.20	0.11	-0.09	-0.14	0.21	0.38	
	Nu	0.21	0.14	0.03	0.05	0.00	0.00	0.09	0.16	
San Remo	**N**	29	29	29	29	29	29	29	29	29
	Na	5	11	6	10	7	9	9	9	8.25
	Ra	4.35	9.24	5.34	7.57	6.21	7.66	8.51	7.87	7.09
	Ho	0.31	0.66	0.52	0.72	0.79	0.76	0.66	0.55	0.62
	UHe	0.72	0.84	0.72	0.81	0.78	0.77	0.88	0.85	0.8
	Fis	0.57***	0.22	0.29	0.11	-0.02	0.01	0.26***	0.36*	
	Nu	0.23	0.09	0.08	0.02	0.00	0.00	0.12	0.16	
Cala Gonone	**N**	19	20	20	20	20	20	20	20	20
	Na	5	10	6	5	7	12	9	6	7.5
	Ra	4.73	8.93	5.67	4.97	6.59	10.08	8.38	5.38	6.84
	Ho	0.21	0.75	0.6	0.6	0.85	0.9	0.55	0.5	0.62
	UHe	0.72	0.87	0.75	0.76	0.8	0.79	0.88	0.75	0.79
	Fis	0.71***	0.14	0.21	0.21	-0.07	-0.14	0.38***	0.34	
	Nu	0.29	0.04	0.08	0.10	0.00	0.00	0.17	0.13	
Terrasini	**N**	20	20	20	20	20	20	20	20	20
	Na	7	13	6	7	8	15	10	10	11
	Ra	6.23	11.29	5.65	6.23	7.51	12.54	9.2	8.61	8.41
	Ho	0.35	0.8	0.7	0.6	0.8	0.9	0.5	0.75	0.68
	UHe	0.74	0.87	0.72	0.72	0.83	0.88	0.87	0.83	0.81
	Fis	0.54*	0.08	0.03	0.18	0.04	-0.02	0.43	0.10	
	Nu	0.22	0.03	0.01	0.09	0.00	0.00	0.18	0.07	
PSP South	**N**	53	53	53	55	55	55	54	55	55
	Na	7	16	9	8	12	12	9	10	12
	Ra	5.73	9.5	5.84	5.76	7.68	7.33	8.11	7.95	7.24
	Ho	0.6	0.72	0.64	0.58	0.76	0.62	0.61	0.71	0.66
	UHe	0.77	0.87	0.69	0.7	0.79	0.7	0.84	0.84	0.78
	Fis	0.22***	0.18	0.07	0.18	0.04	0.11	0.28*	0.15*	
	Nu	0.10	0.07	0.05	0.06	0.00	0.06	0.12	0.08	
PSP North	**N**	24	23	24	24	24	24	24	24	24
	Na	6	10	7	7	10	9	9	10	18
	Ra	5.16	9.09	5.87	5.93	8.26	7.45	8.26	9.72	7.47
	Ho	0.46	0.7	0.88	0.54	0.83	0.58	0.75	0.83	0.7
	UHe	0.76	0.88	0.69	0.74	0.86	0.78	0.86	0.9	0.81
	Fis	0.40***	0.21	-0.27	0.28	0.03	0.26	0.13	0.08	
	Nu	0.17	0.10	0.00	0.10	0.00	0.11	0.07	0.04	
Sant'Antioco	**N**	26	26	26	26	26	26	26	26	26
	Na	5	13	5	8	8	11	11	12	15
	Ra	4.52	10.07	4.78	6.28	7.13	9.05	9.22	9.22	7.53

	Ho	0.27	0.69	0.54	0.65	0.69	0.81	0.62	0.62	0.61
	UHe	0.74	0.85	0.72	0.7	0.82	0.84	0.85	0.84	0.8
	Fis	0.64***	0.19	0.26	0.07	0.15	0.03	0.28	0.27*	
	Nu	0.27	0.06	0.11	0.03	0.05	0.00	0.11	0.11	
Cagliari	**N**	17	17	17	17	17	17	17	16	17
	Na	6	13	5	6	7	9	10	10	12
	Ra	5.94	11.9	4.65	5.47	6.65	7.91	9.44	9.71	7.71
	Ho	0.24	0.71	0.47	0.65	0.82	0.65	0.94	0.69	0.64
	UHe	0.75	0.9	0.58	0.73	0.83	0.66	0.88	0.9	0.78
	Fis	0.69***	0.22	0.19	0.12	0.01	0.01	-0.07	0.24	
	Nu	0.29	0.09	0.05	0.02	0.00	0.00	0.00	0.10	
Vapore	**N**	14	16	16	16	16	16	16	16	16
	Na	5	8	6	5	7	9	11	8	7.38
	Ra	5	7.87	5.74	4.74	6.85	8.59	10.59	7.74	7.14
	Ho	0.64	0.56	0.69	0.56	0.69	0.69	0.88	0.88	0.7
	UHe	0.81	0.88	0.7	0.61	0.82	0.76	0.9	0.84	0.79
	Fis	0.21	0.37	0.02	0.08	0.17	0.09	0.02	-0.04	
	Nu	0.08	0.15	0.00	0.00	0.06	0.00	0.01	0.00	
All populations	**Mean N**	24.67	24.89	25	25.22	25.22	25.22	25.11	25.11	25.05
	Na	10	21	9	11	12	18	15	12	13.5
	Ra	5.28	10.06	5.53	6.05	7.61	8.64	8.98	8.44	7.57
	PIC	0.71	0.86	0.65	0.69	0.79	0.75	0.86	0.83	0.77
	Mean Ho	0.38	0.69	0.62	0.62	0.79	0.76	0.69	0.67	0.65
	Mean UHe	0.75	0.87	0.69	0.73	0.82	0.78	0.87	0.84	0.8

Linkage equilibrium

There was no linkage disequilibrium between all pairs of loci in all populations and, consequently, the loci were treated as independent genetic markers.

5.3.1.2 AMOVA, pairwise *Fst* and Bayesian approach

Differentiation between populations can be estimated with the Analysis of Molecular Variance (AMOVA), based on data of gene frequencies and heterozygosity using the program *Arlequin v.3.1.1*. Were originally estimated indices of variance within and between populations as reported in Table 5.III, including all population into a unique group.

AMOVA revealed that the greatest proportion of global genetic variance was attributable to differences among individuals (99.33%), and the proportion of genetic variation among sampling sites was not significant (F_{ST}=0.00673, P-value=0.06723$\pm$0.00076).

Tab. 5.III Results from analysis of molecular variance (AMOVA)

Source of Variation	*d.f.*	*Sum of Squares*	*Variance components*		*Percentage of Variation*	*Fixation Indices*
Among sampling sites	8	33.342	0.02118	Va	0.67	F_{ST}=0.00673*
Within sampling sites	445	1391.065	3.12599	Vb	99.33	
Total	453	1424.407	3.14717			

* P-value = 0.06723+-0.00076 (100,172 permutations)

Furthermore, a 3 levels AMOVA analysis, including groups of samples, was tested (table 5.IV).

Samples were pooled in three groups (figure 5.2):

- Northern group: San Remo sample;
- Central group: Sardinian samples;
- Southern group: Algeria and Terrasini samples.

The principles used to subdivide samples into these three groups were Euclidian distances and marine current patterns (MCP) described as “more or less steady paths” in Millot (1999). As A. antennatus is a species with a very dispersive larval phase, gene flow should be dependent of planktonic larvae dispersion driven by marine currents. For this reason, I decided to group samples on a possible connection pattern based on marine currents, that could have affected genetic subdivisions, rather than just simple Euclidian distances.

Following these principles, San Remo resulted enough distant (both on a Euclidian and MCP basis) to be considered as a single group. Sardinian samples were pooled together as they turned out to be near and strictly connected by marine currents (as seen in Millot, 1999). Algeria and Terrasini have been considered as a single group as they also are near enough and strictly inter-connected as for the Sardinian samples but well separated from the Central group. In fact, these samples seem near to Sardinian samples on an Euclidian basis, but result very distant when they are

connected following the MCP. No significant genetic differentiation was found among the different areas (P > 0.05).

Fig. 5.2 Representation of sampling subdivision in 3 groups on a geographical basis.

Tab. 5.IV Results from analysis of molecular variance (AMOVA) with a 3-groups structure

Source of Variation	***d.f.***	***Sum of Squares***	***Variance components***		***Percentage of Variation***	***Fixation Indices***	***P-values***
Among groups	2	8.394	-0.00126	Va	-0.04	FCT -0.00040	P-value: 0.49959±0.00158
Within groups among sampling sites	6	25.484	0.02382	Vb	0.76	FSC 0.00763	P-value: 0.07218±0.00088
Within sampling sites	445	1,377.991	3.09661	Vc	99.28	FST 0.00723	P-value: 0.06354±0,00078
Total	453	1,411.870	3.11917				

After sequential Bonferroni corrections, none of the F_{ST} pairwise values were significant, both when the original and the ENA-corrected (Excluding Null Alleles) databases were used.

Results from the Bayesian analysis carried out through *Structure* are in line with previous analyses. The software actually revealed the lowest value of 'LnP(D)' for *K*=1. This result shows that *A. antennatus* individuals analyzed in this study are part of the same genetic population.

5.3.2 Growth and reduction of population size

In this study, none of the sampled populations showed excess of heterozygosity (table 5.V) or exhibited shifted frequency distributions of alleles. Bottleneck tests indicated that the analyzed samples had not experienced a recent reduction in size. From the microsatellite dataset, lnθH and lnθV for each sample were calculated, and the resulting lnβ values were always > 1 (range, 5.6–7.04; mean, 6.67). lnβ is < 1 for expanding populations whose pre-expansion history is stable, but it is > 1 in populations with a reduction in size that precedes a detectable growth phase (Kimmel *et al*., 1998). Our results indicate that the sampled populations may have experienced, in the past, a growth phase following an earlier reduction. Nevertheless, the more conservative g test did not show any evidence of expansion for either the overall data set or for each sampling site studied. The g statistic was 0.8 for the global sample and ranged from 0.94 in PSP South to 5.38 in Vapore (table 5.V). These values did not reach a g=0.14–0.16, which (according to table 1 in Reich *et al*.,1999) should correspond to a population expansion.

Tab. 5.V Results of bottleneck and interlocus g tests. The column TPM reports the Wilcoxon sign-rank test P-value for heterozygosity excess under the TPM model (Two-Phased Model of mutation).

Sampling location	***TPM***	***g-test (value)***
Algeria	0.81	0.99
San Remo	0.58	1.57
Cala Gonone	0.10	4.47
Terrasini	0.99	1.47
PSP South	0.98	0.94
PSP North	0.42	3.61
Sant'Antioco	0.96	1.10
Cagliari	0.68	4.66
Vapore	0.47	5.38
All	**0.01**	0.80

5.4 Sex-biased dispersal

Sex-biased dispersal was tested for all specimens for which the sex was directly determined (for 6 of the 9 geographic locations; 158 specimens: 88 females and 70 males; see table 4.I). Individuals from the remaining three samples (Algeria, San Remo, Terrasini) were excluded from these analyses because only pereiopods were sent to the laboratory, making sex determination impossible. For all the analyses, males and females were pooled into two different groups (group M and group F, respectively). Table 5.V shows the F_{IS}, F_{ST}, *r*, *c*, and *vAIc* values for males and females.

All measurements but F_{ST} point towards a female-biased dispersal: F_{IS} and *vAIc* values were higher in F than in M, *r* and *mAIc* values were negative in F but positive in M. However, only the Mann-Whitney U test results were significant: the distribution of *AIc* values for M and F (figure 5.3a, b) were statistically different (Z = 2.54, one-tailed P = 0.0056). The relative power of these indices to detect a sex bias in the dispersal depends on the magnitude of the bias, dispersal rates, sampling scheme, and markers examined (Goudet *et al*., 2002). When the gene flow is quite high, individual-based assignment tests are expected to be more powerful than summary statistics (i.e., F_{IS} and F_{ST}, which represent a population average); in fact, individual-based assignment tests provide a qualitative and quantitative idea of the scale of dispersal because they do not average over the sample.

Tab. 5.VI Deviation from Hardy–Weinberg expectation (F_{IS}), fixation index (F_{ST}), relatedness (*r*), variance assignment (*vAIc*), and mean assignment (*mAIc*) values estimated separately for male (M) and female (F) *A. antennatus* specimens.

