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"ANALISI DELLA VARIABILITÀ GENETICA DI *PATELLA FERRUGINEA, PATELLA ULYSSIPONENSIS* (MOLLUSCA: GASTROPODA) E *PINNA NOBILIS* (MOLLUSCA: BIVALVIA): IL CONTRIBUTO DEI DATI MOLECOLARI ALLA CONSERVAZIONE DI SPECIE MINACCIATE"

"ANALYSIS OF THE GENETIC VARIABILITY OF *PATELLA FERRUGINEA*, *PATELLA ULYSSIPONENSIS* (MOLLUSCA: GASTROPODA) AND *PINNA NOBILIS* (MOLLUSCA: BIVALVIA): CONTRIBUTION OF MOLECULAR DATA IN THREATENED SPECIES CONSERVATION STUDIES"

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INTRODUCTION

All around the planet the endangered species are precipitately disappearing as direct or indirect result of the anthropization. The use of fossil fuels and deforestation has increased over the last three centuries the concentration of carbon dioxide (CO_2) by 30%; other human activities have doubled the concentration of methane and increased the concentration of other gases involved in global warming. The expansion of urban and agricultural areas has incorporated grasslands, forests and wetlands pouring into water basins increasing amounts of water nutrients, responsible for changes at ecological level of estuarine and coastal ecosystems.

In marine environment, three-quarters of harvested fish populations monitored by the FAO are already overexploited, or will become so without stringent management intervention (Balmford *et al.,* 2005; FAO 2000). Indeed the introduction of aggressive alien species are giving the final jab to exhausted natural (eco)systems. Incontrovertible evidences of the ongoing loss of biodiversity can be found looking at The Red List of Threatened Species, redacted by the International Union for Conservation of Nature (IUCN) that documents the extinction risk of 47,677 species: 17,291 are threatened, including 12% of birds, 21% of mammals, 30% of amphibians, 27% of reef-building corals, and 35% of conifers and cycads. The Living Planet Index reveals that populations of wild species have declined by 30% since 1970; mangrove forests have lost a fifth of their area since 1980, and 29% of seagrass beds are gone (Marton-Lefèvre, 2006).

In marine environment the biodiversity is also threatened by the inaccurate ascertainment to consider it an environment with high phyletic but low specific diversity, inhabited by few, wide ranging species, but where unrecorded majority

of them are vanishing before their existence, much less their biological importance or economic utility, is established (Eckholm, 1978).

A significant difficulty to appreciate the levels of marine biodiversity arises not only because the number of described species falls far short of actual diversity (Blaxter, 2004), but also because species are often exceedingly difficult to recognize using morphological approaches, as an important hidden source of diversity in marine systems lies in cryptic species complexes - species that are difficult to distinguish morphologically (Mayr, 1963) - which are common in marine groups and may be important and underestimated components of the ecosystem (Knowlton, 1993, 2000).

In spite of "sins of mankind" all these species now require human assistance to improve their chances of survival and population recovery, outstandingly, the conservation of natural populations directly in their original environment need to assess the genetic resources that are really available and to investigate the evolutionary mechanisms that regulate their maintenance. Genetic factors make important contributions to extinction risk, for these reasons they require an appropriate management.

Conservation genetics encompasses genetic management of these threatened populations, resolution of taxonomic uncertainties and the use of molecular tools (Frakham, 2003) in order to study genetic patterns or processes that informs conservation efforts (Avise, 2008).

Not all the genomic regions show identical potential in depicting population dynamics of species. Different levels of evolutionary patterns could be only pointed out by means of molecular markers with different specific prerequisites. Genes coding for proteins essential for life generally tend to slowly fix mutations being useful for reconstruction of phylogeny at higher taxonomic level. Conversely, non-coding regions and DNA portions of poor biological interest, accumulating mutations with neutral value, are

generally expected to vary with higher rates thus representing a useful tool in phylogeographic studies devoted to found structuring, if any, among species, populations or even individuals.

In such context, DNA-sequencing techniques, including genotyping of microsatellite and DNA barcoding, and Inter-simple sequence repeat markers (ISSR - Zietckiewicz et al. 1994) were used in the following articles (published, submitted or in prep.) for estimations of different genomic parameters, as well restriction-enzyme assays of mtDNA have been developed in order to solve a cryptic species case.

Patella ferruginea

Patella ferruginea Gmelin, 1791 is an endangered marine gastropod endemic to the Western Mediterranean. Its range is restricted to the Sardinian-Corsican region, North Africa, a few scattered sites in Southern Spain, and Sicily. The occurrence of shells in Palaeolithic and Neolithic deposits suggests that *P. ferruginea* was commonly distributed in the Western Mediterranean throughout the Pleistocene to modern times (Espinosa and Ozawa 2006), but intense human exploitation of the species during the centuries has led to the fragmentation of its range (Paracuellos et al. 2003). How this fragmentation has affected the genetic population structure of the species need to be cleared (Espinosa and Ozawa 2006; Casu et al. 2006).

Pinna nobilis

The fan mussel *Pinna nobilis* Linnaeus, 1758 is the largest endemic Mediterranean bivalve; it is a long-living species, which on average may reach a maximum age of 20 years (Butler et al. 1993), albeit a 27-year-old specimen has been found (Galinou-Mitsoudi et al., 2006).

P. nobilis occurs in coastal areas, between 0.5 and 60 m depth, principally on soft sediments colonized by seagrass meadows, but also on bare sand, mud, maërl beds, among boulders or pebbly bottoms. General knowledge of the main constraints which control the population structure of *P. nobilis* is still incomplete, human activities strongly affected its populations leading to a reduction of its distribution in the last decades, previous papers have highlighted specific features concerning spatial distribution, but consistent genetic data on the species are still lacking.

Patella ulyssiponensis

The limpet *Patella ulyssiponensis* Gmelin, 1791 (Mollusca: Gastropoda) is widely distributed in the Mediterranean as well as in the north-eastern Atlantic, from southern Norway to north-western Africa, including Macaronesia. This limpet is widespread in the shallow subtidal and low intertidal zones where it can be found on the more exposed shores as well as in mid-shore rock pools (Guerra and Gaudêncio 1986; Hawkins et al. 1990). In the Mediterranean *P. ulyssiponensis* is one of the most challenging species, whose abundance estimates may be affected by troublesome distinction between this species and the endemic *Patella caerulea* L. 1758 as well as in the Atlantic coasts and in Macaronesia with *Patella vulgata* Linnaeus, 1758 and *Patella candei* d'Orbigy, 1839 respectively . The application of molecular methods has recently contributed to reveal the occurrence of complexes of cryptic species even in taxa assumed easy to identify in the field, such in genus *Patella* (Sá-Pinto et al. 2010).

In the present study we have identified specific PCR-RFLP (Restriction Fragment Length Polymorphism PCR-based) markers on the COI sequences which could be used as an alternative, fast and cost-effective diagnostic tool for the discrimination of *P. ulyssiponensis s.l.* from *P. caerulea*, *P. vulgata* and *P. candei* (Sanna et al. *in press*), in

order to explain the patterns of spatial genetic structure in the species/form spread along the European Mediterranean and Atlantic coasts.

Ruditapes phillippinarum

In order to achieve a cross priming test using microsatellite primers developed on *Ruditapes philippinarum* Adams and Reeve, 1850, to investigate the endangered species *Ruditapes decussatus* Linnaeus, 1758, we have approached a new line of research in population genetics of these species, by means of our fingerprinting tecniques experience. Bivalves are one of the most invasive group among invertebrates (Sousa et al., 2009) and the examples in support of these evidence are multiple. Manila clam *R. philippinarum* is one of this invaders, belonging to the family *Veneridae* Rafinesque, 1815. Its naturally distribution is the Indo-Pacific region Japan, Korea and China. It was introduced into the west coast of North America, Hawaii, Portugal, French Atlantic coast, French Mediterranean coast (Thau Lagoon), Ireland and Italy (Adriatic Sea and Sardinia) due to its acclimatization and fast population spreading. In Italy, Manila clam was intentionally introduced in Northern Adriatic Sea for aquaculture purposes in 1983 to support a clam fishery suffering a crisis due to overexploitation of native clam *R. decussatus* (Chiesa et al. 2010 and reference therein). In our work we have also investigate the genetic variability of microsatellites among the populations of *R. philippinarum* from North Adriatic Sea and from the Gulf of Olbia (North-East Sardinia) where they have been recently introduced.

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Patterns of spatial genetic structuring in the endangered limpet *Patella ferruginea*: implications for the conservation of a Mediterranean endemic

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Introduction

The limpet *Patella ferruginea* Gmelin, 1791 (Mollusca: Gastropoda) is endemic to the Western Mediterranean, where it is considered the most endangered marine macroinvertebrate by the European Council Directive 92/43/EEC on the Conservation of Natural Habitat of Wild Fauna and Flora, 1992. It is distributed along rocky shores of different lithologies that are exposed to medium to strong wave energy and are between the high midlittoral and supralittoral zones (Biagi and Poli 1986; Laborel-Deguen and Laborel 1991; Ramos 1998). *Patella ferruginea* is a protandrous species (Espinosa et al. 2008) in which the male achieves sexual maturation at the age of 2–3 years (Laborel-Deguen and Laborel 1991). Females produce relatively large-sized eggs, suggestive of lecithotrophic development (Laborel-Deguen and Laborel 1991). The species has trochophora and veliger phases (Espinosa et al. 2010); however, few data are available with regard to the biology and duration of its larval phases.

The occurrence of shells in Palaeolithic and Neolithic deposits suggests that the species was commonly distributed in the Western Mediterranean throughout the Pleistocene to modern times (Espinosa and Ozawa 2006). Since prehistoric times, intense human exploitation of *P. ferruginea* has led to the fragmentation of the species' range (Paracuellos et al. 2003). It is currently restricted to the following areas: Sardinia, Corsica, Tuscan Archipelago, few scattered sites in Sicily, Pantelleria, and the North African shoreline from Tunisia to the Strait of Gibraltar (Biagi and Poli 1986; Guerra-García et al. 2004).

The extent to which this fragmentation has affected the genetic population structure of the species is not clear (Espinosa and Ozawa 2006; Casu et al. 2006). An analysis of the mitochondrial DNA (mtDNA) region coding for Cytochrome c Oxidase subunit I (COI) has shown a lack of genetic population structure in the populations

located in Southern Spain and North Africa (Espinosa and Ozawa 2006). In contrast, Inter-simple sequence repeat (ISSR) markers have shown significant genetic structuring between populations in two Sardinian marine protected areas (MPAs) located only 150 km apart (Casu et al. 2006).

The lack of genetic data from across the entire extant range of *P. ferruginea* represents an issue in managing its conservation because the knowledge of the amount of genetic variability and distribution in space and time is critical for a correct diagnosis of the status, threats and viability of populations (Frankham 1995; Escudero et al. 2003). Indeed, genetic approaches are particularly useful in addressing issues such as the determination of the dispersal capabilities of marine species. In fact, a direct determination of dispersal ability in marine species is difficult to quantify (Kinland and Gaines 2003) and therefore estimates of dispersal are often indirectly inferred by assessing the genetic differentiation among populations (Chambers et al. 2006; Pannacciulli et al. 2009).