	F_{IS}	F_{ST}	*r*	*vAIc*	$mAIc^1$	$mAIc^2$
Females	0.19	0.0052	-0.020	8.87	-0.299	-0.251
Males	0.15	-0.0013	0.003	7.28	0.304	0.330

([1] calculated with FSTAT, [2] calculated with GENECLASS)

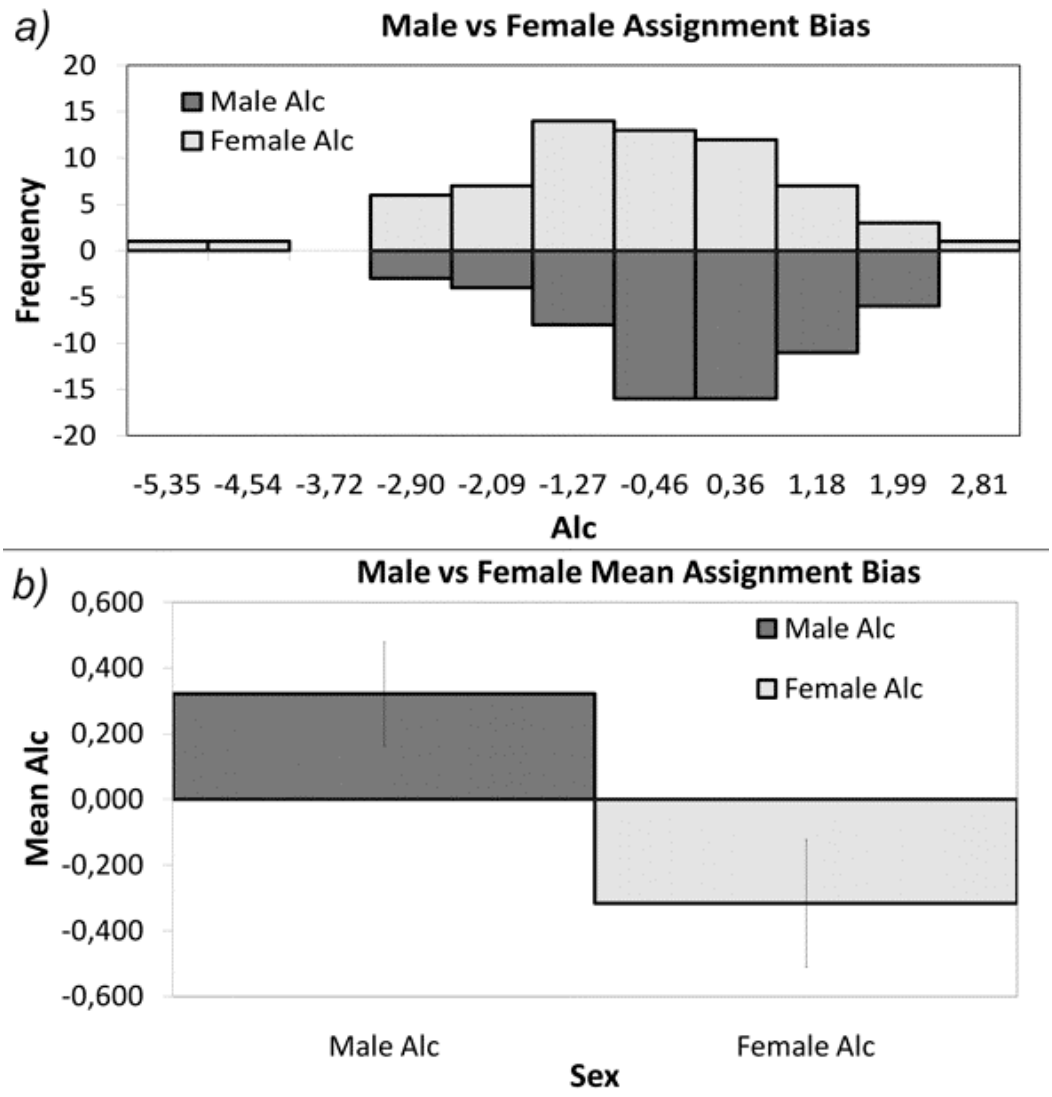


Fig. 5.3 Sex-specific *AIcs* (Assignment Indices corrected) for *Aristeus antennatus*. The calculated single values are shown in (a) and the mean values for the two sexes are shown in (b).

5.5 Comparing results with similar study on *Aristaeomorpha foliacea*

My research activity was carried out at the Centre of Competence in Marine Biodiversity, Com.Bio.Ma. (Cagliari) where it was possible for me to collaborate and compare my results with those obtained from another study of population structure and dynamics, conducted on a species that is very close to *Aristeus antennatus*, the giant red shrimp *Aristaeomopha foliacea*.

Population genetic structure was analyzed in *Aristaeomopha foliacea* with the same procedure followed for the blue and red shrimp (Appendix II). At a first stage we performed the isolation of microsatellite loci using the FIASCO protocol, followed by loci characterization and a survey on the population structure and dynamics of individuals from sampling areas overlapping those of *A. antennatus* (figure 5.4).

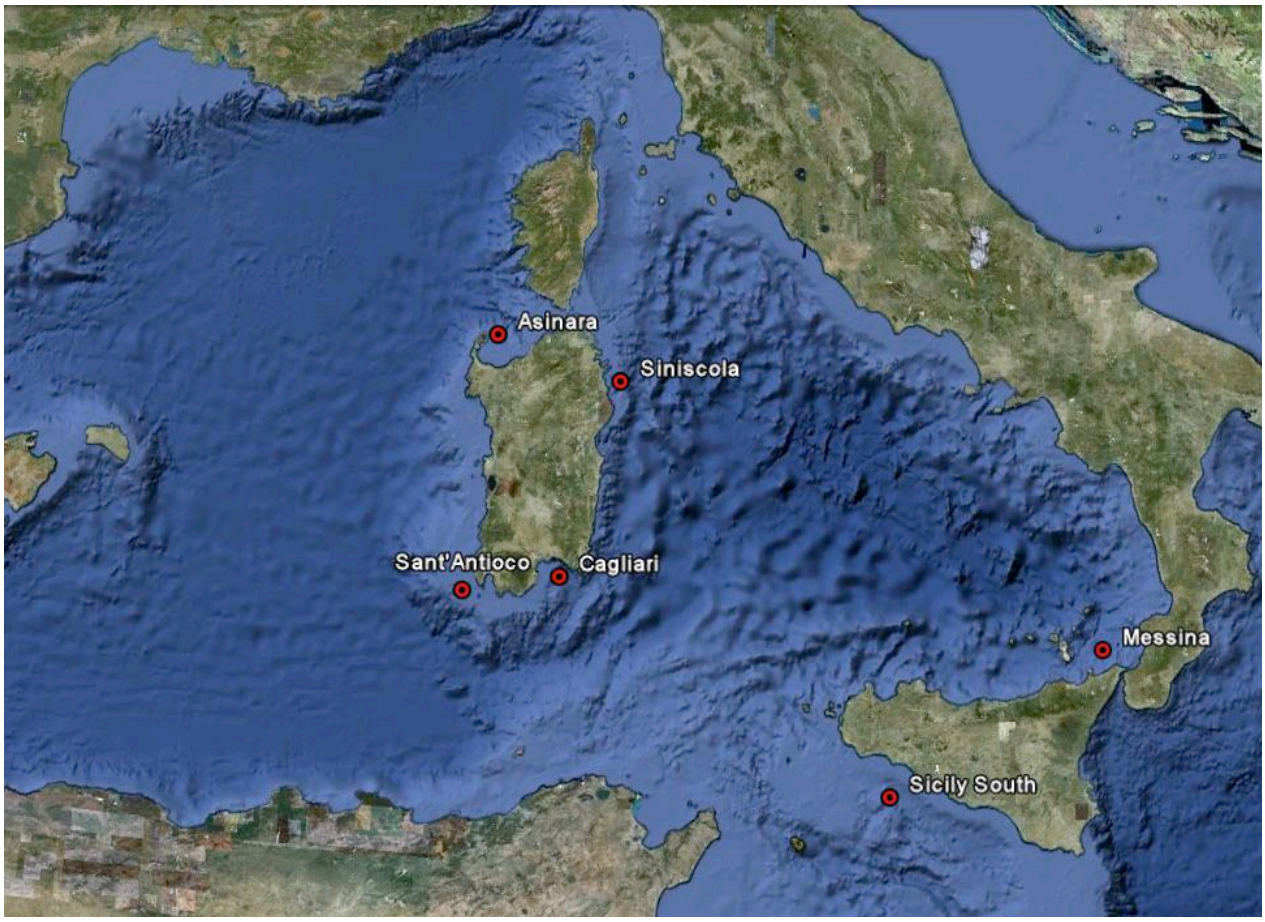


Fig. 5.4 Sampling sites for *A. foliacea* population structure analysis.

5.5.1 Isolation and characterization of microsatellite loci

In *Aristaeomopha foliacea*, from the genomic library enriched for AC repeat, 96 colonies (121 in *A. antennatus*) putatively containing microsatellite were selected and then sequenced. 43 sequences (44 in *A. antennatus*) comprised a repeat, but only 14 showed flanking regions adequate for primer design (18 in *A. antennatus*).

Once verified the functionality of the 14 primer pairs in amplification, loci were amplified for 30 individuals from two separate samples. Over 14 microsatellite loci, 4 loci showed technical difficulties in amplification (3 in *A. antennatus*) and a locus was found to be monomorphic (4 loci in *A. antennatus*). Moreover, in *Aristaeomopha foliacea*, 3 uninformative and poorly variable loci (2 loci in *A. antennatus*) were not considered for population structure analysis, reducing the number of valuable

microsatellite loci to 6. Descriptive statistic of the 6 loci chosen for further analyses are described in table 5.VII.

Tab. 5.VII Number of alleles (*Na*), *Allelic range*, Observed (*Ho*) and expected (*He*) heterozygosity, *P-value* for HW equilibrium are reported for each locus. ***, P <0.001.

Locus	*Na*	*Allelic range*	*Ho*	*He*	*P-value*
Cia 17	33	156-234	0.722	0.943	0***
Cia 19	22	158-212	0.922	0.883	0.1698
Cia 67	10	148-170	0.472	0.521	0.1574
Cia 82	32	187-295	0.444	0.814	0***
Cia 253	5	179-205	0.389	0.428	0.2556
Cia 257	17	171-209	0.806	0.845	0.066

Success in microsatellite isolation for *Aristaeomorpha foliacea* was similar, in terms of percentage of sequences including microsatellite over all sequenced colonies, and of useful loci over all tested primers pairs, to values obtained in *Aristeus antennatus*.

On the whole, in fact, in aristeid shrimps, 1 informative microsatellite locus for population structure studies can be obtained through the sequencing of at least 15-16 white colonies (6 useful loci over 96 colonies in *Aristaeomopha foliacea* and 8 useful loci over 121 colonies in *Aristeus antennatus*).

5.5.2 Genetic population structure

Four populations of the giant red shrimp were sampled off the Sardinian coast (Sant'Antioco, Cagliari, Siniscola and Asinara) and two off Sicily (Messina and Sicily Strait). Overall, a total of 115 individuals were analyzed for the 6 microsatellite loci.

Genetic variability

The software *GeneAlex* allowed us to test the polymorphism of each locus Results indicate that all loci are polymorphic, with the highest degree of polymorphism shown by Cia 17 and Cia 82. For Cia 17, 33 allelic variants were retrieved, their number ranging from 13 in the Messina population to 20 alleles for Asinara and South Sicily samples.

Cia 82 showed a total of 32 alleles distributed from a minimum of 9 alleles (Cagliari) to a maximum of 17 alleles (Sant'Antioco). The less polymorphic loci were found to be Cia 253 and Cia 67 with 5 and 10 alleles, respectively. The highest number of private alleles was scored in south Sicily and Asinara populations with 9 and 8 alleles, respectively, whereas the population with the lowest number was found to be Messina with only 5 private alleles.

Number of alleles per sampling site (2-19) and *PIC* values (0.36-0.91) were very similar to those found in the red and blue shrimp.

Population structure

Populations do not appear genetically differentiated, as evidenced by the low and non-significant pairwise Fst values and by AMOVA analyses (table 5.VIII). AMOVA results showed that genetic variability is mainly due to differences between individuals (differences between individuals component was 99, 31%, a percentage almost identical to the one scored in *A. antennatus*,i.e. 99.33%), rather than to differences between sampling sites, 0.69% (0.67% in *A. antennatus*).