To perform a comprehensive assessment of the genetic population structure of *P. ferruginea*, we sampled individuals across the entire extant range. In doing so, we investigated the presence of genetic discontinuities across the sampled area and explored the potential causes of any genetic structuring. To achieve these goals, we used both nuclear ISSR markers and partial sequences of the following three mtDNA genes: COI, 12S (small-subunit ribosomal RNA gene) and 16S (large-subunit ribosomal RNA gene). As molecular markers of choice we used ISSRs because this technique is affordable and less laborious compared with other fingerprinting methods. Simulations indicate that dominant markers, such as ISSRs, may be as efficient as the codominant ones in estimating genetic diversity (Mariette et al. 2002). Furthermore, ISSRs generate highly reproducible bands, and detect high levels of genetic variability in populations of

marine organisms, even at a small spatial scale (*e.g.*, Casu et al. 2005, 2006; Hassan et al. 2003; Lai et al. 2008; Machkour et al. 2009). On the other hand, sequence data from the mitochondrial genome have been used to infer the occurrence of phylogeographic patterns of limpets (Bird et al. 2007, 2011; Gonzáles-Wevar et al. 2011a; Gonzáles-Wevar et al. 2011b). Furthermore, studies on the COI gene of other *Patella* species, mainly aimed to shed light on the phylogeny of the genus, also showed its potential use in detecting major geographical discontinuities (Sá-Pinto et al. 2008, 2010).

Materials and methods

Sampling and DNA extraction

A total of 213 specimens of *Patella ferruginea* were collected from the intertidal zones of 33 localities of the Western Mediterranean (see Table 1, Fig. 1 for details). For some sampling sites, the number of individuals analysed is very small due to the extremely low density of *P. ferruginea* at those localities (*e.g.*, Nido d'Aquila, Tizzano, Bonifacio, Pantelleria, Marettimo, Favignana) (Table 1). Whenever possible, at least ten individuals were collected from each sampling site using the following nonlethal protocol: the individual was gently removed from the substrate by means of a wood chisel, and a 30-60 mg sample of foot muscle was excised using a sterilised surgical forceps. The individual was then repositioned in its so-called 'home scar', a depression in the rock formed by abrasion by the shell, resulting in a tighter fit to the rock and reduced risk of desiccation. Genomic DNA was extracted from the tissue using the QIAGEN DNeasy Tissue kit.

ISSR analysis

Seven primers tested in a previous study (Casu et al. 2006) were used for genotyping the 213 specimens (Table 2). The PCR mixture, amplification and electrophoretic techniques were performed as described in Casu et al. (2006).

To overcome potential problems due to small sampling size at a number of localities, the underlying genetic population structure was inferred using an individual-based approach (Luikart et al. 2003) using the Bayesian model-based clustering

algorithms implemented in the software package BAPS 5.2 (Corander and Tang 2007) and STRUCTURE 2.2.3 (Pritchard et al. 2000).

BAPS analysis is used to infer the number of genetic clusters (*K*) in the data set, where *K* is treated as an unknown parameter to be estimated. To compare the BAPS results with the STRUCTURE results, the module for the nonspatial clustering of individuals was used. We performed ten independent analyses to assess the reliability of results. In each analysis the number of clusters (*K*) was estimated by a range of possible values (*K* = 2 up to 35). The algorithm was reiterated on each predefined value of *K* five times. The clustering solution with the highest mean posterior probability computed over the ten independent runs was chosen as the correct partitioning on which the admixture analysis was subsequently performed (Vähä et al. 2007).

STRUCTURE analysis, which assumes that *K* is known in advance, was used to assess the occurrence of hierarchical levels of genetic structure. The method is hereafter described: in a given dataset STRUCTURE identifies the uppermost hierarchical structure that corresponds to the minimum number of clusters that captures the major structure in the data (Pritchard and Wen 2004); after this first round data are partitioned into smaller datasets according to the best clustering solution, and subsequent rounds are performed on each subset of data. The procedure is reiterated until it is not possible to partition the data any further. To detect the uppermost hierarchical structure present in a given partition of data we used the method of Evanno et al. (2005). Next, we took into account the posterior probability of data for a given *K*. If both methods failed, the individual assignment patterns were examined using *q* value thresholds of 0.2/0.8 to denote membership in the cluster (Vähä et al. 2007). For each data partition and for each value of *K* ten independent runs of STRUCTURE were performed by applying the admixture model with correlated allelic frequencies (Falush et al. 2003, 2007). Each run

consisted of 100,000 iterations that followed a burn-in period of 100,000 iterations to assess whether the results were consistent across different runs for each inferred value of *K*. STRUCTURE analyses were performed on the CBSU Web Server, and graphical displays of the results were generated using the software package DISTRUCT 1.1 (Rosenberg 2004).

In addition, we compared the results of the model-based clustering with a Principal Coordinate Analysis (PCA) performed by the program GENALEX 6.3 (Peakall and Smouse 2006) on a matrix of interindividual distances via a covariance matrix with a data standardisation method. The ordination was carried out on the entire dataset as well as on subsets of data corresponding to the groups identified by Bayesian-model based clustering.

Genetic structuring was also investigated by a hierarchical Analysis of Molecular Variance (AMOVA) using the software package ARLEQUIN 3.52 (Excoffier and Lischer 2010). The total variance was partitioned into covariance components due to differences within populations, between populations within groups, and among groups. Three different schemes of grouping were tested, two of which were defined *a priori*: one scheme with two groups corresponding to SCR and SAS groups; a second scheme with three groups, because of the further subdivision of SAS group into Alboran Sea and Siculo-Tunisian Strait regions; the third scheme took into account the results of model-based clustering analysis, corresponding to the highest hierarchical genetic structuring. The significance of the fixation indices associated with the different levels of genetic structure was assessed by a non parametric permutation test with 10,000 replicates (Excoffier et al. 1992).

The relationship between geographical and individual pairwise genetic distances were investigated using Mantel correlograms (Oden and Sokal 1986). The Mantel

correlograms were applied to the entire dataset as well as to subsets of data (partitioned according to model based-clustering) using the multivariate, multilocus approach of Smouse and Peakall (1999) implemented in the program GENALEX 6.3. This method combines the information generated from multiple genetic markers to strengthen the spatial signal by reducing stochastic (allele-to-allele and locus-to-locus) noise. Individual pairwise genetic distances are used to estimate the autocorrelation coefficient *r*, which measures the genetic similarity between pairs of individuals whose geographic separation falls within a specified distance class. The number and size of distance classes were set to compare similar sample size within each class. The significance of positive autocorrelation was determined using both a permutation test (1,000 random permutations, 95% confidence interval) and bootstrap (1,000 reps, 95% confidence interval) estimates of *r*. Significant spatial genetic structure was inferred either if the calculated *r* value fell outside this confidence interval and if the bootstrap confidence interval did not include $r = 0$ for a given distance class.

Mitochondrial DNA analysis

The 12S and 16S genes were sequenced for 174 individuals, whereas the COI gene was sequenced for 110 individuals, being 64 COI sequences available on GenBank (accession # AY996038-AY996043; GQ469872-GQ469874; GQ469876) (Table 1). Universal primers were used for COI (Folmer et al. 1994), 12S and 16S (Nakano and Ozawa 2004).

PCR amplification was performed as described in Espinosa and Ozawa (2006) with the following annealing temperatures: COI, 54 °C; 12S and 16S, 49 °C. PCR products were purified with ExoSAP-IT (USB Corporation) and were sequenced using

an external sequencing core service (Macrogen Inc., Seoul, Korea). The sequences obtained were aligned using the software CLUSTAL W Multiple alignment tool in BIOEDIT 7.0.5.2 (Hall 1999).

The number of haplotypes (h), number of polymorphic sites (S), nucleotide diversity (π), and haplotype diversity (H) were calculated to obtain estimates of the genetic divergence among populations using the software package DNASP 5.0 (Rozas and Rozas 1999). The genetic relationships among the haplotypes were inferred using the Median Joining Network analysis (Bandelt et al. 1999) with the software package NETWORK 4.5.0.1 (www.fluxus-engineering.com).

The departures from mutation-drift equilibrium were assessed using Tajima's D (Tajima 1989) and Fu's Fs (Fu 1997) neutrality tests. Significant negative values for both tests are expected under recent population expansion following a bottleneck, selective sweeps or purifying selection (Fu 1997; Ramos-Onsins and Rozas 2002). The significance was tested by performing coalescent simulations (10,000 iterations) in DNASP 5.0.

Results

ISSR

The number and size of bands resolved per primer ranged from ten to 11 and from 500 bp to 1,800 bp, respectively (Table 2). The Bayesian model-based clustering analyses showed a greater degree of genetic structuring in the SCR group, whereas less genetic differentiation was found in the region encompassing the SAS group (Fig. 2a, b). The uppermost hierarchical level of genetic structuring (as estimated from the first round of STRUCTURE analyses) resulted in a clustering solution of $K = 3$: cluster A corresponded to samples from North-Western Sardinia (with the exception of one specimen from Isola dei Porri and those from Argentiera, which were grouped in cluster B); cluster B corresponded to samples from Corsica, North-Eastern Sardinia, Argentiera and one specimen from Isola dei Porri; cluster C corresponded to samples from the SAS group (Fig. 2a).

The second and third round of STRUCTURE analyses (overall $K = 7$ and $K = 10$, respectively) showed a further substructuring that was highly consistent with the clustering solution found by BAPS analysis (Fig. 2b), which identified eight genetic clusters, seven of which corresponded to groups of individuals found after the third round of STRUCTURE analyses (Fig. 2a). The results from both analyses showed a clear spatial genetic structuring in North-Western Sardinia, where cluster A was partitioned in subclusters corresponding to Asinara Island, Coscia di Donna, Mal di Ventre Island, and Isola dei Porri (except one specimen) (Fig. 2a, b). Furthermore, cluster B was partitioned in subclusters corresponding to the samples from Corsica and the samples from North-Eastern Sardinia, which formed two further groups (Fig. 2a, b).

BAPS and the second round of STRUCTURE analyses resulted in the individuals from Argentiera (North-Western Sardinia) being grouped with the population of North-Eastern Sardinia (Punta li Francesi, Nido d'Aquila, Molara, and Molarotto), whereas the third round of STRUCTURE analyses grouped the individuals from Argentiera into a distinct cluster (Fig. 2a, b). In cluster C (Fig. 2a, b), two main clusters were defined by both STRUCTURE (from the second round) and BAPS analyses, roughly corresponding to the westernmost (Strait of Gibraltar and Alboran Sea) and the easternmost (Siculo-Tunisian Strait) samples from the SAS group (see Table 1). However, these two clusters did not completely match with spatially separated areas. In fact, the Cape Bon sample (except for one specimen) grouped with the westernmost samples; similarly, some individuals from the Alboran Island grouped with the easternmost samples.

The first two principal coordinates that resulted from PCA carried out on the entire dataset accounted for 60.24% of the total variation, and identified three groups of individuals (Fig. 3a). These groups are consistent with the highest hierarchical structure found in the first round of STRUCTURE analyses (Fig. 2a). Furthermore, PCA ordination based on subsets of individuals corresponding to the aforementioned groups produced plots which tended to resemble the genetic groupings retrieved in the subsequent rounds of STRUCTURE and BAPS analyses (Fig. 3b, c, d). According to these results, genetic groups retrieved by Bayesian-model based clustering are unlikely to be artefacts due to violation of the model assumptions (Hardy-Weinberg and linkage equilibrium) or isolation by distance (Guillot et al. 2009).

All the three grouping schemes tested with the AMOVA (SAS and SCR; Alboran Sea, Siculo-Tunisian Strait, and SCR; clusters A, B and C) showed a significant genetic differentiation ($P < 0.001$) at the highest level of genetic structure

(differentiation among groups) (Table 3). Nevertheless, the grouping matching the first round of STRUCTURE maximised the Φ_{CT} value (Φ_{CT} = 0.301). Among the remaining groupings, that corresponding to SAS and SCR groups showed the highest Φ_{CT} value (Table 3).

The Mantel correlogram that included all of the samples did not display a clear monotonic decrease, thus suggesting a more complex spatial pattern than a simple isolation-by-distance (IBD) or a clinal variation (Fig. 4a). The pattern was partly clinal, with significant positive values that decreased to significant negative values in the 200 km distance class. Following an upward fluctuation, spatial autocorrelation decreased to significantly negative values for distances from 600 up to 1,000 km, after which they displayed a stochastic pattern. These two troughs involved many pairwise comparisons between samples from the clusters A and B in the SCR group as well as between the SCR group and the cluster C (the SAS group).

When the samples were divided into the three main genetic clusters identified by the first round of STRUCTURE analyses (Fig. 2a), the autocorrelation indices showed different spatial patterns for each of these groups (Fig. 4b, c, d). In the first distance class (within population comparisons) clusters A and B (the SCR group) showed a positive spatial autocorrelation (r = 0.151 and r = 0.128, respectively) greater than that found in cluster C (the SAS group) (r = 0.047). Nevertheless in SCR group spatial autocorrelation fell more quickly than in SAS group. In cluster A spatial autocorrelation fell to non significant values in the second distance class (up to 15 km) whereas in cluster B autocorrelation values were positive in the first two distance classes (up to 15 km) (Fig. 4b, c). Significantly negative autocorrelation values were observed at distance classes that involved pairwise comparisons of specimens from different subclusters. On

the other hand, cluster C (the SAS group) showed a spatial positive autocorrelation for distances up to 40 km (Fig. 4d).

Mitochondrial DNA

Sequences of 581 bp (COI), 365 bp (12S), and 488 bp (16S) were obtained. The most common COI haplotype was shared by 165 of the 174 individuals (about 95%), the remaining nine distributed as follow: ALB 3 and CHA 5; CDN 2; CHA 2; PAR 1; PAR 2; MVE 6; ARG 7; LIT 3. Compared to the results of Espinosa and Ozawa (2006), only three new COI haplotypes were found, (Fig. 5).

The three nucleotide substitutions observed in our COI dataset (110 sequences) (Fig. 5) produced the following two amino acid changes: one in the individual ARG 7 (SER-ASN) and one in CDN 2 (ALA-THR) (see also Espinosa and Ozawa 2006). For 12S, only two haplotypes (cf. previous sequences from GenBank: Accession Nos. FJ767829 and FJ767830) were detected, the less common of which was found in three individuals (ARG 9, CGR 1, ACS 7). The unique nucleotide substitution observed produced no amino acid change. For 16S, only one haplotype (cf. previous sequences from GenBank: Accession Nos. FJ767822). was found. Therefore, no further analysis on the 12S and 16S genes was performed. Considering together the 12S, 16S and COI genes we found a 92% of mitochondrial haplotypes homology in *P. ferruginea*.

The following estimates of COI genetic variability were low: the number of haplotypes $h = 9$, the number of polymorphic sites $S = 8$, the nucleotide diversity $\pi = 0.00018$ (± 0.00006), and the haplotype diversity $H = 0.101$ (± 0.032). The network (Fig. 5) based on COI sequences has a star-like configuration with a very common central haplotype that was spread across all locations. The eight additional haplotypes were directly connected to the central one by only one mutation. Both Tajima's (D = -

2.078, $P < 0.001$) and Fu's (Fs = -16.495, $P < 0.001$) neutrality tests on COI displayed a significant departure from mutation-drift equilibrium.

Discussion

ISSR markers show a high level of genetic variability throughout the study area, indicating the presence of three major genetic discontinuities. The uppermost hierarchical structure, detected by STRUCTURE analyses, identified a genetic cluster corresponding to samples from the SAS group and two genetic clusters in the SCR group (Fig. 2a). Remarkably, grouping samples into the three clusters maximised the portion of genetic differentiation among groups with respect to alternative grouping schemes, which, although significant, showed lower fixation indices (Table 3).

The genetic break observed between the two main geographic areas considered in this study (the SAS group and the SCR group) is probably linked to the presence of a barrier to gene flow represented by the Sardinian Channel. No data are available regarding the actual level of larval dispersal for *Patella ferruginea*; however, a stretch of approximately 180 km of open sea, which separates North Africa from Sardinia, is likely to be the most serious hindrance to gene flow between the two regions. Indeed, a low potential for dispersal may enhance the perception of a species to barriers (Baus et al. 2005), particularly in organisms living in fragmented habitats (see Mejri et al. 2009 and references therein). Furthermore, the clear North-South geographic separation of these two groups coincides with some sub-basins of the Mediterranean that have different biological and ecological conditions (Bianchi 2007). Interestingly, the genetic structuring found between the SCR and the SAS groups fits with the surface isotherms recorded in the Mediterranean during February: populations from the SAS group lie between the isotherms of 15° C and 14° C, whereas the SCR group lie between the isotherms of 14° C and 13° C (Bianchi 2007). The population history of *P. ferruginea* from SCR and SAS might thus include fragmentation due to physical-chemical

boundaries, followed by restricted gene flow among sub-basins. Regardless to the causes of such differentiation, other marine invertebrates show a similar pattern of genetic distribution (see Calvo et al. 2009 and references therein).

Conversely, it is not easy to identify in the SCR group the causes for the genetic structuring occurring between cluster A, roughly corresponding to North-Western Sardinia, and cluster B, which encompasses North-Eastern Sardinia and Corsica (Fig. 2a). This fact may be related to the presence of an inhospitable sandy shore extending almost continuously for about 100 km from the Asinara Island to Punta li Francesi that may represent a hindrance to constant and conspicuous level of gene flow in West-East and *viceversa* direction. However, all specimens from Argentiera (North-Western Sardinia) grouped in cluster B, representative of North-Eastern Sardinia and Corsica (Fig. 2a, 3a). The genetic makeup of Argentiera sample may indicate that occasional long distance dispersal events, in a background of reduced gene flow, may occur along the coasts of North of Sardinia and South of Corsica. Although the circulation pattern in this area is very variable due to the complex coastal geometry, nonetheless the two prevailing currents flow eastward and westward, respectively (Gerigny et al. 2011). Nonetheless, our data do not allow to rule out the possibility that sample from Argentiera is representative of a relic population and cluster B had a wider geographical distribution in the past. Notably, the Argentiera site is the only one among those of SCR group characterised by high cliff; additionally, the high hydrodynamic regimes occurring in this area make almost impossible to reach it from the mainland, and harvesting, even by sea, is hazardous. These facts may have contribute to the conservation of traits of an ancient genetic variability.

A second, interesting results is the observed high degree of genetic structuring within SCR group. Indeed, a clear genetic structuring in North-Western Sardinia and, at

a lesser extent, in North Eastern Sardinia and Corsica, was evidenced by Bayesian model based clustering (Fig. 2a, b) and PCA ordination plots (Fig. 3b, c, d). In North-Western Sardinia there is no evident geographic barrier to gene flow which may explain the highly fragmented genetic pattern. On the other hand, its rocky shores are easily accessible (except those of Argentiera), and *P. ferruginea* is subject to regular, high levels of collection for human consumption. These facts lead to hypothesise that past and present human pressure is the main cause of genetic drift of these populations. Indeed, several sites are presently characterised by both very low density and reduced specimen size (Casu, pers. obs.). Although *P. ferruginea* is known to be able to adjust the timing of sex change according to the density of large individuals (Rivera-Ingraham et al. 2011a), the lack of a population of large-sized individuals affects the reproductive rate (Espinosa et al. 2006).

In Corsica and North-Eastern Sardinia, a less marked spatial genetic structure than that found in North-Western Sardinia has been observed (Figs 2a, b, 3c). These findings suggest that gene flow is sufficient enough to prevent high level of genetic differentiation in this area. However, the pattern of genetic distribution in North-Eastern Sardinia is quite complex, due to the overlap of two Bayesian subclusters that do not match any discrete geographic areas. The high small-scale geographical complexity of this area, which mostly encompasses an archipelago with complex hydrodynamic regimes (Pracchi and Terrosu-Asole 1971; Gerigny et al. 2011), may shape the distribution of genetic variability.

In contrast to the patterns observed for the SCR group, the pattern of ISSR genetic variability found in the SAS group is rather homogeneous (Figs 2a, b, 3d). The Mantel correlogram obtained for the SAS group (Fig. 4d) displayed a shape that resembled IBD or an almost clinal variation. Although we cannot exclude the possibility

that this pattern might reflect, in part, the less-dense geographic coverage of sampling sites in some areas of the SAS group, we nonetheless hypothesise that a high level of gene flow occurs in the SAS group. The different degree of genetic structuring may also depend on the larger population sizes encountered in the SAS group than in the SCR group (Casu, Rivera-Ingraham, pers. obs.; Espinosa 2009). The lack of strong population fragmentation may allow a stepping-stone fashioned gene flow (see Kimura and Weiss 1964) along the coastline, which may be favoured by the surface directional current. For instance, the influence of the Algerian Current, which flows eastward (Robinson et al. 2001), may contribute to promote the spread of *P. ferruginea* larvae. Indeed, this current has been cited as responsible of the dispersion by rafting of the vermetid gastropod with direct development *Dendropoma petraeum* (Calvo et al. 2009) which shows low genetic structuring in a region encompassing the Northern African and South-Eastern Spanish coasts from the Atlantic to the Siculo-Tunisian Strait. Nonetheless, the presence of individuals sharing the same genetic makeup as those found in the Zembra and the Sicilian islands in Alboran Island suggests that local currents, gyres and eddies (Send et al. 1999) may also favour an occasional larval dispersal westward.

Unlike the ISSR analysis, mtDNA sequences show no significant genetic differentiation throughout the entire *P. ferruginea* distribution range. Several reasons may be invoked to explain the differences observed between nuclear and mtDNA markers. First, ISSRs depict patterns of multilocus variations at many independent loci, whereas mtDNA reflects variations at a single locus or a small number of concatenated loci. Furthermore, ISSRs may detect higher levels of genetic variability and/or genetic structuring with respect to mtDNA markers. This may be related to the high potential for detecting differences using random or semirandom primers that amplify nuclear

noncoding DNA sequences, which evolve faster and are less constrained by selection than mtDNA genes (*e.g.,* De Aranzamendi et al. 2009). Indeed, mtDNA may be affected by natural selection (Ballard and Whitlock 2004), which may drastically reduce the number of haplotypes. In particular, Bazin et al. (2006) suggested that the mtDNA variation in invertebrates is consistent with the 'genetic draft theory', which implies a reduction of genetic diversity due to positive selection (Gillespie 2001). *Patella ferruginea* mainly occurs in the rocky intertidal zone exposed to wave action, where challenging ecological conditions may enhance the processes of rapid selection for a restricted number of haplotypes. The hypothesis that selection is affecting mtDNA diversity in *P. ferruginea* is supported by the significant departure from mutation-drift equilibrium, as evidenced by the negative values of selective neutrality tests. Although such a signature may also reflect bottlenecks or population expansions, we are persuaded that these demographic processes should be ruled out because, if that were the case, the ISSRs and mtDNA would have displayed similar genetic patterns. Whatever the reason that led to the observed lack of mtDNA genetic variability, our study evidenced the scarce contribute of this molecule to infer on phylogeographic patterns of *P. ferruginea*.