Tab. 5.VIII Results from analysis of molecular variance (AMOVA)

Source of Variation	***d.f.***	***Sum of Squares***	***Variance components***		***Percentage of Variation***	***Fixation Indices***
Among sampling sites	5	14.044	0.01541	Va	0.69	F_{ST}=0.00690*
Within sampling sites	224	497.108	2.21923	Vb	99.31	
Total	229	511.152	2.234923			

* P-value = 0.19208+-0.00373 (100,100 permutations)

In line with these results, Bayesian analysis identified the most likely grouping configuration in K=1 confirming that all individuals belong to one population, as seen in *A. antennatus*.

The PCA analysis (figure 5.5) performed with *Adegenet* (Jombart T. 2008) graphically shows a substantial genetic homogeneity even among groups of individuals hundreds of kilometers apart, suggesting that individuals of giant red shrimp in the western Mediterranean represents a single panmitic stock.

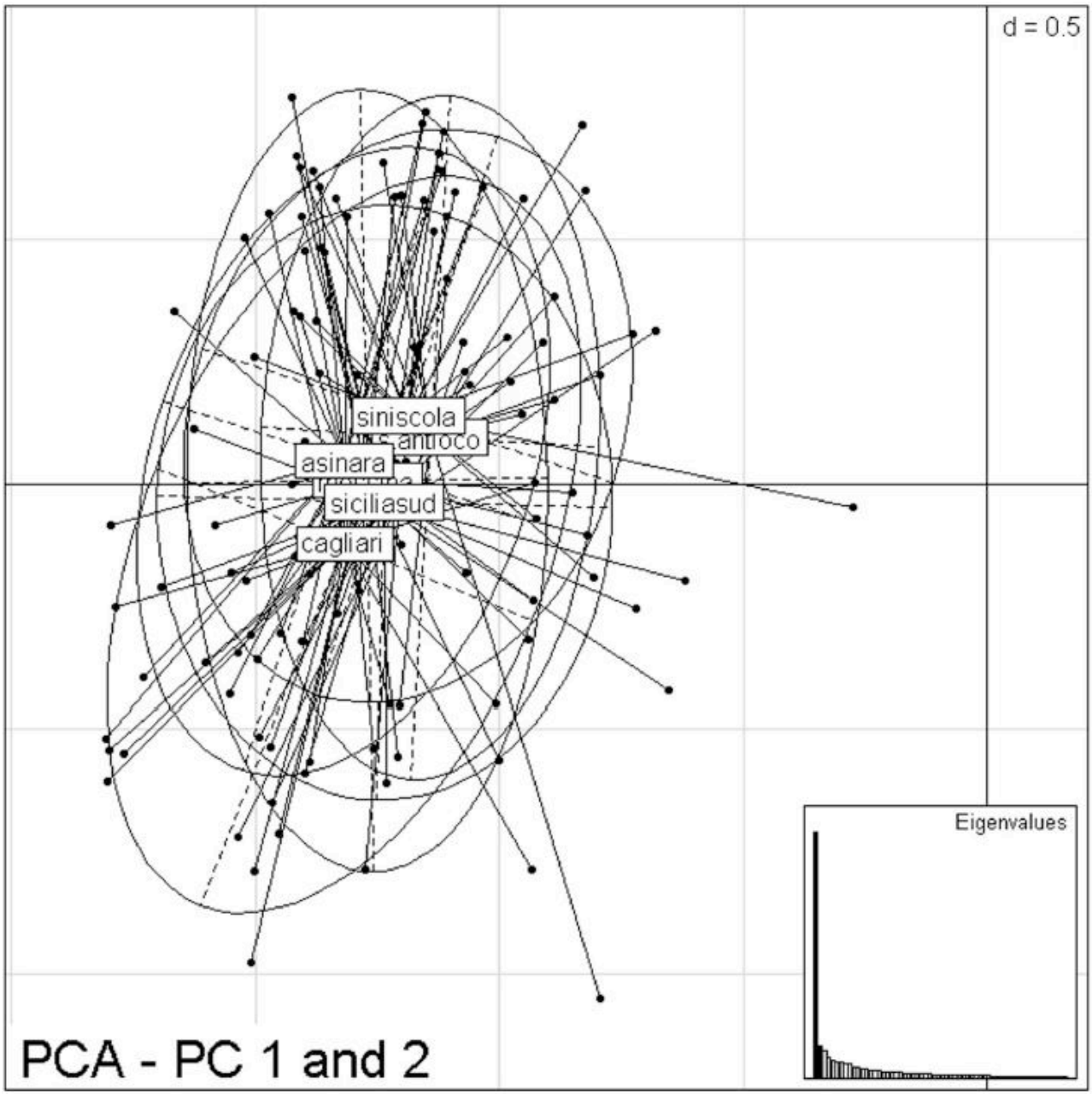


Fig. 5.5 PCA analysis, performed with *Adegenet*, of *A. foliacea* samples. Black dots represent individuals, while ovals represent sampling location means.

Finally, the bottleneck and interlocus g test revealed no signs of recent demographic reductions or increases in *A. foliacea*. These results are consistent with those reported for *A. antennatus* in this study.

5.5.3 Sex-biased dipersal

The giant red shrimp *Aristaeomopha foliacea* does not have an imbalanced sex ratio as a function of depth, as it happens in *Aristeus antennatus*. Both sexes, in fact, maintain a balanced ratio throughout the water column. This aspect was analyzed also under the genetic aspect through an analysis of sex-biased dispersal.

Figure 5.6 shows the distribution of corrected assignment indices (*AIc*), calculated for *A. foliacea*. As evidenced by the graph the two distributions are not significantly different (Z = 0.119, P-value = 0.905) and dispersal mediated by one sex it is not assumed. Under this aspect the two species clearly differ under dispersive strategies, nevertheless, producing the same homogeneous structure.

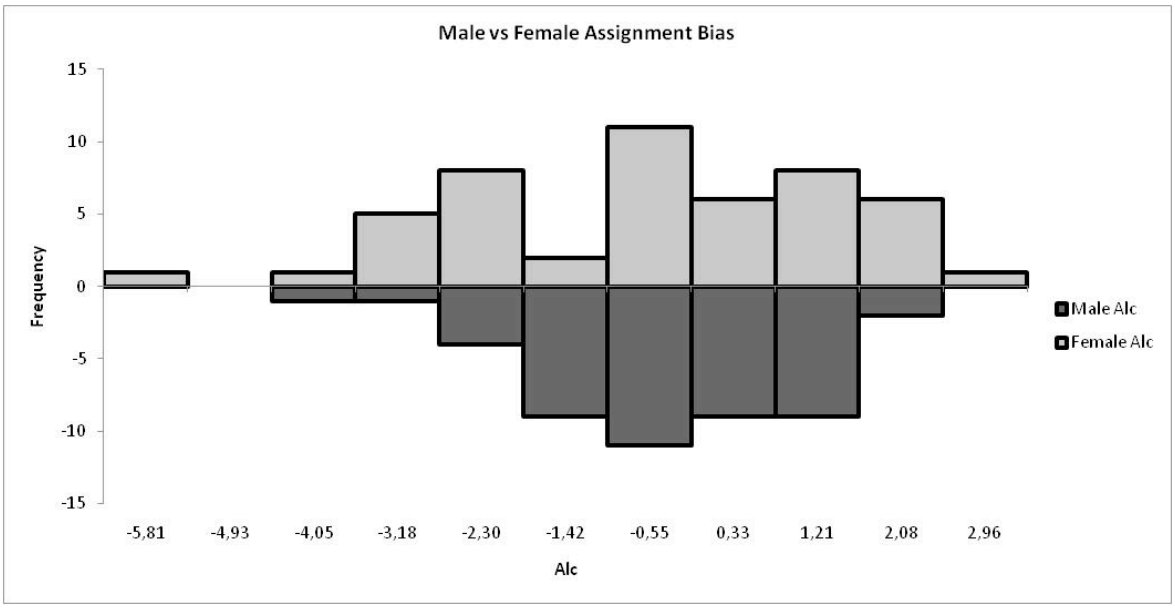


Fig. 5.6 Sex-specific *AIcs* (Assignment Indices corrected) distribution for *A. foliacea*.

6 Discussion

6.1 Isolation of nuclear microsatellite markers

To date, little is known of *A. antennatus* population structure and genetic diversity. To elucidate the amount of genetic variation among blue and red shrimp populations, we isolated microsatellite loci. The fast isolation by amplified fragment length polymorphism (*AFLP*) of sequences containing repeats (*FIASCO*) protocol (Zane *et al*. 2002) was used to isolate microsatellites from this species.

The success of isolation can be determined as the percentage of informative genetic markers obtained in time and effort. In this study, 8 polymorphic microsatellite loci resulted from a screening of 121 cloning colonies. This resulted in a number of approximately 15 sequenced colonies to obtain 1 informative and valuable marker.

Our results were in line with many other studies published at the moment the thesis project outline was assessed, demonstrating effectiveness of the applied method. Nowadays, other and more efficient protocols have been developed. Above all, Leese *et al* (end 2008) developed a new "reporter genome protocol (*RGP*)" that utilizes naturally occurring repeat motifs in genomes of distantly related organisms as hybridization probes. This method was proved to be very successful in all 13 enrichment reactions from eight marine target species (comprising a dinoflagellate, a diatom, and six arthropod species), yielding on average 85.5% positive colonies. This success rate places the *RGP* method above *FIASCO*, that was nevertheless the best choice at the time for isolation of microsatellite loci in *A. antennatus* revealing a satisfactory success rate of 36,4% (44 sequences containing repeats over 121 colonies).

6.2 Polymorphism and genetic indices of isolated loci

The availability of informative markers for genetic analyses in Penaeoid species (which includes aristeid shrimps) has been demonstrated to be problematic because it

is hard to isolate microsatellite loci due to the occurrence of large and complex repeat arrays and insufficient flanking sequences, which make primer design very difficult (Ball *et al*., 1998; Tassanakajon *et al*., 1998; Xu *et al*., 1999). Nevertheless, all eight loci used in this study showed high polymorphism; the *PIC* value per locus ranged from 0.65 to 0.86, which was much greater than the standard value of 0.5 (Botstein *et al*., 1980). This result indicates that these loci were suitable for population analyses.

6.3 Population genetics

Genetic diversity

The genetic diversity of *A. antennatus* populations was high, as shown by both *Na* (mean 13.5 alleles per locus) and mean *Ho* (over all loci, 0.65, which was less than the 0.80 mean value for *UHe*). These diversity parameters were in the range of those reported for other Penaeoid species analyzed with microsatellites (Brooker *et al*., 2000; Supungul *et al*., 2000; Xu *et al*., 2001; Maggioni *et al*., 2003; Ward *et al*., 2006; Borrell *et al*., 2007; Li *et al*., 2007; Robainas-Barcia *et al*., 2008; You *et al*., 2008; Meng *et al*., 2009). The average heterozygosity levels of microsatellite data for *A. antennatus* were about 10 times higher than those obtained in previous studies based on allozyme data where *He* ranged from 0.077 to 0.095 in *A. antennatus* populations from Sardinia and Sicily (Marchi *et al*., 1995), and even lower values (0.043–0.066) were found in *A. antennatus* from several localities in the Atlantic and western Mediterranean (Sardà *et al*., 1998). Similar differences in variability between genetic markers were obtained also for *P.monodon* (Brooker *et al*., 2000; Xu *et al*., 2001), *L. schmitti* (Maggioni *et al*., 2003) and in most of the works on the genetic diversity of penaeid prawns reviewed by Benzie (2000) who concluded that estimates of variability from DNA-based markers showed much higher levels of diversity in natural populations than those inferred from allozymes.

The high-level polymorphism observed in our study confirmed that microsatellites are suitable markers to analyze the genetic variation of *A. antennatus* populations.

HWE Equilibrium

We detected significant deviations from *HWE* in the present study, (table 5.II), and heterozygote deficiencies were always responsible for these deviations. Similar deficits have been widely observed and are regarded as quite common in shrimp species (Li *et al*., 2007; Robainas-Barcia *et al*., 2008; You *et al*., 2008; Meng *et al*., 2009). Causes of deviation include scoring errors, inbreeding, selection, population admixture, and null alleles. Stutter-related scoring errors were absent in our analysis because we were able to score the alleles of the eight microsatellites unambiguously. Inbreeding likely does not explain the observed heterozygote deficiency because our samples came from wild populations that are supposed to be outbred. Furthermore, when F_{IS} was calculated, it was significantly positive in only a few cases (mostly in locus Ant93). Microsatellites are supposed to be neutral markers, which means that selection should not be involved in the heterozygote deficiency. In fact, the Ewens-Watterson neutrality test confirmed that all loci were selectively neutral. The Wahlund effect, which describes heterozygote deficiency caused by subpopulation structure, is not plausible in our case because genetically different sources of individuals were not confirmed by the analyses. Therefore, arbitrary admixture of individuals does not explain the observed heterozygote deficiency. The presence of null alleles remains the most plausible reason for the departures from *HWE* in all populations. The analysis of *HWE* assuming the presence of null alleles (genotypes corrected with the INA method) showed that all sampling localities were in equilibrium. Finally, throughout the *HWE* analyses, one locus (Ant93) was mainly responsible for almost all of the observed deviations, suggesting the possibility to exclude it in future studies.