Implications for conservation

As the most endangered macroinvertebrate in the Mediterranean, *P. ferruginea* urgently requires a conservation plan (Guerra-García et al. 2004). With the exception of some North African *P. ferruginea* populations (Templado 2001), most others appear to be in clear regression and below viable limits (Templado and Moreno 1997). To alleviate this problem, Templado (2001) suggested the translocation of individuals from well-settled

areas as a valuable management practice. Recent studies also suggest that patellid larvae can be recruited on artificial surfaces, which can later be successfully translocated (Rivera-Ingraham et al. 2011b). However, although low-diversity mtDNA lineages are typically regarded as unimportant from a conservation viewpoint, our results based on multilocus nuclear markers suggest that great caution should be exercised when translocations are planned due to the genetic heterogeneity observed even on a very small local scale. In this context, the ISSR genetic differentiation found within the SCR group may be of crucial importance to conservation efforts. Whatever the causes, a strong genetic structuring in the North-Western Sardinia has been observed. In this area the Argentiera sample, in which individuals show the genetic makeup of samples from North-Eastern Sardinia and Corsica (cluster B) (Fig 2a, b), may represent a conservation unit.

Another population that may be regarded as a conservation unit is that from the protected area of Asinara Island (North-Western Sardinia). The three subsamples analysed (Cala Sant'Andrea, Punta Sabina, and Pedra Bianca, Table 1, Fig. 1) show a high degree of identity, suggesting an efficient gene flow within the island. Furthermore, the scattering of its individuals is greater than that of other samples from both the SCR group and the SAS group, as evidenced by the PCA ordination (Fig. 3a, b), which suggests the presence of a preserved population on this island. However, this population appears to be isolated from the neighbouring, non protected areas of North-Western Sardinia (such as Coscia di Donna and Isola dei Porri), leading to potential determination of the Asinara population as a peripheral isolate (Frey 1993). More studies on larval recruitment in this area may help to better understand whether the Asinara population also represents a "source population" (*sensu* Pulliam 1988) of genetic variability. This variability must be preserved to assure the viability of the

neighbouring populations, provided that the harvesting of *P. ferruginea* is totally forbidden.

Our data from ISSRs also suggest that in the SAS the populations from the Zembra and Sicilian islands may fit the definition of “source-sink populations” (*sensu* Pulliam 1988), with the small populations from Pantelleria, Marettimo and Favignana derived from the Zembra island by the effect of the main marine currents. Thus, recovery of these populations could be linked to an effective plan to protect the population of Zembra.

The presence of different possible conservation units reflects the absence of effective gene flow among some populations. Such populations are at high risk of extinction because the immigration rates are close to zero, hindering the rate of population recovery, and often their population sizes are low to very low. Consequently, specific protection measures should be applied to these populations. The success of such measures clearly depends primarily on the reinforcement of the ban on further removal of *P. ferruginea* individuals.

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FIGURES

Fig. 1 Sampling locations of *P. ferruginea*. In the upper box, the main geographic areas analysed in the western Mediterranean are shown. In the following boxes (a, b, c, d) the collection sites at each area are reported. a) Sardinia and Corsica; b) Siculo-Tunisian Strait; c) Strait of Gibraltar; d) Alboran Sea; e) the position of the Western Mediterranean in the world map. Abbreviations are given in Table 1.

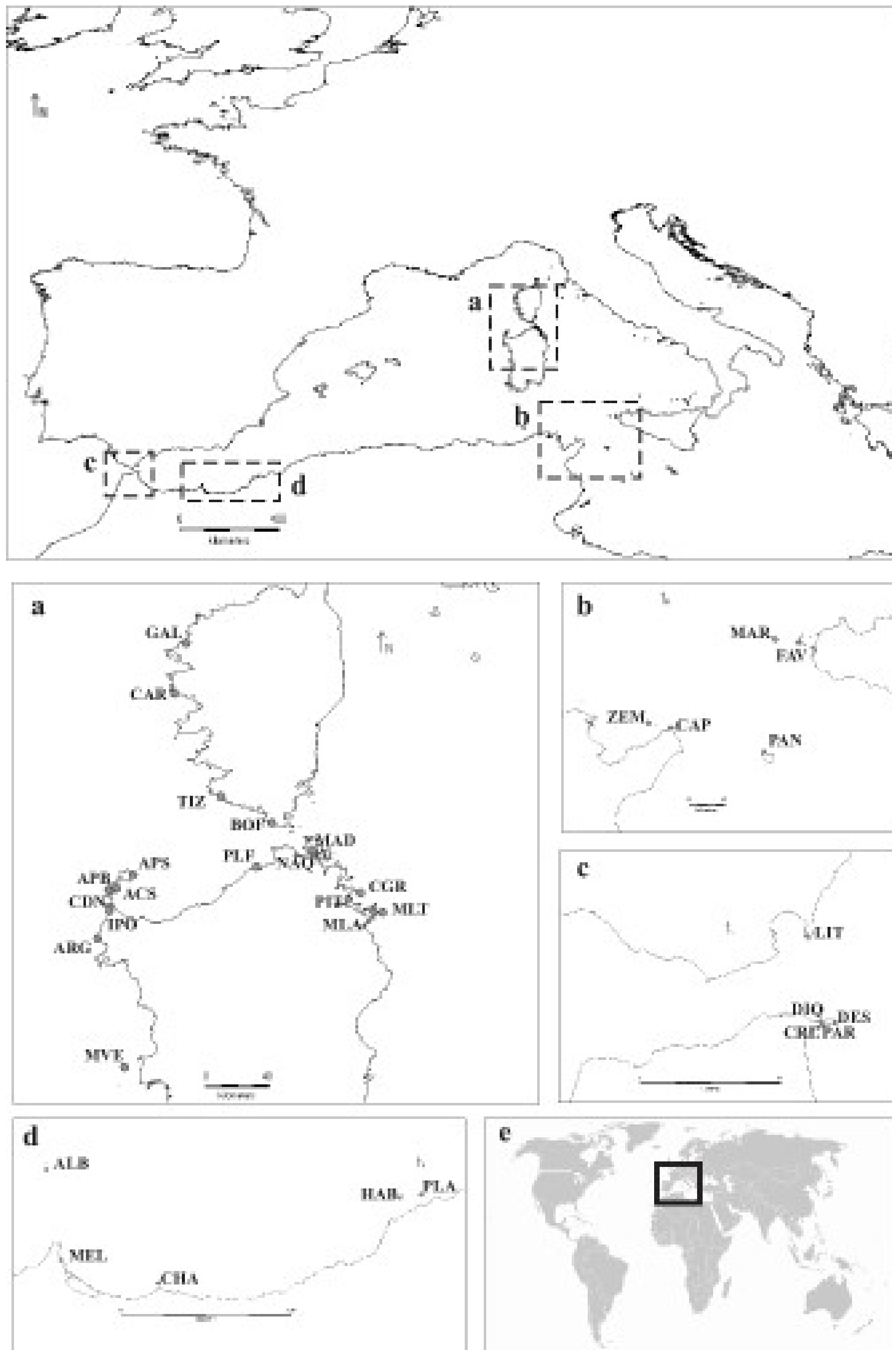
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Fig. 2 ISSR dataset: estimated genetic structure in *P. ferruginea* as inferred using the Bayesian model-based clustering analysis. a) Results of STRUCTURE and b) results of BAPS analyses.

Each individual is represented by a thin vertical line, which is partitioned into *K*-coloured segments (*K* = number of clusters). The height of each segment is proportional to the individual estimated membership in the corresponding cluster. Black lines separate individuals from different sampling sites ordered as in Table 1. Abbreviations are given in Table 1.

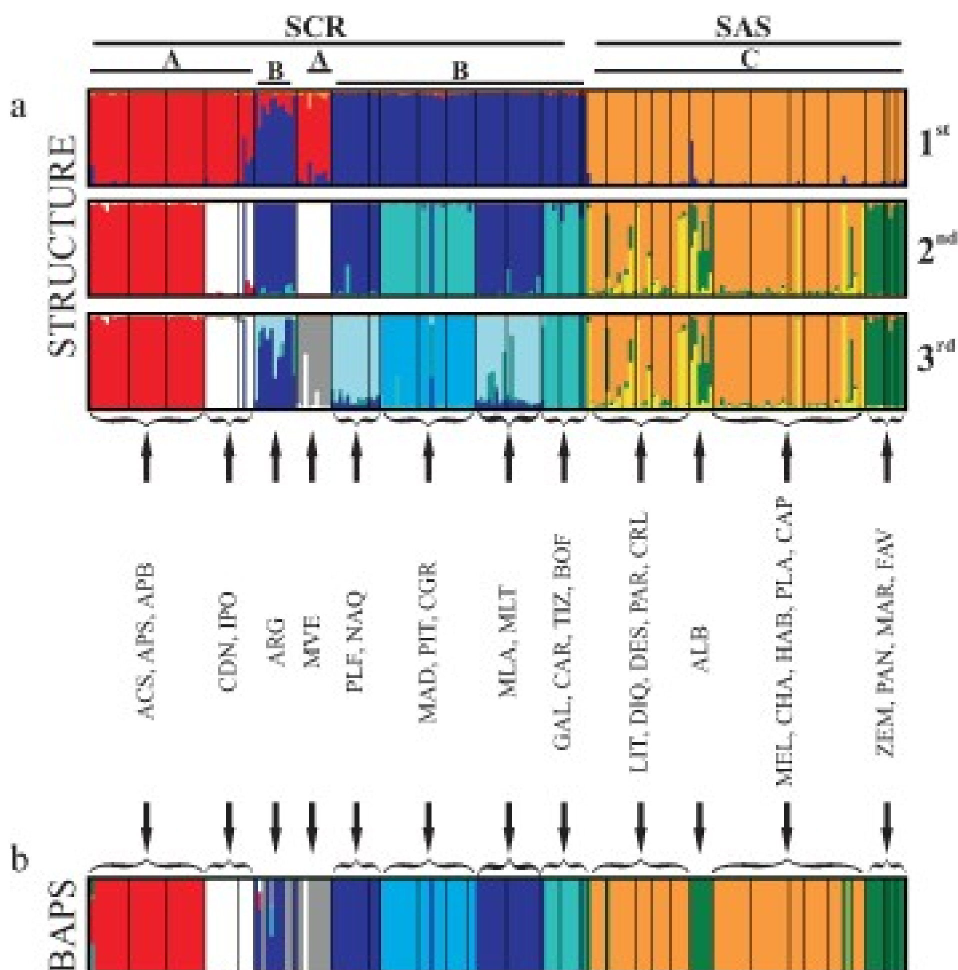


Fig. 3 ISSR dataset: Principal Coordinate Analysis (PCA) of *P. ferruginea*. The ordination plots displays the relationships between individuals according to the first two axes of variation. The first and second principal coordinates are plotted on the x- and y-axes, respectively. PCA plots include: a) all samples; b) cluster A; c) cluster B; and d) cluster C, as retrieved by STRUCTURE analyses. Abbreviations are the same as those in Table 1.