Spatial and Temporal Variation

We found no significant differences in mean *Na*, *Ho*, *He*, and *Ra* among sampling sites. It is important to highlight the absence of differences between commercial stocks (samples from exploited grounds) and deeper stocks that are regarded by

several researchers as virgin stocks (such as the PSP samples not yet exploited by trawl fishing). Similarly, no differences were found between temporal samples. This result seems to indicate a high genetic diversity that was stable over the three years of analysis. However, three years are a very short period of time to draw final conclusions about genetic stability of *A. antennatus* populations; it will be important to extend genetic monitoring over longer periods. In fact, results of previous studies on prawns highlighted the importance of analyzing a large number of spatial and temporal samples. For example, in the pink prawn *Farfantepenaeus notialis*, significant changes over years have been detected, with very unstable and highly pronounced genetic structure (Robainas-Barcia *et al*., 2005; Robainas-Barcia *et al.*, 2008). The *F. notialis* population structure has been altered by environmental factors. In particular, hurricanes (causing significant changes in marine current patterns and hence in larval dispersal and recruitment) determined the occurrence genetically different populations, then substituted by a single panmictic population, from one year to the other.

In organisms such as marine shrimps that are characterized by an high fecundity and a significant early mortality, another possible cause for changes in allele frequencies is the high variance in reproductive success. This variance combined with a rapidly increasing fishing pressure may contribute to a reduction in effective population sizes and thus lead to measurable fluctuations in gene frequencies over either geographical or temporal scales (Ball and Chapman, 2003). Populations of the white shrimp *Litopenaeus setiferus* sampled within a period of two years showed significant temporal changes in allele frequencies (Ball and Chapman, 2003). The authors suggested that these populations could have experienced great demographic fluctuations caused by different factors that probably affected the temporal stability of allele frequencies. In *A. antennatus*, marked interannual fluctuations in abundances and yields are known to exist and have been attributed to environmental factors that promote variations in mobility patterns (see paragraph 2.8. Evaluation and exploitation). Therefore, it will be important to extend the genetic analyses of this

species over several years to determine if the year-to-year differences in abundances could alter the genetic structure of populations.

Hierarchical analysis of molecular variance

The AMOVA analyses revealed a substantial lack of genetic structure, that is no significant differentation among sampling locations. No spatial structure was found even though the samples were collected from sites that are hundreds of kilometers apart. These results were in perfect agreement with recent studies of *A. antennatus* that utilized mtDNA sequence analyses in the western Mediterranean (Maggio *et al*., 2009; Roldán *et al*., 2009). The AMOVA analysis showed that most of the observed genetic variation was attributable to interindividual differences (99.33%). Similarly, intrasample differences calculated from mtDNA data ranged from 96.5% (Roldán *et al*., 2009) to 98.3% (Maggio *et al*., 2009). Recently, high levels of genetic homogeneity were detected among samples from different depths of *A. antennatus* from the Catalan Sea (Sardà *et al*., 2010). All these results are consistent with the biology of this species that uses both a larval dispersal strategy and an adult migration behavior to widely disperse individuals and genetic information. Moreover, this result is not surprising because in shrimps, genetic differentiation is thought to be progressively less appreciable in species whose life cycles provide greater opportunities for the mixing of populations (Mulley and Latter, 1980). Following Dall *et al*.'s (1990) life-history classification of Penaeoid shrimps, *A. antennatus* is characterized by a type 4 life history, which is typical of species that spend all their life in offshore regions with great potential opportunities for mixing.

Reduction and growth of populations

Bottleneck and interlocus g tests failed to detect patterns consistent with variations in size in the contemporary gene pools. Both of these analyses indicated that today *A. antenn*atus populations are stable. However, lnβ was always > 1, which indicates that before the population reached its contemporary size there was likely a previous reduction in size. This last result is consistent with mtDNA data from previous

studies of *A. antennatus* in which mismatch analyses indicated a sudden population expansion after a bottleneck event that could have occurred about 2,000 years ago (Maggio *et al*., 2009; Roldán *et al*, 2009).

The discrepancy between lnβ and the bottleneck test is not new; Bos *et al*. (2008) stated that similar results may be explained by the severity and the age of the bottleneck and/or the stability of the molecular signal (King *et al*., 2000; Garza and Williamson, 2001). In fact, while the lnβ metric is known for its ability and power to detect historic signals of expansion (King *et al*., 2000), the bottleneck test is suited for the detection of recent reductions in size (leading to shifts from *HWE*), but it is insensitive to earlier events (Cornuet and Luikart, 1996). The bottleneck test is known to be sensitive to gene flow, which rapidly degrades the signal of past bottlenecks (Busch et al., 2007).

6.4 Sex-biased Dispersal

In the present study, samples caught at very different depths were analyzed. It is plausible that individuals that live at different depths are subject to different marine currents and environmental conditions that influence their dispersal capabilities. Furthermore, these populations have a different composition of males and females: *A. antennatus* males are more prevalent at depths > 800 m while females are usually found in shallower waters. This peculiar depth distribution suggests that males and females might encounter different systems of marine currents (Guijarro *et al*., 2008). Adult *A. antennatus* females seem to be strictly associated with the warm and saline Levantine Intermediate Waters, whereas adult males are more correlated with the cold Western Mediterranean Deep Waters (Guijarro *et al*., 2008). Thus, Guijarro *et al*. hypothesized two different mobility patterns for the different sexes in relation to the observed differences. The important role of water masses for *A. antennatus,* distribution, and in particular the deep waters that influence male distribution, was also discussed by Ghidalia and Bourgois (1961), Bombace (1975), Relini and Orsi Relini (1987), and Demestre and Martín (1993).

For marine species that are particularly vagile, estimating dispersal by direct observations is not feasible. However, genetic methods can be used to provide insights on how dispersal translates into effective dispersal and gene flow. Differences in the sex dispersal can be indirectly tested using microsatellite data, that permit only detection of short-term (instantaneous) dispersal because the signal disappears after the dispersing individuals mate due to Mendelian segregation of biparental markers (Goudet *et al*., 2002). In the dispersing sex, individuals sampled from one single patch will be a combination of residents and immigrants, hence a heterozygote deficit (a higher *Fis*) is expected due to the Wahlund effect (Goudet *et al*., 2002). Furthermore, a relatively higher frequency of rarer genotypes is expected, which is indicated by a negative *mAIc* value (Paetkau *et al*., 1995; Prugnolle and de Meeus, 2002) and a larger *vAIc* value (Favre *et al*., 1997). For the same reason, dispersal leads to lower relatedness (*r*) and interpopulation differentiation (*Fst*) (Goudet *et al*., 2002; Prugnolle and de Meeus, 2002).

In our study, four measurements indicated female-biased dispersal: *Fis* and *vAIc* values were higher in F than in M, and *r* and *mAIc* values were negative in F but positive in M. Therefore, we hypothesize that *A. antennatus* females can more easily disperse, thanks to the Levantine Intermediate Waters, compared to males, which are tied to the more stable and weak Western Mediterranean Deep Water Circulation and hence contribute less to the mixing of red and blue shrimp populations. The mechanisms that influence the genetic flow and structure of *A. antennatus* are fundamental to understand its biology, and such an understanding ultimately will lead to sustainable management of this precious resource.

6.5 Comparing results with similar study on *Aristaeomorpha foliacea*

Results from the genetic survey on *Aristeus antennatus* showed a complete absence of population structure, in line with the biological characteristics of the species. These results are in line with those obtained in *Aristaeomorpha foliacea* emphasizing

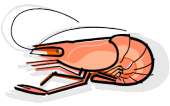
the relationship between high dispersive capacity, both at larval and adult stage, and a complete genetic mixing in aristeid shrimps species from the western Mediterranean.

Moreover, for *Aristeus antennatus*, it was determined that sexes contribute differently to gene flow. The species in fact has a sex ratio unbalanced towards males under 800 m depth, while this ratio is unbalanced towards females in the upper layers, where they are likely to be subject to more dispersive oceanographic conditions.

Through genetic analyses, using microsatellite loci, it has been possible to assess indirectly the degree of dispersion of sexes. The results from *Aristeus antennatus* showed that the analyzed males were represented by more sedentary individuals, while females mostly by migrants. Therefore it can be argued that females disperse more, thus contributing to a stronger gene flow, compared to males.

In *Aristaeomorpha foliacea*, the two sexes do not show different sex-ratio along the water column. In fact, in this case there is no evidence of sex-mediated genetic dispersal by performing the same analysis conducted in *Aristeus antennatus*.

In conclusion, despite the studies of population structure in the two species have led to almost identical conclusions, both qualitatively and quantitatively, it is interesting to note that this result is achieved with very different population dynamics between the two species.



7 Conclusions and Future studies

Our genetic assessment of red and blue shrimps in the western Mediterranean Sea provides a baseline from which *A. antennatus* populations can be appraised in the future. The high level of genetic diversity in this species may help to buffer it from the detrimental genetic effects of increasing fishing pressure. In theory, large population sizes and high fecundities tend to make *A. antennatus* scarcely susceptible to population collapse. However, a very high rate of exploitation (higher than the present one) could exacerbate the magnitude of natural population fluctuations and thereby reduce the effective population size over time, with subsequent detrimental genetic effects. Genetic monitoring could help to promptly identify negative effects on genetic diversity caused by fisheries and to provide data for developing sound management and conservation measures.

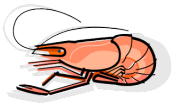
The present genetic survey on aristeid shrimps was conducted with the use of microsatellite markers due to their important features that make this genetic markers an optimal instrument for population structure and dynamics investigation purposes.

Nevertheless, recent studies show that the variation in adaptive traits can reveal finer population structures and dynamics than neutral markers, such as microsatellites. In fact, the variability of adaptive traits (also known as "quantitative" because they are linked to the phenotype), *Qst*, is almost always larger than the variability of neutral markers, *Fst* (Conover *et al*., 2006).

For this reason I decided to invest my foreign training period, included in the PhD program, at the Institute for Clinical and Molecular Biology (Christian Albrechts University, Kiel, Germany) where, from 1st November 2009 to 31st January 2010 I did a practical term in the working group of “Marine medicine” of Prof. Philip Rosenstiel and Dr. Eva Philipp. The aim of the term was to get an introduction in high-throughoutput sequencing technology and data handling. I learned how to assemble large sequence data sets using different bioinformatic tools. I also got a first

experience in the analysis of this kind of data searching for genes of interest within a in-house sequence database as well as in databases from the NCBI. From the identified sequences, primers were designed and first products were gained by PCR. I further got some introduction in gene expression techniques (semi-quantitative PCR, qPCR) and gene sequence characterization (RACE).

All thes~~e~~ methodologies learned aimed to enable me to develop adaptative and more efficient molecular markers in the future. These new markers, implemented in new-coming analysis of genetic population structure and dynamics in aristeid shrimps, could allow to resolve many interesting aspect on the ecology of such important and cryptic species, from a brand new point of view.



8 References

AA.VV. (1990). Risorse demersali Tirreniche. Distribuzione dei rendimenti di cattura di *Merluccius merluccius Aristeu antennatus e Aristaeomorpha foliacea. Oebalia* **16** (2): 791-797

Arculeo M., Baino R., Abella A. and Riggio S. (1994) Distribution and growth of *aristeus antennatus* in Southern Tyrrenean Sea. *NTR-ITPP, Special Publication*.

Ardizzone G.D., Belluscio A., Schintu P. (1994) Considerazioni sullo stato di sfruttamneto delle risorse demersali (Isola di Giannutri – Foce di Garigliano). *Biol. Mar. Medit.*, **1,** 43-46.

Ball A. O., Leonard S. and Chapman R. W. (1998). Characterization of (GT)(n) microsatellites from native white shrimp (Penaeus setiferus). *Molecular Ecology* **7**, 1251-1253.

Ball A. O. and Chapman R. W. (2003). Population genetic analysis of white shrimp, *Litopenaeus setiferus*, using microsatellite genetic markers. *Molecular Ecology* **12**, 2319-2330.

Beghi L. (1992) Metodi matematici e statistici per la biologia. Edizioni Libreria Progetto, Padova.

Bekkevold D., Hansen M.M. and Mensberg K.-L.D. (2004). Genetic detection of sex-specific dispersal in historical and contemporary populations of anadromous brown trout *Salmo trutta*. *Molecular Ecology* **13**, 1707-1712.

Benham C. (2001) From DNA structure to gene expression: mediators of nuclear compartmentalization and dynamic. *Chromosome Researches* **11**, 5/July, 2003 435-445

Benzie J.A.H. (2000) Population genetic structure in penaeid prawns. *Aquaculture Research* **31**, 1: 95–119.

Berry O., Tocher M.D. and Sarre S. D. (2004). Can assignment tests measure dispersal? *Molecular Ecology* **13**, 551-561.

Bianchini M.L. and Ragonese S. (1994) Life cycles and fisheries of the deep-water red shrimps *Aristaeomorpha foliacea* and *Aristeus antennatus*. *Proc. Int. Workshop N.T.R.-I.T.P.P.*, 3.

Bilgin R. (2007). Kgtests: a simple Excel Macro program to detect signatures of population expansion using microsatellites. *Molecular Ecology Notes* **7**, 416-417.

Bombace G. (1975). Considerazioni sulla distribuzione delle popolazioni di livello batiale con particolare riferimento a quelle bentoniche. *Pubblicazioni della Stazione Zoologica di Napoli* **39 (Suppl. 1)**, 7-21.

Bos D.H., Gopurenko D., Williams R.N., and DeWoody J.A. (2008) Inferring population history and demography using microsatellites, mitochondrial DNA, and Major Histocompatibility Complex (MHC) genes. *Evolution* **62(6)**: 1458–1468.

Borrell Y.J., Arenal F., Mbemba Z. M., Santana O., Díaz-Férnandez R., Vázquez E., Blanco G., Sánchez J. A. and Espinosa G. (2007). Spatial and temporal genetic analysis of the Cuban white shrimp *Penaeus (Litopenaeus) schmitti*. *Aquaculture* **272**, S125-S138.

Botstein D., White R. L., Skolnick M. and Davis R. W. (1980). Construction of a genetic linkage in man using restriction fragment length polymorphism. *The American Journal of Human Genetics* **32**, 314-331.

Brian A. (1931) La biologia del fondo "a scampi" del Mar Ligure: *Aristaeomorpha, Aristeus* e altri macruri natanti. *Boll. Mus. Zool. e Anat. Comp. Univ. Genova,* **11**, 1-6.

Brooke H.E. (1999). The stock concept revisited: perspectives on its history in fisheries. *Fisheries Research*, **43**(1): 9-11.

Brooker A. L., Benzie J.A.H., Blair D. and Versini J.J. (2000). Population structure of the giant tiger prawn *Penaeus monodon* in Australian waters, determined using microsatellite markers. *Marine Biology* **136**, 149-157.

Busch J.D., Waser P.M. and DeWoody J.A. (2007). Recent demographic bottlenecks are not accompanied by a genetic signature in banner-tailed kangaroo rats (*Dipodomys spectabilis*). *Molecular Ecology* **16**, 2450-2462.

Caddy J.F. (1993). Some future perspectives for assesment and management of Mediterranean fisheries. *Sci. Mar.* **57:** 121-130.

Campillo A. (1994) Bioecology of *Aristeus antennatus* in the French Mediterranean. *N.T.R.-I.T.P.P.* **3**: 25-26

Carbonell A., Dos Santos A., Alemany F. and Vélez-Belchi P. (2010). Larvae of the red shrimp *Aristeus antennatus* (Decapoda: Dendrobranchiata: Aristeidae) in the Balearic Sea: new occurrences fifty years later. *Marine Biodiversity Records* **3**, e-103.

Carbonell A., Lloret J. and Demestre M. (2008). Relationship between condition and recruitment success of red shrimp (*Aristeus antennatus*) in the Balearic Sea (Northwestern Mediterranean). *Journal of Marine Systems* **71**, 403-412.

Cartés J.E. and Sardà F. (1989). Feeding ecology of the deep-water aristeid crustacean *Aristeus antennatus*. *Mar. Ecol. Prog. Ser.* **54**: 229-238

Cartés J.E., Papiol V. and Guijarro B. (2008). The feeding and diet of the deep-sea shrimp *Aristeus antennatus* off the Balearic Islands (Western Mediterranean): Influence of environmental factors and relationship with the biological cycle. *Progress in Oceanography* **79**, 37-54.

Cartés J.E., Maynou F., Fanelli E., Papiol V. and Lloris D. (2009). Long-term changes in the composition and diversity of deep-slope megabenthos and trophic

webs off Catalonia (western Mediterranean): Are trends related to climatic oscillations? *Progress in Oceanography* **82**, 32-46.

Cartés J.E. and Sardà F. (1989), Feeding ecology of the deep-water aristeid crustacean *Aristeus antennatus*, *Mar. Ecol. Prog. Ser.,***54:** 229-238.

Carvajal-Rodriguez A., de Una-Alvarez J. and Rolan-Alvarez E. (2009). A new multitest correction (SGoF) that increases its statistical power when increasing the number of tests. *BMC Bioinformatics* **10**, 209.

Cau A., Carbonell A., Follesa M.C., Mannini A., Orsi Relini L., Politou C.-Y., Ragonese S., Rinelli P. (2002) MEDITS-based information on the deep-water red shrimps *Aristaeomorpha foliacea* and *Aristeus antennatus* (Crustacea: Decapoda: Aristidae). *SCI. MAR.,* **66** (Suppl. 2): 103-124.

Cau A., Sabatini A., Murenu M., Follesa MC, Cuccu D. (1994) Considerazioni sullo stato di sfruttamento delle risorse demersali (Mari di Sardegna) , *Biol. Mar. Medit.*, **1**(2): 66-76

Chapuis M.-P. and Estoup A. (2007). Microsatellite Null Alleles and Estimation of Population Differentiation. *Molecular Biology and Evolution* **24**, 621-631.

Colloca F., Gentiloni P., Agnesi S., Schintu P., Cardinale M., Belluscio A., Ardizzone G.D. (1998) Age and growth of the mediterranean hake (*Merluccius merluccius*) in the Central Mediterranean sea, *Fish otolith research and application,* 2nd International Symposium Bergen (Norway) 20-25 June 1998

Company J.B., Puig P., Sardà F., Palanques A., Latasa M. and Scharek R. (2008). Climate Influence on Deep Sea Populations. *Plos One* **3**.

Conover D.O., Clarke L.M., Munch S.B., Wagner G.N. (2006) Spatial and temporal scales of adaptive divergence in marine fishes and the implications for conservation. *Journal of Fish Biology* **69:** 21–47.

Cornuet, J.M. and Luikart, G. (1996). Description and Power Analysis of Two Tests for Detecting Recent Population Bottlenecks From Allele Frequency Data. *Genetics* **144**, 2001-2014.

Cowen R.K., Gawarkiewicz G., Pineda J., Thorrold S.R. and Werner F.E. (2007). Population connectivity in marine systems: an overview. *Oceanography* **20(3)**, 14-21.

Crow J.F. and Kimura M, (1970) An Introduction to Population Genetics Theory. *Harper and Row, New York***.**

Cruz F., Perez M. and Presa P. (2005). Distribution and abundance of microsatellites in the genome of bivalves. *Gene***, 346,** 241–247.

D'Onghia G., Matarrese A., Maiorano P. and Panza M. (1997) Commercial trawling, discars and recruitment from fishery of the North-Western Ionian Sea, *Biol. Mar. Medit.*, **4**(1): 291-297

Dall W., Hill B.J., Rothlisberg P.C. and Sharples, D.J. (1990). *Advances in Marine Biology: The Biology of the Penaeoidae*. Academic Press Limited.

De-Xing Zhang, Godfrey M. Hewitt (2003). Nuclear DNA analyses in genetic studies of populations: practice, problems and prospects. Invited review. *Molecular Ecolog,* **12**: 563-584.

Demestre M. and Fortuno J.-M. (1992) Reproduction of the deep-water shrimp *Aristeus antennatus* (Decapoda: Dendrobranchiata). *Mar. Ecol. Prog. Ser.* **84**: 41-51.

Demestre M. and Martín, P. (1993). Optimum exploitation of a demersal resource in the western Mediterranean: the fishery of the deep-water shrimp *Aristeus antennatus* (Risso, 1816). *Scientia Marina* **57**, 175-182.

Demestre M and Lleonard J (1993) Population dynamics of *Aristeus antennatus* (Decapoda: Dendrobranchiata). Mar Ecol Prog Ser 57(2–3):183–189

Demestre M. (1995) Moult activity-related spawning success in the Mediterranean deep-water shrimp *Aristeus antennatus* (Decapoda: Dendrobranchiata). *Mar. Ecol. Prog. Ser*. **127**: 57-64.

Dempster A.P., Laird N.M. and Rubin D.B. (1977). Maximum Likelihood from Incomplete Data via the EM Algorithm. *Journal of the Royal Statistical Society. Series B (Methodological)* **39**, 1-38.

Di Rienzo A, Peterson A C, Garza J C, Valdes A M, Slatkin M, and Freimer N B (1994). Mutational processes of simple-sequence repeat loci in human populations. *Proc. Nath. Acad. Sci U.S.A.* **91**: 3166-3170

Dos Santos A. (1998). On the occurrence of larvae of *Parapenaeus longirostris* (Crustacea: Decapoda: Penaeoidea) off the Portuguese coast. *Journal of Natural History* **32**, 1519-1523.

Eisen J.S. (1999) A case of mistaken identity. *Neuron* **23**(4):626-627 *Review*.

Ellegren (2004) Is There Evidence for Convergent Evolution Around Human Microsatellites?. *Mol Biol Evol.*2007; **0**: msm051v1-1

Excoffier, L., Laval, G. and Schneider, S. (2005). Arlequin (version 3.0): An integrated software package for population genetics data analysis. *Evolutionary bioinformatics online* **1**, 47-50.

Excoffier L., Smouse P.E. and Quattro J.M. (1992). Analysis of molecular variance inferred from metric distances among dna haplotypes: application to human mitochondrial DNA restriction data. *Genetics* **131**, 479-491.

FAO (1987), Mediterranée et Mer Noire, zone de peche **37,** Vol.1, 1987

Favre L., Balloux F., Goudet J. and Perrin N. (1997). Female-biased dispersal in the monogamous mammal *Crocidura russula*: evidence from field data and microsatellite patterns. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **264**, 127-132.

Fiorentino F., Massi, D., Zamboni, A. and Relini, G. (1995) Rendimenti dello strascico batiale del Mar Ligure Centro-Orientale in una serie pluriennale di osservazioni. *Biol. Mar. Medit.*, **1**(2): 191-197

Garza J.C. and Williamson E. G. (2001). Detection of reduction in population size using data from microsatellite loci. *Molecular Ecology* **10**, 305-318.

Gerber S., Mariette S., Streiff R., Bodenes C. and Kremer A. (2000). Comparison of microsatellites and amplified fragment length polymorphism markers for parentage analysis. *Mol. Ecol*., **9**, 1037–1048.

Ghidalia W. and Bourgois F. (1961). Influence of temperature and light on the distribution of shrimps in medium and great depths. *FAO General Fisheries Council for the Mediterranean Studies and Reviews* **16**, 1-49.

Glenn T.C. and Schable N.A. (2005) Isolating microsatellite DNA loci. In: Molecular Evolution: Producing the Biochemical Data, *Part B (eds Zimmer, E.A. and Roalson, E.). Academic Press*, San Diego, USA, pp. 202–222.

Goudet J., Perrin N. and Waser P. (2002). Tests for sex-biased dispersal using bi-parentally inherited genetic markers. *Molecular Ecology* **11**, 1103-1114.

Guijarro B., Massuti E., Moranta J. and Diaz P. (2008). Population dynamics of the red shrimp *Aristeus antennatus* in the Balearic Islands (western Mediterranean): Short spatio-ternporal differences and influence of environmental factors. *Journal of Marine Systems* **71**, 385-402.

Guo S., Thompson E. (1992). Performing the exact test of Hardy Weinberg proportion for multiple alleles. *Biometrics*, **48**: 361-372.