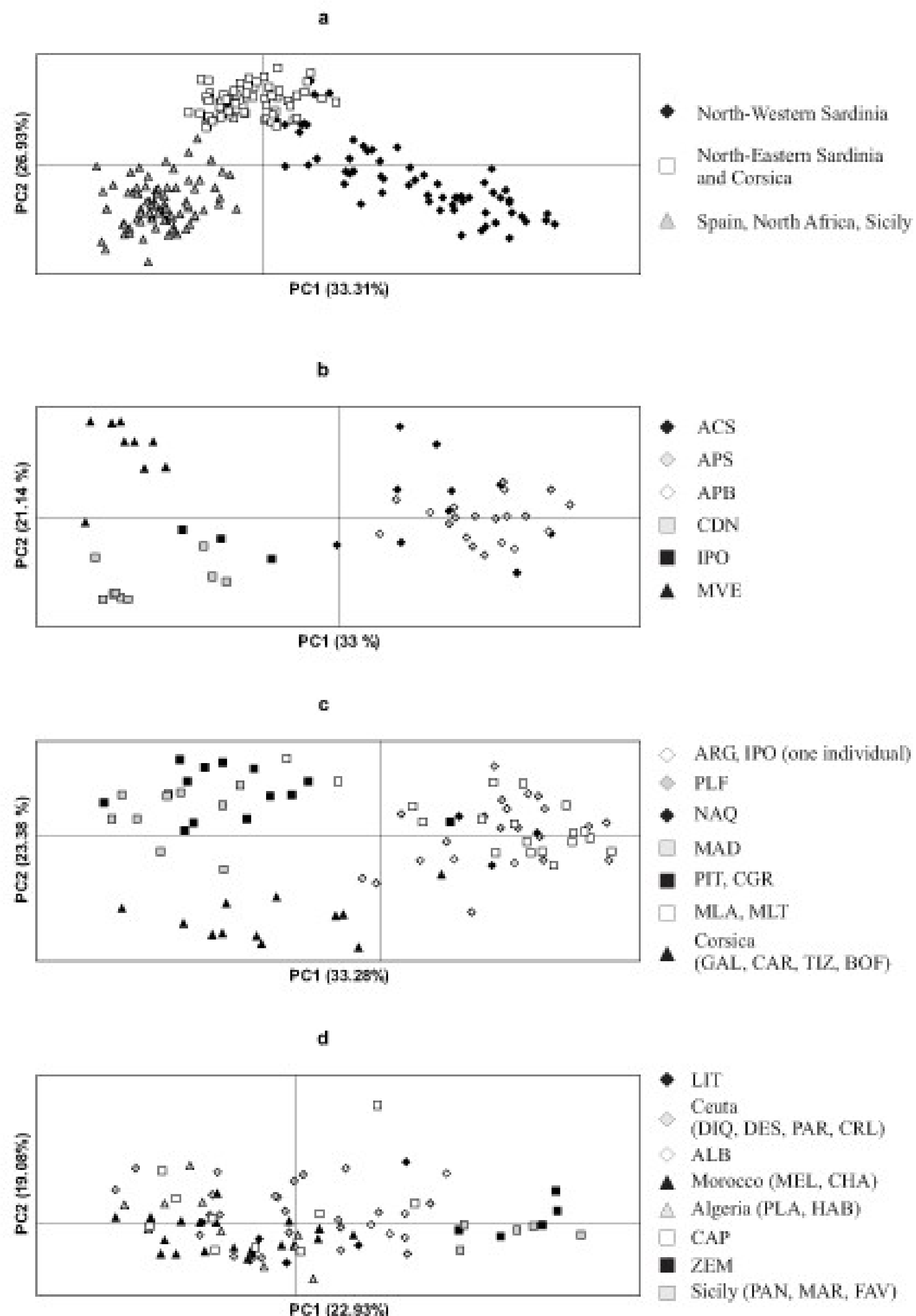


Fig. 4 ISSR dataset: spatial autocorrelation analyses of *P. ferruginea* based on Mantel correlograms.

Dotted black lines bound the 95% confidence interval of the distribution of permuted *r*-values.. Error bars about *r* indicate 95% confidence limits of each *r*-value estimated by bootstrap. Mantel correlograms include: a) all samples; b) cluster A; c) cluster B; and d) cluster C, as retrieved by STRUCTURE analyses. Distance classes are plotted on the x-axis; genetic correlation is plotted on the y-axis.

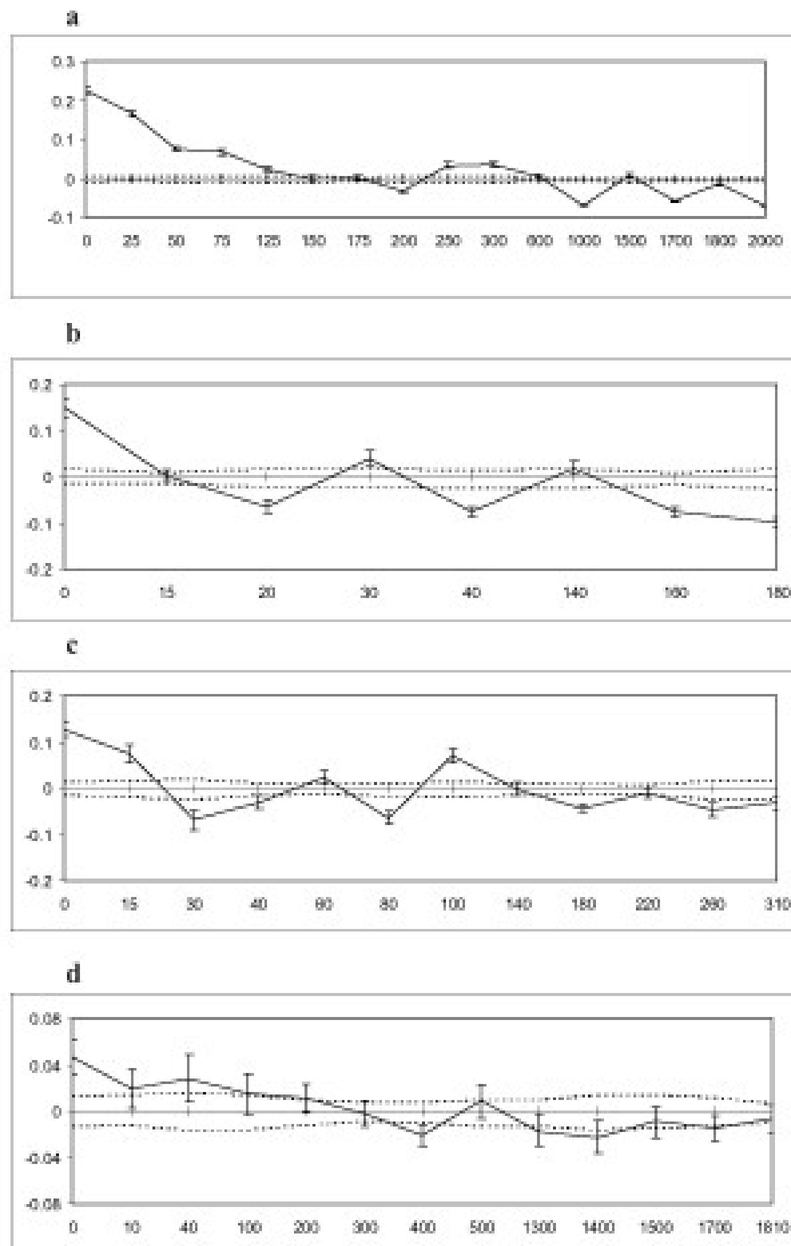
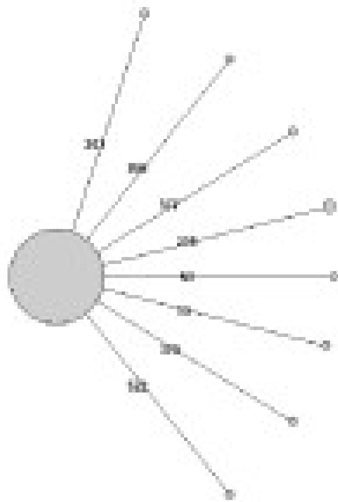


Fig. 5 Mitochondrial DNA dataset: network based on COI sequences.

Circle size is proportional to the number of individuals sharing the same haplotype. The largest circle contains 169 specimens of *P. ferruginea*. Labels on branches indicate the position at which mutations have occurred (mutated position: ARG 7: 13; MVE 6: 53; CDN 2: 252; CHA 2: 266; ALB 3 and CHA 5: 311; PAR2: 370; LIT 3: 522; PAR 1: 577).



TABLES

Table 1 Sampling details of *P. ferruginea*. Asterisks (*) follow individuals whose COI sequences were obtained from GenBank

Sampling station	Code	Geographical location of sampling station	Latitude; Longitude	Sampling period	No. of individuals analysed
					ISSR/mtDNA
Cala Sant'Andrea	ACS	NW Sardinia	41°00'52.17"N; 08°15'08.65"E	Mar. 2005	10/7 (3*)
Punta Sabina	APS	NW Sardinia	41°05'21.35"N; 08°21'03.07"E	Mar. 2005	10/8 (4*)
Pedra Bianca	APB	NW Sardinia	41°00'09.00"N; 08°12'34.44"E	Mar. 2005	10/8 (4*)
Coscia di Donna	CDN	NW Sardinia	40°54'00.83"N; 08°13'09.85"E	Aug. 2006	9/8
Isola dei Porri	IPO	NW Sardinia	40°52'53.61"N; 08°12'52.04"E	Aug. 2006	4/4
Argentiera	ARG	NW Sardinia	40°43'30.69"N; 08°08'50.30"E	Feb. 2007	11/9 (4*)
Mal di Ventre	MVE	NW Sardinia	39°59'23.75"N; 08°17'52.32"E	Mar. 2005	9/8 (5*)
Punta li Francesi	PLF	NE Sardinia	41°08'09.56"N; 09°03'09.44"E	Feb. 2007	10/7
Nido d'Aquila	NAQ	NE Sardinia	41°12'52.84"N; 09°22'47.56"E	Feb. 2007	3/2
Madonnetta	MAD	NE Sardinia	41°13'50.30"N; 09°22'34.28"E	Sept. 2009	10/8
Pittulongu	PIT	NE Sardinia	40°57'06.31"N; 09°34'40.81"E	Nov. 2009	7/6
Cala Greca	CGR	NE Sardinia	40°59'14.72"N; 09°39'12.99"E	Nov. 2009	8/8
Molara	MLA	NE Sardinia	40°52'35.66"N; 09°43'48.04"E	Jun. 2008	8/7
Molarotto	MLT	NE Sardinia	40°52'27.52"N; 09°46'43.64"E	Jun. 2008	9/7 (4*)
Galeria	GAL	NW Corsica	42°24'55.70"N; 08°39'08.29"E	Jan. 2009	5/5
Cargese	CAR	NW Corsica	42°07'44.24"N; 08°35'13.82"E	Jan. 2009	5/5
Tizzano	TIZ	SW Corsica	41°32'19.26"N; 08°51'03.41"E	Jan. 2009	1/1
Bonifacio	BOF	SW Corsica	41°23'14.17"N; 09°08'37.96"E	Jan. 2009	1/1
Gibraltar	LIT	Strait of Gibraltar	36º07'13.10"N; 05º21'09.38"W	Sept. 2004	5/4*
Dique de Poniente	DIQ	Strait of Gibraltar	35º53'53.61"N; 05º19'06.02"W	Oct. 2004	8/6*
Desnarigado	DES	Strait of Gibraltar	35º53'38.57"N; 05º16'52.34"W	Sept. 2008	4/3
Parque Mediterráneo	PAR	Strait of Gibraltar	35º53'23.89"N; 05º18'37.60"W	Sept. 2004	5/4*
Chorrillo	CRL	Strait of Gibraltar	35º52'52.84"N: 05º19'25.62"W	Sept. 2008	5/5
Alboran Is.	ALB	Alboran Sea	35º54'02.59"N; 03º02'01.45"W	Sept. 2004	6/4*
Melilla	MEL	Alboran Sea	35º19'28.23"N; 02º57'21.16"W	Sept. 2004	10/5*
Chafarinas Is.	CHA	Alboran Sea	35º10'54.04"N: 02º25'54.18"W	Sept. 2004	10/8*
Plane Is.	PLA	Alboran Sea	35º19'08.48"N; 01º28'45.00"W	Apr. 2008	4/4
Habibas Is.	HAB	Alboran Sea	35º43'09.69"N: 01º08'16.33"W	Apr. 2008	6/4
Cape Bon	CAP	Siculo-Tunisian Strait	36º27'12.19"N; 10º47'29.90"E	Apr. 2008	10/9*
Zembra Is.	ZEM	Siculo-Tunisian Strait	37°07'44.65"N; 10°47'32.70"E	Jun. 2009	5/4
Pantelleria	PAN	Siculo-Tunisian Strait	36°50'22.32"N; 11°57'14.26"E	Jun. 2009	1/1
Marettimo	MAR	Siculo-Tunisian Strait	37°58'14.41"N: 12°04'15.90"E	Jun. 2009	2/2
Favignana	FAV	Siculo-Tunisian Strait	37°57'08.48"N; 12°18'12.73"E	Jun. 2009	2/2