Hedgecock D., Launey S., Pudovkin A., Naciri Y., Lapègue S. and Bonhomme F. (2007). Small effective number of parents (*Nb*) inferred for a naturally spawned cohort of juvenile European flat oysters *Ostrea edulis*. *Marine Biology* **150**, 1173-1182.

Heldt J.H. (1954). Stades larvaires d'*Aristeomorpha foliacea* (Risso) et *Aristeus antennatus* (Risso). Décapodes Penéides. *Comptes Rendus Académie de Sciences* **239**, 1080-1082.

Heldt J.H. (1955). Contribution à l'étude de la biologie des crevettes pénéides *Aristaeomorpha foliacea* (Risso) et *Aristeus antennatus* (Risso) (Formes larvaires). *Bulletin de la Société des Sciences Naturelles de Tunisie* **VIII 1-2**, 1-29.

Jombart T. (2008). Adegenet: a R package for the multivariate analysis of genetic markers. *Bioinformatics* 24: 1403-1405.

Kalinowski, S.T. (2002). How many alleles per locus should be used to estimate genetic distances? *Heredity***, 88**, 62–65.

Kapiris K. (2004). Biology and fishery of *Aristaeomorpha foliacea* (Risso, 1827) and *Aristeus antennatus* (Risso 1816) (Decapoda: Dendrobranchiata). Ph.D. Dissertation, University of Athens, Athens, Greece.

Kimmel M., Chakraborty R., King J.P., Bamshad M., Watkins W.S. and Jorde L.B. (1998). Signatures of population expansion in microsatellite repeat data. *Genetics* 148, 1921-1930.

Kimura M., Crow J. (1964) The number of alleles that can be maintained in a finite population. *Genetics* 1964.

Kimura M. and Ohta T. (1978) Stepwise mutation model and distribution of allelic frequencies in a finite population. *PNAS*. **75**/6: 2868-2872

King J.P., Kimmel M. and Chakraborty R. (2000). A Power Analysis of Microsatellite-Based Statistics for Inferring Past Population Growth. *Molecular Biology and Evolution* **17**, 1859-1868.

Lagardère J.P. (1972) Recherches sur l'alimentation des crevettes de la pente continentale marocaine. *Tethys*, **3** (3): 655-675

Lawson Handley L.J. and Perrin N. (2007). Advances in our understanding of mammalian sex-biased dispersal. *Molecular Ecology* **16**, 1559-1578.

Leberg P.L. (2002). Estimating allelic richness: Effects of sample size and bottlenecks. *Molecular Ecology* **11**, 2445-2449.

Leese F., Mayer C. and HeldC. (2008) Isolation of microsatellites from unknown genomes using known genomes as enrichment templates. *Limnol. Oceanogr.: Methods* **6**, 2008, 412–426

Li C.M, Campbell S.J., Kuraratne D.S., Bellamy R., Ruwende C., McAdam K.P.W.J., Hill A.V.S. and Lammas D.A. (2002) Association of a polymorphism in the P2X7 gene with tuberculosis in a Gambian population**.** *JSTOR*.

Li Y., Wongprasert K., Shekhar M., Ryan J., Dierens L., Meadows J., Preston N., Coman G. and Lyons R. E. (2007). Development of two microsatellite multiplex systems for black tiger shrimp *Penaeus monodon* and its application in genetic diversity study for two populations. *Aquaculture* **266**, 279-288.

Luikart G., Allendorf F., Cornuet J.-M. and Sherwin W. (1998). Distortion of allele frequency distributions provides a test for recent population bottlenecks. *Journal of Heredity* **89**, 238-247.

Maggio T., Lo Brutto S., Cannas R., Deiana A.M. and Arculeo M. (2009). Environmental features of deep-sea habitats linked to the genetic population structure of a crustacean species in the Mediterranean Sea. *Marine Ecology-an Evolutionary Perspective* **30**, 354-365.

Maggioni R., Rogers A.D. and Maclean N. (2003). Population structure of *Litopenaeus schmitti* (Decapoda: Penaeoidae) from the Brazilian coast identified using six polymorphic microsatellite loci. *Molecular Ecology* **12**, 3213-3217.

Manly B.F.J. (1985). *The statistics of natural selection on animal populations*: Chapman and Hall, London.

Marchi A.L.(1995).Genetic variation in *Aristeus antennatus* (Crustacea: Aristidae) in the Mediterranean basin: analysis of 16 enzyme loci. *Biol. Mer. Medit.,* **2** (2): 495-498.

Massuti E., Monserrat S., Oliver P., Moranta J., Lopez-Jurado J.L., Marcos M., Hidalgo M., Guijarro B., Carbonell A. and Pereda P. (2008). The influence of oceanographic scenarios on the population dynamics of demersal resources in the western Mediterranean: Hypothesis for hake and red shrimp off Balearic Islands. *Journal of Marine Systems* **71**, 421-438.

Matarrese A., D'Onghia G., Tursi A. (1992); Struttura e dinamica dello stock di *Aristeus antennatus* (Risso, 1816) (Crustacea, Decapoda) nel Mar Jonio, *Oebalia* **17** (2): 61-67

Matarrese A., D'Onghia G., De Florio M., Panza M. and Costantino G. (1995). Recenti acquisizioni sulla distribuzione batimetrica di *Aristaeomorpha foliacea* ed *Aristeus antennatus* (Crustacea Decapoda) nel Mar Ionio. *Biologia Marina Mediterranea* **2(2)**, 299-300.

Matarrese A., D'Onghia G., Tursi A. and Maiorano P. (1997). Vulnerabilità e resilienza di *Aristaeomorpha foliacea* (Risso,1827) e *Aristeus antennatus* (Risso, 1816) (Crustacea, Decapoda) nel Mar Ionio, *S.It.E.*, Atti, **18**: 535-538

Maynou F. (2008a). Environmental causes of the fluctuations of red shrimp (*Aristeus antennatus*) landings in the Catalan Sea. *Journal of Marine Systems* **71**, 294-302.

Maynou F. (2008b). Influence of the North Atlantic Oscillation on Mediterranean deep-sea shrimp landings. *Climate Research* **36**, 253-257.

Meglécz E., Péténian F., Danchin E., D'Acier A.C., Rasplus J.Y. and Faure E. (2004) High similarity between flanking regions of different microsatellites detected within each of two species of Lepidoptera: *Parnassius apollo and Euphydryas aurinia*. *Mol Ecol* **13:** 1693–1700.

Meng X.H., Wang Q.Y., Jang I.K., Liu P. and Kong J. (2009). Genetic differentiation in seven geographic populations of the fleshy shrimp *Penaeus (Fenneropenaeus) chinensis* based on microsatellite DNA. *Aquaculture* **287**, 46-51.

Miller S.A., Dykes D.D, Polesky H.F. (1988). A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res*.; **16**(3): 1215.

Millot C. (1999) Circulation in the Western Mediterranean Sea. *Journal of Marine Systems* **20:** 423-442

Mulley J.C. and Latter B.D.H. (1980). Genetic variation and evolutionary relationships within a group of thirteen species of Penaeoid prawns. *Evolution* **34**, 904-916.

Mura M., Campisi S. and Cau A. (1992). Osservazioni sulla biologia riproduttiva negli Aristeidi demersali nel Mediterraneo Centro-Occidentale, *Oebalia* **17** (2): 75-80

Mura M. and Cau A. (1989). Sul dimorfismo sessuale e sex-ratio in *Aristeus antennatus* (Risso, 1816). *Oebalia* **15(2)**, 811-814.

Nei M. (1987). *Molecular Euolutionaly Genetics*: Columbia University Press, New York.

Neigel J.E.(1997). A comparison of alternative strategies for estimating gene flow from genetic markers. *Ann. Rev. Ecol. Syst.,* **28**, 105-128

Nouar A. (2001). Bio-écologie de *Aristeus antennatus* (Risso, 1816) et de *Parapenaeus longirostris* (Lucas, 1846) des côtes algériennes. *Rapports et procès-verbaux des réunions / Commission Internationale pour l'Exploration Scientifique de la Mer Méditerranée,* **36**, 304.

Orsi Relini, **L. (1980).** Aspetti riproduttivi in *Aristeus antennatus* (Risso, 1816) (Decapoda, Aristeidae). – *Memoria della Biologia Marina e Oceanographia* 10 (Suppl.)*:* 285–289.

Orsi Relini L., and Pestarino M. (1981). Riproduzione e distribuzione di e *Aristeus antennatus* (Risso, 1816) sui fondi batiali liguri. *Nota preliminare. Quad. Lab. Tecnol.*, **3** (suppl.1): 123-133

Orsi Relini L., and Relini G. (1979). Pesca e riproduzione del gambero rosso *Aristeus antennatus* (Risso, 1816) nel Mar Ligure. *Quad. Civ. Staz. Idrobiol. Milano,* **7** : 39-62

Orsi Relini L. and Relini G. (1985). The red shrimps fishery in the Ligurian sea: mismanagement or not. *FAO Fish. Rep.* **336**: 99-104.

Orsi Relini L. and Relini G. (1996). Seventeen instars of adult life in female *Aristeus antennatus* (Risso, 1816). A new interpretation of life span and growth. *Proc. 6th Colliquim Crustacea Decapoda Mediterranea,* Firenze, settembre 1996.

Orsi Relini L. and Relini G. (1998). Long-term observations of *Aristeus antennatus:* size structures of the fished stock in growth parameters with some remarks about the "recruitment". *Cah. Opt. Medit.,***35**: 311-322.

Orsi Relini L., Cima C., Palandri G., Relini M., Torchia G. (1995). I crostacei decapodi mesopelagici nell'alimentazione dei crostacei decapodi batiali. *Biol. Mar. Medit.*, **2** (2): 227-232.

Ouborg N.J., Piquot Y., Van Groenendael J.M. (1999). Population Genetics, Molecular Markers and the Study of Dispersal in Plants. *Journal of ecology* **87**: 551-568.

Paetkau D., Calvert W., Stirling I. and Strobeck C. (1995). Microsatellite analysis of population structure in Canadian polar bears. *Molecular Ecology* **4**, 347-354.

Papaconstantinou C. and Kapiris K. (2001). Distribution and population structure of the red shrimp (*Aristeus antennatus*) on an unexploited fishing ground in the Greek Ionian Sea. *Aquatic Living Resources* **14**, 303-312.

Park S.D.E. (2001). Trypanotolerance in west African cattle and the population genetic effects of selection.Ph.D. thesis, University of Dublin.

Peakall R.O.D. and Smouse P.E. (2006). Genalex 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* **6**, 288-295.

Piry S., Luikart G. and Cornuet J. (1999). BOTTLENECK: A computer program for detecting recent reductions in the effective population size using allele frequency data. *Journal of Heredity* **90(4)**, 502-503.

Piry S., Alapetite A., Cornuet J.-M., Paetkau D., Baudouin L. and Estoup A. (2004). GENECLASS2: A Software for Genetic Assignment and First-Generation Migrant Detection. *Journal of Heredity* **95**, 536-539.

Politou C.-Y., Karkani M. and Dokos J. (1998). Distribution of Decapods caught during MEDITS surveys in Greek waters. In: J.A. Bertrand and G. Relini (eds.), Demersal resources in the Mediterranean, Proceedings of the Symposium held in Pisa, 18-21 March 1998, Actes des Colloques, 26: 196-207. IFREMER, Plouzané.

Pla C., Roldán M. and García-Marín J. (1995). Biochemical genetics of the pink prawn, *Aristeus antennatus* Risso, in the western Mediterranean. *Rapports et procès-verbaux des réunions / Commission Internationale pour l'Exploration Scientifique de la Mer Méditerranée,* **34**, 39.

Primmer C.R., Raudsepp T., Chowdhary B.P., Møller A.P. and Ellegren H. (1997) Low Frequency of Microsatellites in the Avian Genome. *Genome Res.* 1997 **7**: 471-482

Pritchard J.K., Stephens P., Donnelly P. (2000). Inference of population structure using multilocus genotype data. *Genetics*, **155**: 945–959.

Prugnolle, F. and de Meeus, T. (2002). Inferring sex-biased dispersal from population genetic tools: a review. *Heredity* **88**, 161-165.

Queller D. C. and Goodnight K. F. (1989). Estimating relatedness using genetic markers. *Evolution* **43**, 258-275.

Ragonese, S. and Bianchini, M.L. (1996). Growth, mortality, yield-per-recruit of the deep-water shrimp *Aristeus antennatus* of the straight of Sicily (Mediterranean Sea). *Fishery research* **26**: 125-137.