Table 2 ISSR dataset: primer names and sequences, number of polymorphic bands *per* primer and range of molecular weight in base pairs (bp)

Primer	Sequence (5'-3')	No. of bands	Size range of bands (bp)
IT1	$(CA)_8GT$	9	650-1500
IT2	$(CA)_8AC$	6	600-1700
IT3	$(CA)_8AG$	11	500-1600
SAS1	$(GTG)_4GC$	8	550-1500
SAS3	$(GAG)_4GC$	9	600-1500
UBC811	$(GA)_8C$	7	500-1800
UBC827	$(AC)_8G$	10	500-1600

Table 3: ISSR dataset: three-level analysis of molecular variance (AMOVA). Groups for three-level AMOVA were defined *a priori* (SAS vs SCR, or SCR, Alboran sea and Siculo-Tunisian strait), either inferred according to PCoA (Principal coordinate Analysis) and model-based clustering analyses. d.f.: degrees of freedom, SS: sum of squares, var. comp.: variance component, %var: percentage of variation. ** $p < 0.01$, *** $p < 0.001$

Source of variation	d.f.	SS	Var. comp.	%var	Φ-*statistics*
SAS (Spain, Africa, Sicily) vs SCR (sardinian-Corsican region)					
Among groups	1	238.461	2.101	21.07	Φ_{CT} = 0.211***
Among populations within groups	26	638.136	2.542	25.49	Φ_{SC} = 0.330***
Within populations	185	985.901	5.329	53.44	Φ_{ST} = 0.466***
Alboran sea, Siculo-Tunisian strait, Sardinian-Corsican region					
Among groups	2	257.173	1.829	18.80	Φ_{CT} = 0.188***
Among populations within groups	25	619.424	2.570	26.42	Φ_{SC} = 0.325***
Within populations	185	985.901	5.329	54.78	Φ_{ST} = 0.452***
Cluster A, cluster B, cluster C					
Among groups	2	451.701	2.971	30.14	Φ_{CT} = 0.301***
Among populations within groups	25	424.826	1.557	15.80	Φ_{SC} = 0.226***
Within populations	185	985.901	5.329	54.06	Φ_{ST} = 0.459***

Analysis of the genetic structure of the Fan Mussel *Pinna nobilis* (Mollusca: Bivalvia): a mtDNA perspective

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Introduction

Bivalves (Mollusca: Bivalvia) represent one of the most common organisms in marine and freshwater environments (Morton, 1996), whose free-swimming pelagic larval phase (veliger) may be involved in the high potential for dispersal of their species (Gosling, 2003). Even if different environmental parameters among marine basins may shape dispersal patterns influencing biodiversity (Bianchi 2007), marine habitats, when opposing a low level of physical barriers to genetic exchange, could play an important role in the dispersion even for bivalve sessile species with a benthonic life cycle. In fact, high rates of gene flow mediated by elevated levels of dispersal could affect evolutionary genetic processes independently from geographical factors. In addition the efficiency of species' potential for dispersal and recruitment is strongly involved in capability of ocean currents to transport larvae (Domeier, 2004; Peharda and Vilibić, 2008). Therefore, the knowledge of potential for dispersal and genetic settlement of species along their range of distribution, could be helpful in depicting the population dynamics which contributed to set their biogeographic background tracing signatures in the spatial distribution of molecular variation (Avise et al. 1987).

Under a molecular perspective, different authors tried to shed some light on the early origin of the class of bivalves. However, conflicting results produced a confused perspective on this topic, probably as consequence of the bivalves rapid genome evolution (Huvet et al. 2000; Launey and Hedgecock 2001, Knudsen et al. 2006; Plazzi and Passamonti 2010). Only recently Plazzi and Passamonti (2010), using four mitochondrial markers, evidenced that all main deep events in bivalves radiation took place in a relatively short 70 Myr time during late Cambrian/early Ordovician. Among the class of Bivalvia, mussels (*Mytilus, Pinna*), scallops (*Pecten, Chlamys*), oysters

(*Ostrea, Crassostrea*) and arks (*Arca, Scapharca*) are representatives of the monophyletic species-rich subclass Pteriomorphia (Matsumoto, 2003).

The taxonomic resolution of this subclass is puzzling (Purchon, 1987; Waller, 1990; Morton, 1996; Salvini-Plawen and Steiner, 1996; Cope, 1996, 1997), and led some authors to hypothesize a polyphyly (Carter, 1990; Starobogatov, 1992; Giribet and Distel, 2003), probably due to a rapid radiation event in its early evolution (Campbell, 2000; Steiner and Hammer, 2000; Matsumoto, 2003).

One of the best known species included within this subclass is the Mediterranean endemic fan mussel, *Pinna nobilis* Linnaeus, 1758. It belongs to the superfamily Pinnoidea which shows its closer genetic relationship (on mitochondrial bases) with the Pteriomorphia superfamilies Pterioidea and Osteoidea (Matsumoto, 2003). It occurs at depths between 0.5 and 60 m, in soft and sandy bottoms mostly characterized by seagrass meadows (Zavodnik, 1967; Zavodnik et al., 1991; Katsanevakis, 2005); individuals attain lengths whose maximum values of 1 m (Zavodnik et al., 1991) led to consider the species as one of the largest among bivalves.

In the western Mediterranean, *P. nobilis* reproduces in the summer period, from June to August (De Gaulejac, 1995). Embryos diffuse in the water column as veliger larvae stadium (Butler et al., 1993) before settling in the sediment and anchoring by means of byssus produced by juveniles. A very little is known about the length of larval life cycle of *P. nobilis* which can be considered equivalent to a period of 5–10 days (De Gaulejac and Vicente, 1990). Anyway, according to what found for bivalves' larvae by Gosling (2003), it is supposed that *P. nobilis* veliger exhibits daily migration across the water column tending to pass daylight hours in less illuminated and deeper waters and night time on superficial ones (Peharda and Vilibić, 2008). Taking into account such trend *P. nobilis* larvae could diffuse by means of tidal currents especially during the

night hours restricting their movement to about eight hours per day (Peharda and Vilibić, 2008). Furthermore, the larval life cycle of this species, as it happens in almost all species of class Bivalvia, could be strongly affected by environmental conditions of its habitat such as temperature, salinity and food ratio (Gosling, 2003). For this reason, potential for dispersal and gene flow characterizing Mediterranean populations of *P. nobilis* should be evaluated in the light of their geographic distribution and environmental conditions across the basin.

Even if the species is distributed throughout the whole Mediterranean, till now only populations belonging to few areas have been investigated. In particular the literature provides works dealing with ecological aspects of populations from the Gulf of Lion (Vicente, 1990), the Ionian Sea (Giacobbe and Leonardi, 1987; Centoducati et al., 2007), the Balearic Sea (García-March, 2005), the Adriatic Sea (Peharda and Vilibić, 2008), the Sardinian (Porcheddu et al. 1998; Caronni et al. 2007; Addis et al. 2009) and Tunisian coastline (Tlig-Zouari, 1993; Rabaoui et al., 2008, 2010) and the Aegean Sea (Katsanevakis, 2005; Galinou-Mitsoudi et al., 2006).

The only two studies carried out on the molecular aspects of this species, analyzed Mediterranean populations from Aegean Sea (Katsares et al., 2008) and Tunisian coasts (Rabaoui et al., 2011).

The former study analysed the genetic variability of four Greek populations obtained by means of the mitochondrial markers Cytochrome c Oxidase subunit I (COI) and 16S rDNA. High levels of haplotypic diversity were found for COI gene even considering the lack of genetic structuring among populations. On the other hand, Rabaoui et al.(2011) used COI sequences to describe the genetic variability of five populations from northern, eastern, and southern Tunisian coastline and further evaluated wether the two morphological forms of this species were genetically differentiated.The authors

found a North-East decreasing gradient of genetic variability among populations that was explained in the light of the variance in hydrographic parameters of the area analysed. No evidence of molecular divergence was found between the two morphological forms.

Starting from such studies, increasing the researchers' efforts in improving molecular analysis on the *P. nobilis* populations could be helpful to better clarify how environmental parameters and anthropogenic stress could have acted on the population dynamics of this species together with stochastic forces such as founder effects or genetic bottlenecks.

Up two decades ago the populations of *P. nobilis* throughout the whole Mediterranean have been endangered by the reduction of their natural habitat as main consequence of marine pollution and human activities principally involved in recreational and commercial fishing (Vincente, 1990; Zavodnik et al., 1991; Vicente and Moreteau, 1991; Richardson et al., 2004; Rabaoui et al., 2010).

In order to cope with the decreasing population density of this species, in 1995 *P. nobilis* has been included in the list of Mediterranean endangered species (Annex IV of the Habitat Directive and Annex II of the Barcelona Convention).

In such context, the aim of this work is to provide the first insight on genetic variability and phylogeographic patterns characterizing populations of *P. nobilis* from both western and eastern Mediterranean areas. Furthermore, since bivalve's fecundity usually increases with size and age (Gosling, 2003) with important implications on recruitment effect, a specific non-lethal sampling method was developed in order to collect samples without killing individuals which can live for over 20 years (Richardson et al., 1999; Šiletić and Peharda, 2003; Richardson et al., 2004). Mitochondrial markers

(COI and 16S) were used with the aim to compare results with those obtained by Katsares (2008) and Ramboui (2011) for Aegean and Tunisian populations respectively.

Materials and Methods

Sampling

A total of 212 specimens belonging to 24 Mediterranean populations (Table 1 and Fig. 1) were collected. Where possible, at least ten individuals were analysed for each sample. A non-lethal sampling method was applied by abscising a small portion of mantle tissue in situ without sacrificing the individual.

Analytical methods

DNA extraction: DNA was isolated using the Qiagen DNeasy tissue kit. The DNA concentration was estimated by means of fluorimetric measurements (average value per sample: 100 ng/μl).

PCR amplification: COI and 16S rDNA mitochondrial regions were amplified using specific primers designed by the Authors for the COI gene (L: 5'-ggttgaactathtatccncc-3' and H: 5'-gaaatcatyccaaaagc-3') and primers provided by Wilson and colleagues (2001) for 16S gene. Each 25 μl PCR mixture contained about 100 ng of total genomic DNA, 0.32 μM of each primer and 2.5 U of EuroTaq DNA Polymerase (Euroclone) 1× reaction buffer and 200 μM of each dNTP. The $MgCl_2$ concentration was set at 3 mM and 25 μg of BSA were added to the reaction mix.

PCR amplifications were performed according to the following steps: 1 cycle of 2 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 50°C and 1 min 30 s at 72°C each. At the end, a post-treatment of 5 min at 72°C and a final cooling at 4°C were applied. For all PCR reactions negative controls and replicates were included. Electrophoretic

runs were carried out at 4 V/cm for 20min on 2% agarose gels, made using 0.5× TBE buffer with ethidium bromide (10 mg/ml) for staining of DNA fragments. PCR products were purified using ExoSAP-IT (USB Corporation), and sequenced using an external sequencing core service (Macrogen Inc., Korea). Each mitochondrial region was sequenced in both forward and reverse directions and the corresponding sequencing runs were repeated twice in order to verify the reliability of results.

Data analysis: Sequences were aligned using the program Clustal W (Thompson et al. 1994), implemented in BioEdit 7.0.5.2 software package (Hall 1999), and deposited in GenBank (Accession Nos.XXXX).

The best probabilistic model of sequence evolution was determined for each mtDNA region using the program jModeltest 0.1.1 (Posada 2008) with a maximum likelihood optimised search, using the Akaike Information Criterion (AIC).

Within population genetic variability –was estimated taking into accout insertion/deletions; number of polymorphic sites (S), number of haplotypes (H), nucleotide diversity (π), haplotype diversity (h), and mean number of pairwise differences (d), were estimated with the software package DnaSP 5.10 (Librado and Rozas 1999).

Genetic relationships among individuals were checked by a Median-Joining network (Bandelt et al. 1999), using the software package Network 4.5.0.1 (www.fluxus-engineering.com). Transitions and transversions were equally weighted, whereas, for the COI region, mutations were inversely weighted according to the estimated number of mutational changes (more weight was given to mutational events that occurred only once).

The occurrence of genetic structuring among samples was investigated by the Bayesian model-based clustering algorithm implemented in Baps 5.2 (Corander and

Tang 2007). This method uses a stochastic optimization algorithm approach to retrieve the posterior mode of the number of genetically diverged groups (i.e. *K* is estimated as a random variable). The clustering was performed using the module for linked molecular data and applying the codon linkage model which
is the appropriate choice for sequence data. Each analysis was performed ten times with a vector of values (1-22) for *K* each with six replicates.

Haplotypes were grouped into haplogroups that reflected clustering results; for each sampling location we computed the proportion of a given haplogroup in order to build interpolation maps of the haplogroup frequency in the Mediterranean. We used the inversed weighted distance (IDW) method implemented in gstat (Pebesma 2004) and interpolated the estimated values Raster (Hijmans and Van Etten 2011). These computations were made with R 2.13.0 of which these libraries are part (R development core team 2011).The Analysis of Molecular Variance (AMOVA) was performed by using the software package Arlequin 3.5.1.2 (Excoffier and Lischer 2010). Individuals were grouped according to their geographical provenience. Furthermore pairwise Φ_{ST} values based on Tamura and Nei (1993) genetic distances were calculated using the gamma value (γ) obtained in jModelTest. The significance of variance components and *F*-statistics was tested by a permutation test with 1,000 replicates.

The fit of the data to ademographic expansion model was assessed by computing the mismatch distribution (distribution of the pairwise differences). Additionally, Tajima's D (Tajima, 1989; Nei and Kumar, 2000) and Fu's (1997), F_S neutrality tests implemented in Arlequin were used to assess departures from mutation drif-equilibrium model. Significant negative values for both tests are expected under recent population expansion, selective sweeps, or purifying selection.

Principal components analysis (PCA) was carried out on the mitochondrial haplogroups' frequencies distribution using the software package XL Stat (AddinSoft, Paris) in order to obtain a bi-dimensional display of experimental data drawn by the software Surfer 6.04 (Golden Software, Golden, CO).

Results

Dual peaks of similar height, which could be interpreted as evidence of mitochondrial pseudogenes in the nucleus (Numts) (Sorenson and Quinn, 1998) or heteroplasmy were not observed in sequences' electropherograms. Furthermore, PCR products (resolved as electrophoresis bands) did not show any occurrence of aspecificity, excluding the possibility of multiple nuclear mtDNA-like sequences.

COI

We obtained a 338 base pairs (bp) long sequence alignment. Our dataset was enlarged including 26 specimens belonging to four Grecian populations (Katsares et al. 2008) and 49 individuals from five Tunisian populations (Rabaoui et al. 2001) (see Table 1 for details). JModelTest calculated as appropriate model of nucleotide substitution the model TPM2uf+I+G with gamma shape $\gamma = 0.3670$.

The 42 polymorphic sites (S) found defined 58 different haplotypes with an average haplotype diversity (h) equal to 0.700 and a correspondent average nucleotide diversity (π) equal to 0.006. Grecian and Tunisian samples showed the lowest values of h and π (Table 2).

Median-Joining network analysis performed on the pooled dataset provided an overall low level of divergence among the haplotypes (Fig. 2). With the merely exception of a single Grecian individual from Epanomi, the haplotypes diverged each other for one/two point mutations only. furthermore most individuals were grouped into five main haplotypes compatible with a sharp founder effects.. There was no clear evidence of haplotypes geographic structuring at both local (among populations) and regional (among basins) spatial scales among populations was evidenced (Fig. 2a). Nonetheless, grouping samples according to seven Mediterranean macro-areas of

Sardinia (SAR), Sicily (SIC), Elba Island (ELB), Venetian Lagoon (VL), Cyprus (CIP), our Tunisian sample (BIZ), Greece (GRE) and Tunisia (TUN) (Fig. 2b), no haplotype was shared between Tunisian and Grecian samples and the other populations.

The Bayesian analysis evidenced the occurrence of four distinct groups of haplotypes (P1, P2, P3 and P4). When displayed on the network graphic, each of them appears characterised by one or two main haplotypes with derived variability set in a star-like network configuration (Fig. 3). Three of the mitochondrial COI haplogroups found (P1, P2 and P3) belong to the samples from Sardinia, Sicily, Elba Island, Venetian Lagoon and to the single Tunisian individual (BIZ) from our dataset without evidence of geographic structuring, while all the other samples from Tunisia and Greece belong to the fourth group (P4) with the exception of one Grecian individual from Epanomi and three Tunisian specimens from Monastir which grouped with P2 and P3 haplogroups respectively.

The interpolation of haplogroups' frequencies (Table 3) over the Mediterranean map gives a snapshot of their potential geographic distribution (Fig. 4) . The haplogroup P1 is spans across the western Mediterranean, reachs Sicily and spreads into the Ionian sea. The haplogroup did not display a gradient of decreasing frequencies as higher values were found in northern Sardinia, and in both North-West and South-East of Sicily (Fig. 4a). Tough the haplogroup P2 is the most geographically spread across the whole Mediterranean basin it displays a lower average frequency than P1 (0.22 and 0.30, respectively. It reaches its higher frequencies in areas as far as northern Adriatic sea and Sardinian sea (Oristano, western Sardinia) (Fig. 4b). The haplogroup P3 spreads in western Mediterranean, Sicily, Adriatic Sea and sporadically in Tunisia (two individuals from Monastir), and it is characterised by the lowest average frequency (0.06).. Its higher level of frequency correspond to the Elba Island, Molara in the North-

eastern cost of Sardinia and Alghero in the north-western Sardinian coast (Fig. 4c). The haplogroup P4 is the most frequent in eastern Mediterranean and it is found exclusively in Grecian and Tunisian populations in which displays high rates of frequency (Fig. 4d).

PCA carried out on the basis of frequencies' distribution of the COI mitochondrial haplogroups identified by Bayesian analysis (P1, P2, P3 and P4) was displayed by drawing frequencies maps (Fig. 5). The first principal component, which accounts for 47.28% of total variance, displays two centres of expansion in eastern Mediterranean corresponding to Grecian and Tunisian areas (Fig. 5a). The second principal component, which accounts for 21.01% of total variance, displays a centre of expansion in the western Mediterranean with the highest level of variability in the Tyrrhenian Sea (Fig. 5b). The third principal component, which accounts for 18.30% of total variance, displays a centre of expansion in the Adriatic Sea corresponding to the Venetian Lagoon (Fig. 5c).

Grouping populations within seven Mediterranean macro-areas (Sardinia, Sicily, Elba Island, Venetian Lagoon, Greece and Tunisia), AMOVA was performed setting $\gamma = 0.367$ after jModeltest estimation (Table 4). Samples with less than 5 individuals were excluded from the analysis.

The datasets showed a within-population variation higher (81.17%) than that among groups (37.82%). Three groups of populations were identified as divergent with correspondent highly significant *P* values (Table 4): i) the group (SES) correspondent to the Mediterranean westernmost areas (Sardinia and Elba Island) and Sicily; ii) the group (VL) correspondent to the Adriatic Sea area (Venetian Lagoon); and iii) the group (GT) correspondent to the Mediterranean easternmost areas among those considered (Greece) and Tunisia.

The mismatch analysis carried out on the whole dataset (Fig. 6a) showed an incipient bell-shape curve. A similar trend is shown by analysis of SES (Fig. 6b) and VL (Fig. 6c) groups while a starting shape curve occurs in the mismatch distribution of GT group (Fig. 6d).

Outcomes provided by mismatch analysis were confirmed by the statistical significance - with the only exception of Venetian population as probably consequence of its reduced size - of Tajima's D and Fu’s FS negative values of the neutrality tests (Table 5).

16S

A total of 199 individuals were analysed obtaining a 497 bp long sequences alignment. With the only exception of Network analysis, such dataset has been analysed without comparison with other Mediterranean populations since the only sequences of this gene available at the moment for *Pinna nobilis* correspond to the two haplotypes – whose sequences are eighth base pairs shorter than ours (490 bp) - found by Katsares et al.(2008) among Grecian populations.

JModelTest calculated as appropriate model of nucleotide substitution the custom model TrN+G with gamma shape $\gamma = 0.0110$.

The 29 polymorphic sites (S) found defined 34 different haplotypes with an average haplotype diversity (*h*) equal to 0.542 and a correspondent average nucleotide diversity (π) equal to 0.002 (Table 6).

Median-Joining network analysis (Fig. 7) was carried out including in our dataset 25 sequences belonging to Grecian populations (Katsares et al. 2008) and representing the only two 16S haplotypes known till now for this species. The most diffused haplotypes (G1) characterises populations of Epanomi, Aggeloxori and

Korinthiakos, while the less diffused one (G2) characterises populations of Xios. In order to analyse sequences of equal length, we excluded from the analysis the first eight bp fragments from our sequence dataset obtaining a final 490 bp long sequences alignment. Network graphic output provided a star-like configuration with a main haplotype shared by 49% of individuals differing from surrounding haplotypes, including Grecian ones, for one/two point mutations only. No evidence of geographic structuring occurred neither at local scale considering singularly all populations (data not shown) nor at larger scale grouping populations in seven macro-areas (SAR, SIC, ELBV, VL, CYP, BIZ and GRE), with the only exception of the Grecian populations which do not share haplotypes with others. It is interesting to note that a Cyprian haplotype appears included in the Grecian variability differing for a single point mutation from Grecian founder haplotype.