Rannala B. and Mountain J. L. (1997). Detecting immigration by using multilocus genotypes. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 9197-9201.

Reich D. E. and Goldstein D. B. (1998). Genetic evidence for a Paleolithic human population expansion in Africa. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 8119-8123.

Reich D., Feldman M. and Goldstein D. (1999). Statistical properties of two teststhat use multilocus data sets to detect population expansions. *Molecular Biology and Evolution* **16**, 453.

Relini G. and Orsi Relini L. (1987). The decline of red-shrimps stocks in the gulf of Genoa. *Investigacion Pesquera* **51(1)**, 245-260.

Richard G.F. and Paques M. (2000). Mini- and microsatellite expansions: the recombination connection. *EMBO Rep. 2000 August 15*; **1**(2): 122–126

Robainas-Barcia A., Lopez G.E., Hernandez D. and Garcia-Machado E. (2005). Temporal variation of the population structure and genetic diversity of *Farfantepenaeus notialis* assessed by allozyme loci. *Molecular Ecology* **14**, 2933-2942.

Robainas-Barcia A., Blanco G., Sánchez J., Monnerot M., Solignac M. and García-Machado E. (2008). Spatiotemporal genetic differentiation of Cuban natural populations of the pink shrimp *Farfantepenaeus notialis*. *Genetica* **133**, 283-294.

Roldàn M.I., Heras S., Patellani R. and Maltagliati F. (2009). Analysis of genetic structure of the red shrimp *Aristeus antennatus* from the Western Mediterranean employing two mitochondrial regions. *Genetica* **136**, 1-4.

Rousset F. (2008). genepop'007: a complete re-implementation of the genepop software for Windows and Linux. *Molecular Ecology Resources* **8**, 103-106.

Sardà F. and Cartés J.E. (1994). Spatio-temporal structure deep-water shrimps *Aristeu antennatus* (Decapoda, Aristeide) population in the Western Mediterranea. *Fishery bulletin*, **92** (3): 599-607.

Sardà F., Maynou F., Talló L. (1997) Seasonal and spatial mobility patterns of rose shrimp *Aristeus antennatus* in the Western Mediterranean: results of a long-term study. MEPS **159**:133-141

Sardà F., Bas C., Roldán M.I., Pla C. and Lleonart J. (1998). Enzymatic and morphometric analyses in Mediterranean populations of the rose shrimp, *Aristeus antennatus* (Risso, 1816). *Journal of Experimental Marine Biology and Ecology* **221**, 131-144.

Sardà F., D'Onghia G., Politou C.Y., Company J. B., Maiorano P. and Kapiris K. (2004). Deep-sea distribution, biological and ecological aspects of *Aristeus antennatus* (Risso, 1816) in the western and central Mediterranean Sea. *Scientia Marina* **68**, 117-127.

Sardà F., Roldán M.I., Heras S. and Maltagliati F. (2010). Influence of the genetic structure of the red and blue shrimp, *Aristeus antennatus* (Risso, 1816), on the sustainability of a deep-sea population along a depth gradient in the western Mediterranean. *Scientia Marina* **74**, 569-575.

Schlötterer C. (2000) *Drosophila virilis* Has Long and Highly Polymorphic Microsatellites *Molecular Biology and Evolution* **17**:1641-1646.

Seridji A. (1971). Contribution a l'étude des larves crustacés décapodes en baie d'Alger. *Pelagos* **3**, 1-107.

Spedicato M.T., Greco, S., Lembo, G., Perdichizzi, F. (1995) Prime valutazioni sulla struttura dello stock di Aristeus antennatus (Risso, 1816) nel Tirreno Centrale meridionale. *Biol. Mar. Medit*

Sunnucks (2000) SSCP is not so difficult: the application and utility of single-stranded conformation polymorphism in evolutionary biology and molecular ecology *Molecular Ecology* **9** (11), 1699–1710

Supungul P., Sootanan P., Klinbunga S., Kamonrat W., Jarayabhand P. and Tassanakajon A. (2000). Microsatellite Polymorphism and the Population Structure of the Black Tiger Shrimp (*Penaeus monodon*) in Thailand. *Marine Biotechnology* **2**, 339-347.

SYNDEM (1999). Synthesis of the knowledge on Bottom Fishery Resources in Central Mediterranean (Italy and Corsica). *Biol. Mar. Medit***., 6 (Suppl.):** 1-869

Taberlet P., Luikart G. (1999) Non-invasive genetic sampling and individual identification. *Biological Journal of the Linnean Society,* **68**:41-55

Tassanakajon A., Tiptawonnukul A., Supungul P., Rimphanitchayakit V., Cook D., Jarayabhand P., Klinbunga S. and Boonsaeng V. (1998). Isolation and characterization of microsatellite markers in the black tiger prawn *Penaeus monodon*. *Molecular Marine Biology and Biotechnology* **7**, 55-61.

Vaccarella R. (1986) Pesca a strascico nel basso Adriatico: crostacei. *Nova Thalassia* 8 supp. **3**: 663-664.

Vos P., Hogers R. and Bleeker M. (1995). AFLP: a new technique for DNA fingerprinting. *Nucleic Acid Research*, **23**: 4407-4414.

Ward R., Ovenden J., Meadows J., Grewe P. and Lehnert S. (2006). Population genetic structure of the brown tiger prawn, *Penaeus esculentus*, in tropical northern Australia. *Marine Biology* **148**, 599-607.

Weir B.S. and Cockerham C.C. (1984). Estimating F-statistics for the analysis of population structure. *Evolution* **38**.

Wilson G.A. and Rannala B. (2003). Bayesian Inference of Recent Migration Rates Using Multilocus Genotypes Genetics, Vol. **163**, 1177-1191, March 2003

Wright J.M. and Bentzen P. (1994). Microsatellites - genetic-markers for the future. *Reviews in Fish Biology and Fisheries* **4**, 384-388.

Wright S. (1951). The genetical structure of populations. *Ann. Eugen*., **15**: 323-354.

Wright S. (1965). the interpretation of population structure by F-statistics with special regard to system of mating. *Evol*., **19**: 395-420.

Xu Z., Dhar A.K., Wyrzykowski J. and Alcivar-Warren A. (1999). Identification of abundant and informative microsatellites from shrimp (Penaeus monodon) genome. *Animal Genetics* **30**, 150-156.

Xu Z.K., Primavera J.H., de la Pena L.D., Pettit P., Belak J. and Alcivar-Warren A. (2001). Genetic diversity of wild and cultured Black Tiger Shrimp (*Penaeus monodon*) in the Philippines using microsatellites. *Aquaculture* **199**, 13-40.

Yeh F., Rong-Cai Y. and Boyle T. (1998). POPGENE, version 1.31 Microsoft Window-based freeware for population genetic analysis: quick user guide. Edmonton, Alberta, Canada: University of Alberta, Center for International Forestry Research.

You E.M., Chiu T.S., Liu K.F., Tassanakajon A., Klinbunga S., Triwitayakorn K., de la Pena L.D., Li Y. and Yu H.T. (2008). Microsatellite and mitochondrial haplotype diversity reveals population differentiation in the tiger shrimp (*Penaeus monodon*) in the Indo-Pacific region. *Animal Genetics* **39**, 267-277.

Zane L., Bargelloni L., Patarnello T. (2002). Strategies for Microsatellite Isolation: a review. *Molecular ecology* **11**(1): 1-16.

9 Annexes

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PERMANENT GENETIC RESOURCES

Isolation and characterization of 14 polymorphic microsatellite markers for the blue and red shrimp, *Aristeus antennatus* (Crustacea, Decapoda)

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Abstract

Eighteen microsatellite loci were isolated and characterized from the blue and red shrimp, *Aristeus antennatus* Risso 1816, a commercially exploited marine crustacean widely distributed throughout the Mediterranean Sea and in the eastern Atlantic. Polymorphism was assessed in a population (n = 20) from the southwestern Sardinian seas; 14 loci resulted polymorphic and showed from three to 13 alleles. The observed heterozygosity ranged from 0.2 to 0.85. These microsatellites will be potentially useful for the study of *A. antennatus* population genetic structure.

Keywords: Aristeidae, *Aristeus antennatus*, Crustacea, Decapoda, microsatellites, shrimp

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Aristeus antennatus Risso 1816, is a marine deep shrimp distributed in the eastern Atlantic (from Portugal to the Cape Verde Islands) and in the entire Mediterranean Sea (Holthuis 1980). The blue and red shrimp is an economically important crustacean species, one of the main resources of bottom trawling in the Mediterranean region (Cau *et al*. 2002). Subjected to intense fishery exploitation, the resource is considered fully exploited in many areas of its distribution range (Company *et al*. 2008). To date, little is known of *A. antennatus* population structure and genetic diversity. To elucidate the amount of genetic variation among blue and red shrimp populations, we isolated microsatellite loci.

The fast isolation by amplified fragment length polymorphism (AFLP) of sequences containing repeats (FIASCO) protocol (Zane *et al*. 2002) was used to isolate microsatellites. Muscle tissues were sampled from three individuals, stored in absolute ethanol, and then used for the extraction of the genomic DNA using the salting-out protocol (Miller *et al*. 1988). DNA was digested with the endonuclease *Mse*I (New England Biolabs) and ligated to *Mse*I AFLP adaptors (Vos *et al*. 1995). Restricted and ligated fragments were amplified with *Mse*I adapter-specific primers (Vos *et al*. 1995).

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All polymerase chain reactions (PCR) were performed on a Mastercycler ep gradient S (Eppendorf) as described in Zane *et al*. (2002). Amplified DNA was enriched using a biotinylated probe [$(AC)_{17}$] for 15 min at room temperature; DNA fragments hybridized with the biotinylated probe were selectively separated using streptavidin-coated beads (Roche Diagnostics) in the presence of a magnetic field (MagnaRack, Invitrogen). Nonspecific DNA was removed by four nonstringency washes and four stringency washes (for details see Zane *et al*. 2002).

DNA was then eluted from the beads using TE 10:1 buffer at 95 °C for 5 min, precipitated with sodium acetate and isopropanol and finally re-suspended in 50 µL of sterile water.

Five microlitres of the recovered fraction was amplified with AFLP adapter-specific primers under the conditions described above. PCR products were cloned using the TOPO TA cloning kit (Invitrogen); 121 recombinant clones were PCR amplified with universal vector M13 primers, purified with ChargeSwitch PCR CleanUp Kit (Invitrogen). The sequencing service was provided by BMR Genomics srl, University of Padova (see www.bmr-genomics.it/). A total of 44 sequences presented a microsatellite, 30 of which with appropriate flanking length and sufficient quality regions for primer design. Primers pairs were designed using

Table 1 Characteristics of 14 microsatellite loci isolated from *Aristeus antennatus* (n = 20 individuals), with primer sequences, fluorochrome dye of forward primer, touchdown (TD) annealing temperatures (T) in C, repeat motif derived from a sequenced clone, number of alleles observed (N_A), observed allele size ranges, number of individuals assayed (N), observed (H_O) and expected (H_E) heterozygosities (boldface numbers indicate significant deviation from Hardy–Weinberg equilibrium with $^*P < 0.05$, $^{**}P < 0.01$)

Locus name (GenBank Accession no.)	Primer sequence 5′–3′	Dye 5′	T annealing	Repeat sequence 5′–3′	N_A	Allele size range (base pair)	N	H_O	H_E
Ant 99 (EU417954)	F: TGTCATAGCGGCTTCCAT R: ATATCTTGTTACGACCCTCG	HEX	TD, 59 →51	$(TG)_4CT(TG)_5CG(TG)_3$	7	197–244	20	0.700	0.687
Ant 6 (EU417944)	F: AGCAATAATTAGATGATGCC R: GTTTATGGTGGAAGATGAAT	HEX	TD, 59 →51	$(GT)_5AT(GT)_{12}$	3	151–161	20	0.350	0.304
Ant 51 (EU417950)	F: CCTTCCGTTTCTTCTACAGT R: AAAACCCACTTACGCTACTC	TAMRA	TD, 59 →51	$(TG)_{10}$	4	280–288	20	0.550	0.645
Ant 66 (EU417951)	F: TACTGCCTTGAGATCGTTAC R: CCTTCCGTTTCTTCTACAGT	HEX	TD, 59 →51	$(CA)_{10}$	4	314–322	20	0.500	0.619
Ant 93 (EU417952)	F: TGCTGATACAGAAGGTAGGC R: TTGGTACTGTTTCCCCATGC	FAM	TD, 58 →50	$(AC)_{10}$	5	216–226	19	**0.350****	**0.745**
Ant 16 (EU417949)	F: TGAGACCCTCAGACTCAC R: TCTTTCTTTCTACTTCCCCTC	FAM	TD, 58 →50	$(GT)_9$	8	134–170	20	**0.200****	**0.870**
Ant 94 (EU417953)	F: GAGGTGTAGGCAGAGTGA R: GCCTCTTTTACGTTACGCTG	TAMRA	TD, 63 →55	$(TG)_{12}$	10	159–186	20	**0.600***	**0.882**
Ant 9 (EU417955)	F: CGCCTACACCGATGGTTCCT R: GCCTCCCACTGCCAACATGA	HEX	TD, 68 →60	$(GT)_5G(GT)_3$	3	220–224	20	0.200	0.230
Ant 20 (EU417956)	F: TAGTGTTCCATAGACTTATA R: ACTAGACAAATCTAAATGCT	TAMRA	TD, 52 →44	$(CA)_7$	8	165–205	20	**0.550****	**0.793**
Ant 37 (EU417957)	F: TGTACGGGGCGACAGTCTAC R: GGGGAGACGGCGAAGCAAAC	FAM	TD, 68 →60	$(TG)_{11}$	8	256–274	20	0.850	0.823
Ant 34 (EU417958)	F: AACGTGCCAATCAAAGTGAT R: TGAGGTAGAGACAAAGACTG	TAMRA	TD, 58 →50	$(AC)_9$	6	205–249	20	0.650	0.838
Ant 46 (EU417959)	F: AATCAGAGGATCACGACACT R: TACTGTGTCTTTGGCAACTG	FAM	TD, 59 →51	$(AC)_6AG(AC)_6$	13	176–221	20	**0.600****	**0.890**
Ant 82 (EU417960)	F: TGTTACGATTCCTGGTAAGG R: ATGAAGTGGTTGAGTAGTCC	HEX	TD, 59 →51	$(AC)_{10}$	9	192–218	20	0.750	0.882
Ant 194 (EU417961)	F: ATGGATTCAATAATTTCGGC R: ATATTCTGGCATTTTGTAGG	HEX	TD, 54 →46	$(CA)_8$	4	150–164	20	0.500	0.705

programs OLIGO EXPLORER 1.2 and OLIGO ANALYSER 1.1.2 (www.genelink.com); 18 were used to amplify the genomic DNA from 20 *A. antennatus* individuals from Sardinian seas.

The PCRs (25- L total volume) included 40 ng of genomic DNA, 2 mM $MgCl_2$, 0.4 pmol of each primer, 0.2 mM dNTPs, 2.5 L *Taq* DNA polymerase PCR buffer 10× (200 mM Tris-HCl, 500 mM KCl) and 0.8 U of *Taq* DNA polymerase (Invitrogen). A touchdown PCR profile (Don *et al*. 1991) was used for all loci according to the following scheme: initial denaturation step of 94 C for 5 min followed by 40 cycles of 94 C for 30 s, annealing temperature for 35 s at different temperatures for each primer pair (see Table 1) and decreasing –1 C for the first eight cycles, 72 C for 30 s and a final extension step of 72 C for 5 min.

Forward primers labelled by different fluorescent dyes allowed fragment detection on an ABI PRISM 3730xl automated sequencer (ROX 400 as size standard) provided by the BMR Genomics facility (see above for details) and were combined so as to assay three to four loci simultaneously in a single capillary electrophoresis. Allele scoring was carried out using PEAK SCANNER version 1.0 (Applied Biosystems). Among the 18 primer pairs tested, 14 produced polymorphic microsatellite markers (Table 1) (GenBank Accession nos EU417944, EU417949–EU417961), four were monomorphic (GenBank Accession nos EU417945–EU417948). The polymorphic loci showed variability in number of alleles (from three to 13) with a mean number of 6.6.

Allele size calling for all 18 loci was consistent and PCR success across all individuals and loci was 99.6%, with only one null allele for locus Ant 93. The mean observed and expected heterozygosities were 0.525 (range 0.2–0.85) and 0.708 (range 0.23–0.89).

Five loci (Ant 16, Ant 20, Ant 46, Ant 93 and Ant 94) showed significant deviations from Hardy–Weinberg expectations in the Markov chain method (parameters used: 100 000 Markov chain steps; 10 000 dememorization steps) using the ARLEQUIN version 3.11 software package (Excoffier *et al*. 2005) and heterozygote deficit in GENEPOP

version 4.0 (Rousset 2008). It is not excluded that these results may be due to the presence of inbreeding or null alleles.

Genotypic linkage disequilibrium between all pairs of loci was tested; after Bonferroni correction for multiple comparisons, loci Ant 51 and Ant 66 resulted linked.

The newly polymorphic microsatellite markers could be useful to identify valid stocks or management units for the shrimp *A. antennatus*.

References

Cau A, Carbonell A, Follesa MC *et al*. (2002) MEDITS-based information on the deep-water red shrimps *Aristaeomorpha foliacea* and *Aristeus antennatus* (Crustacea: Decapoda: Aristeidae). *Scientia Marina*, **66** (Suppl. 2), 103–124.

Company JB, Puig P, Sardà F, Palanques A, Latasa M, Scharek R (2008) Climate influence on deep sea populations. *PLoS ONE*, **3** (1): e1431. doi: 10.1371/journal.pone.0001431.

Don RH, Cox PT, Wainwright BJ, Baker K, Mattick JS (1991) 'Touchdown' PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Research*, **19**, 4008.

Excoffier L, Laval G, Schneider S (2005) ARLEQUIN ver. 3.0: an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online*, **1**, 47–50.

Holthuis LB (1980) *FAO Species Catalogue*. *Vol. 1. Shrimps and Prawns of the World. An Annotated Catalogue of Species of Interest to Fisheries*. Food and Agriculture Organization of the United Nations, Rome, Italy.

Miller SA, Dykes DD, Polesky HF (1988) A simple salting-out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Research*, **16**, 12–15.

Rousset F (2008) GENEPOP '007: a complete re-implementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Notes*, **8** (1), 103–106.

Vos P, Hogers R, Bleeker M *et al*. (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research*, **23**, 4407–4414.

Zane L, Bargelloni L, Patarnello T (2002) Strategies for microsatellite isolation: a review. *Molecular Ecology*, **11**, 1–16.

MICROSATELLITE MARKERS FOR POPULATION GENETIC STUDIES OF THE GIANT RED SHRIMP *ARISTAEOMORPHA FOLIACEA* (CRUSTACEA, DECAPODA)

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Abstract

Microsatellite markers have been isolated and characterized from the giant red shrimp *Aristaeomorpha foliacea* in order to investigate on the presence of distinct stocks in the Western Mediterranean Sea. Polymorphism of the newly obtained loci was assessed in a restricted sample of 30 specimens, nine loci provided markers with polymorphism (range 2–19 alleles per locus). Six loci, the most polymorphic and easiest to amplify and to score, were tested in a larger sample of 6 populations from the Western Mediterranean Sea. Microsatellite data revealed a substantial genetic homogeneity and no signs of recent bottlenecks, suggesting the existence of a high gene flow that connects all populations.

Keywords: Genetics, Crustacea, Western Mediterranean, Deep Waters, Fisheries

Introduction

The giant red shrimp, *Aristaeomorpha foliacea*, is a cosmopolitan crustacean species, widespread in the eastern and western Atlantic, the western Pacific, the Indian Ocean and the Mediterranean Sea. It is one of the most important exploited crustaceans, having a long traditional and economical significance for the deep fishery of the western and central Mediterranean countries. Despite its commercial importance, little is known about the genetics, population structure, and migration patterns of this species. Since direct tracking movements of individuals is very difficult for marine species, the use of indirect methods, including genetic approaches, for the measurement of connectivity among populations can be very informative. With the aim of studying the population structure and genetic connectivity among giant red shrimp populations from the Western Mediterranean Sea and obtaining new and powerful genetic markers, microsatellites loci were isolated from *Aristaeomorpha foliacea* genome using the FIASCO protocol [1] from a partial genomic library enriched for an AC motif.

Results

Among 96 recombinant clones obtained, a total of 43 sequences had a microsatellite, 14 of which with flanking region of appropriate length and sufficient quality to allow the design of primers that were used to amplify the genomic DNA of a subsample of 30 specimens from two locations. Four out of 14 microsatellites were immidiately discarded because of their low amplification success, while one was found to be monomorphic.

The remaining 9 loci were characterized by a high variability in PIC values (0.36-0.91) and in the number of alleles (range 2 – 19).

In this study only the 6 most polymorphic loci were tested in a larger sample and used to make a preliminary investigation on the genetic structure of Mediterranean giant red shrimps. A total of 115 specimens were analysed from 6 populations: 4 samples were collected off the Sardinian coast (Sant'Antioco, Cagliari, Siniscola, Asinara) and two samples off Sicily (Messina and Sicily Channel). Two loci (Cea17 and Cia82) significantly deviated from HWE and this could be explained by their associated elevated frequencies of null alleles (10% and 19%, respectively) calculated with the algorithm of Dempster [2]. *A. foliacea* populations resulted not genetically differentiated as indicated by the low and not significant pair-wise Fst values calculated with Arlequin 3.1 [3].

Analysis of molecular variance AMOVA clearly showed that genetic variability was largely due to differences among individuals (99.31%) rather than to differences among populations (0.69%).

The absence of population structure was further confirmed by the bayesian clustering method implemented in Structure 2.3 [4] indicating K=1 as the most probable structure.

The PCA performed with Adegenet [5] showed a substantial genetic homogeneity among populations separated by hundreds of kilometres suggesting that western Mediterranean populations could represent a unique panmictic stock (fig 1).

Finally, the bottleneck test [6] and interlocus g-tests [7] did not find signs of recent bottlenecks (reduction of population size) or growth (increase of population size after the recovering from a demographic collapse), leading to hypothesize that, up to now, the Western Mediterranean giant red shrimps have experienced a sustainable fishing pressure.

All these results clearly indicate that the microsatellite markers tested here can represent very useful tools for population genetic studies of *A. foliacea*, for instance for monitoring the detrimental genetic effects of any future increasing fishing pressure.

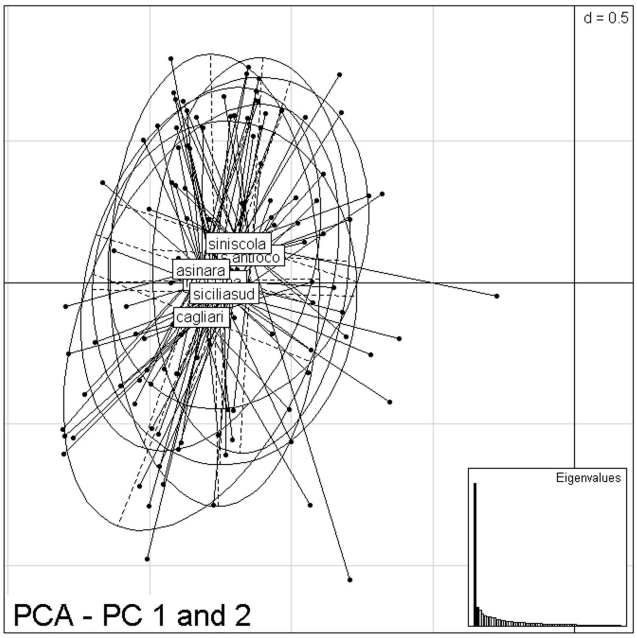

Fig. 1. Typology of giant red shrimp populations obtained by interclass PCA. Eigenvalues corresponding to the represented components are filled in black. Points represent genotypes; samples are labelled inside their 95% inertia ellipses

References

1 - Zane L., Bargelloni L., Patarnello T. 2002., *Molecular ecology* 11(1): 1-16.

2 - Chapuis M-P, Estoup A. 2007., *Mol Biol Evol*. 24: 621–631.

3 - Excoffier, L. G. Laval, and S. Schneider. 2005., *Evolutionary Bioinformatics* Online 1: 47-50.

4 - Falush, D., Stephens, M., and Pritchard, J. K., 2007. *Molecular Ecology Notes* 7(4): 547-578.

5 - Jombart T. 2008., *Bioinformatics* 24: 1403-1405.

6 - Piry S., Luikart G., Cornuet J.M. 1999., *Journal of Heredity*, 90: 502– 503.

7 - Reich D.E., Feldman M.W., Goldstein D.B. 1999., *Mol. Biol. Evol*. 16, 453-466.