The Bayesian analysis evidenced the occurrence of three distinct groups of haplotypes (Fig. 8). The first group, shared by 69% of individuals, is present in almost all populations analysed. The second group, shared by 16% of individuals, is less diffused being totally absent in four Sardinian populations (SAL, MP, MAR NAS VSM), one Sicilian population (SVC) and in the Venetian Lagoon. The third group, shared by 15% of individuals, is almost exclusively of Venetian Lagoon being also present in six Sardinian populations which are mostly located in the North-eastern coastline (MO, SAL, CC, OTT, IMV AND VSM).

The mismatch analysis carried out on the whole dataset (Fig. 9) showed a starting shape curve. The same trend characterises all the populations analysed (data not shown).

Results were confirmed by the statistical significance of the neutrality tests as provided by Tajima's D (D = -2.18578, $P = 0.001$) and Fu’s FS (FS = -29.342, $P = 0.000$) negative values.

Discussion and Conclusion

The present work provides first molecular insights on a Mediterranean large scale for populations of *Pinna nobilis*. High levels of resolution are here presented for Sardinia and Sicily, setting the basis for future studies on population dynamics of these islands. Furthermore, it has been possible including into our dataset previous molecular data obtained for Grecian (Katsares et al. 2008) and Tunisian (Rabaoui et al. 2011) populations, thus obtaining a whole Mediterranean molecular perspective on the mitochondrial genetic variability of the species.

The non-lethal sampling strategy used to collect portions of tissue from individuals makes this research part of a global context of biodiversity conservation (Casu et al. 2006 and 2010), whose genetic analysis contribute to improve the knowledge on species' variability without sacrificing individuals. In such a topic this work triples the amount of genetic information known till now on *P. nobilis* avoiding any damage for the populations analysed.

The mitochondrial COI region showed elevated rates of variability higher than those obtained from the analysis of 16S, thus confirming the goodness of such marker in depicting the population dynamics of *P. nobilis*. On the other hand results obtained from 16S dataset, although evidenced the occurrence of an higher number of haplotypes in respect to what found by Katsares (2008), confirm the scarce variability of this region.

The outcomes of COI pooled dataset suggest a possible occurrence of a pattern of variability which decrease eastward, as evidenced by haplotype and nucleotide diversity values that in Tunisian and Grecian samples, with the exception of Epanomi, are lower than the observed average rates. Nonetheless, taking into account the high level of variability found for the Grecian population of Epanomi and the occurrence in the Aegean Sea of species' richness comparable to what found in the western

Mediterranean (Zenetos 1997; Morri et al. 1999; Logan et al. 2002), such evidence could be considered as not conclusive, thus requiring further investigations.

In the light of the high evolutionary rate found in some species of the genus *Crassostrea* (Huvet et al. 2000; Launey and Hedgecock 2001), which is considered as one the most molecular affine to the genus *Pinna* (Matsumoto 2003), the genetic variability of *P. nobilis* showed in the present research is compatible with a recent demographic expansion of populations. This inference is suggested by the occurrence of very low number of point mutations that differentiate haplotypes each other. In such context, the population expansion dynamics evidenced by star-like network configuration, mismatch analysis and neutrality tests for the whole area analysed could be considered as consequence of the environmental stress which affected the species during last centuries. In fact marine species, after early lowering of diversity and regression of habitat (Pearson and Rosemberg, 1978; Gray, 1989), overcame environmental changing with new population demographic expansion generally affected by evolutionary forces such as founder effect and genetic drift.

Some of these forces seem to have acted on the current genetic structuring of *P. nobilis* in the Mediterranean, which is conceivable as product of repeated bottle-necks occurred on an ancient panmittic Mediterranean population. Although, this hypothesis is not corroborated by the low dispersal capability of the veliger larval stadium of *P. nobilis* evidenced by previous works (Gosling, 2003; Peharda and Vilibić, 2008), the complex pattern of currents in the Mediterranean may have also favoured dispersal and panmixia in the Mediterranean (Bianchi 2007).

Nowadays, the present genetic pattern of *P. nobilis* as inferred by analysis of our data is characterised by a structuring according to the Mediterranean bio-geographic

partitioning proposed by Bianchi and Morri (2000), involved in geological, climatic and hydrologic factors,

The placing the group (SES), including populations from Sardinia, Elba Island and Sicily, corresponds to the bio-geographic Mediterranean sector “Balearic Sea to Tyrrhenian Sea” identified by Bianchi and Morri (2000). It is not surprising that Sicilian southern populations (PAC and OGN) are here included, being Sicily located westward of the boundary between the western and eastern Mediterranean according not only to Bianchi and Morri (2000) but also to Pérès and Picard (1964).

The centre of variability expansion in the Tyrrhenian Sea is located in correspondence of the North-East of Sardinia. It is an interesting finding, that corroborates the theory of island biogeography (MacArthur and Wilson 1967) suggesting that a large extension of marine protected areas (MPA) could represent a source of biodiversity for neighbouring areas. In fact along north-eastern Sardinian coast two MPAs (Parco Nazionale Arcipelago di La Maddalena and Area Marina Protetta Tavolara Capo Coda Cavallo), which could be involved in the high variability occurring in the area, are situated. Mismatch analysis and neutrality test values support the hypothesis of a recently originated population, which probably tends to the equilibrium (Rogers and Harpending 1992).

Grecian and Tunisian samples (group GT) represent part of the genetic variability of eastern Mediterranean corresponding to the bio-geographic sectors “Gulf of Gabès to Levant Sea” and “North Aegean” identified by Bianchi and Morri (2000). Samples from northern Tunisia (N) are included in this group being molecularly similar to the other Tunisian populations analysed. Anyway it is not surprising since two studies on within-Mediterranean distributions of sponges (Pansini and Longo, 2003) and pycnogonids (Chimenz-Gusso and Lattanzi, 2003) found higher faunal affinities

between eastern and south-western Mediterranean localities than between north-western and south-western localities.

Furthermore, a recent study carried out on the black-striped pipefish *Syngnathus abaster* (Syngnatidae), which lives in brackish-water habitats and presents reduced potential for dispersal, evidenced a sharp genetic structuring between Tunisian and French populations (Ben Alaya et al., 2011). Sample from Cyprus, which should be part of the eastern Mediterranean bio-geographic sector "Ionian Sea and South Aegean" identified by Bianchi and Morri, did not show any similarity with Grecian and Tunisian populations, rather it shares haplotypes with populations from Sardinia, Elba Island, Sicily and Venetian Lagoon. Anyway the reduced size of Cyprian sample (two individuals only) did not allow to conclusively infer on these results.

Grecian and Tunisian group (GT) shows two centres of expansion, which correspond to the occurrence of two different bio-geographic sectors within it (Bianchi and Morri, 2000). Anyway, demographic analysis carried out on the whole sample strongly evidenced the occurrence of a homogeneous population in demographic expansion. Such finding represents a possible consequence of phenomena of bottle-neck followed by founder effects as it is suggested by the high rate of haplotype sharing between Grecian and Tunisian populations.

Venetian lagoon (VL) is characterized by the occurrence of an isolated population which although showing a moderate haplotype sharing with SES group, statistically diverges from the other two Mediterranean groups identified (SES and GT). This area corresponds to the bio-geographic Mediterranean sector "North Adriatic" described by Bianchi and Morri. Mismatch analysis and neutrality tests advance the trend of this population to the equilibrium suggesting that it may be evolving independently from others. The occurrence in the Venetian Lagoon of haplotypes

present also in populations from other groups, could be explained in the light of a presumptive past panmictic population of *P. nobilis* in the Mediterranean fragmented by bottle-necks.

In such context, considering the overall sample analysed, *P. nobilis* shows across the all Mediterranean four COI mitochondrial haplogroups probably originated by founder effects. Two of them (P1 and P3) are almost entirely characteristic of Sardinia, Elba Island and Sicily probably and originated recently in western Mediterranean where diffused by larval dispersal; one (P4) characteristic of Grecian and Tunisian populations only, therefore likely recently originated in eastern Mediterranean; and the haplogroup P2, which is the second most diffused, being diffused in populations from SES and VL groups and Cyprus could realistically represent a mark of ancient Mediterranean genetic settlement of *P. nobilis* which has been lost as consequence of bottle-necks and subsequent founder effects.

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FIGURES

Fig. 1. Map of Mediterranean with the localisation of the sampling sites of *Pinna nobilis*. Sampling locations from Sardinia are depicted in the inset. The location codes are given in Table 1.

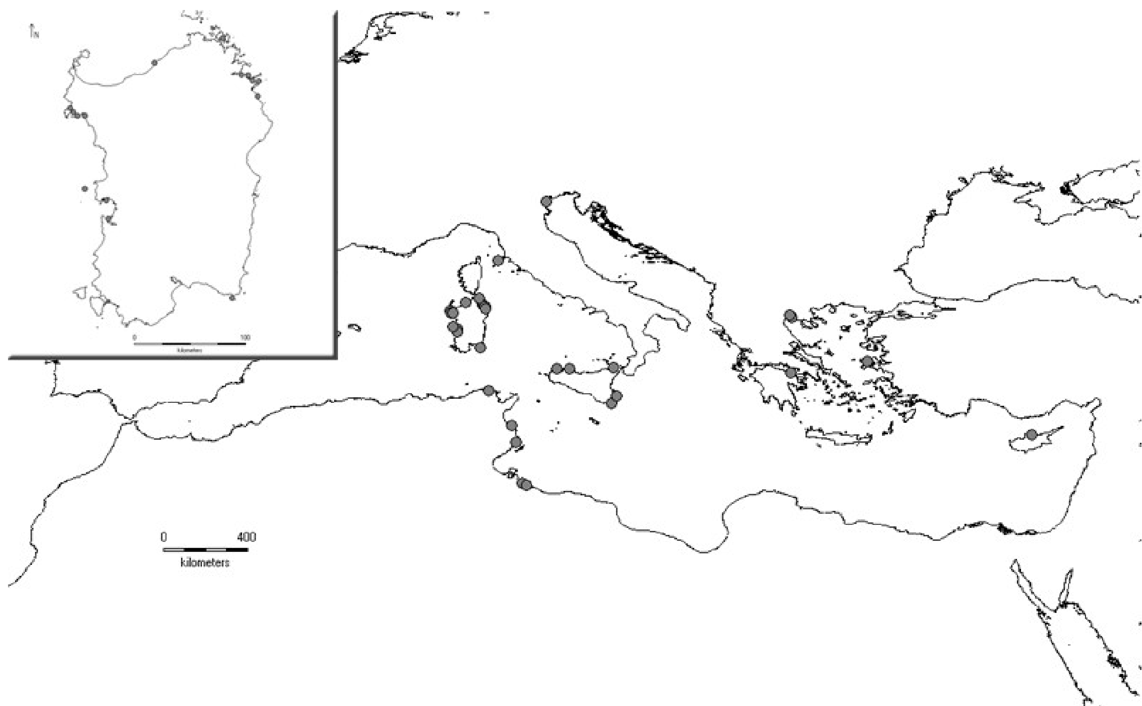


Fig. 2. a) Median-Joining network showing relationships among all *P. nobilis* populations on the basis of COI region analysis. The population codes are given in Table 1. b) Median-Joining network showing relationships among seven Mediterranean macro-areas. SAR: Sardinia; SIC: Sicily; ELB: Elba Island; VEN: Venetian Lagoon; BIZ: Tunisian sample from our dataset; CIP: Cyprus; GRE: Greece; TUN: Tunisia. Asterisks (*) and numbers identify sequences taken from the GenBank database: (1) Katsares et al. 2008; (2) Ramboui et al. 2011.

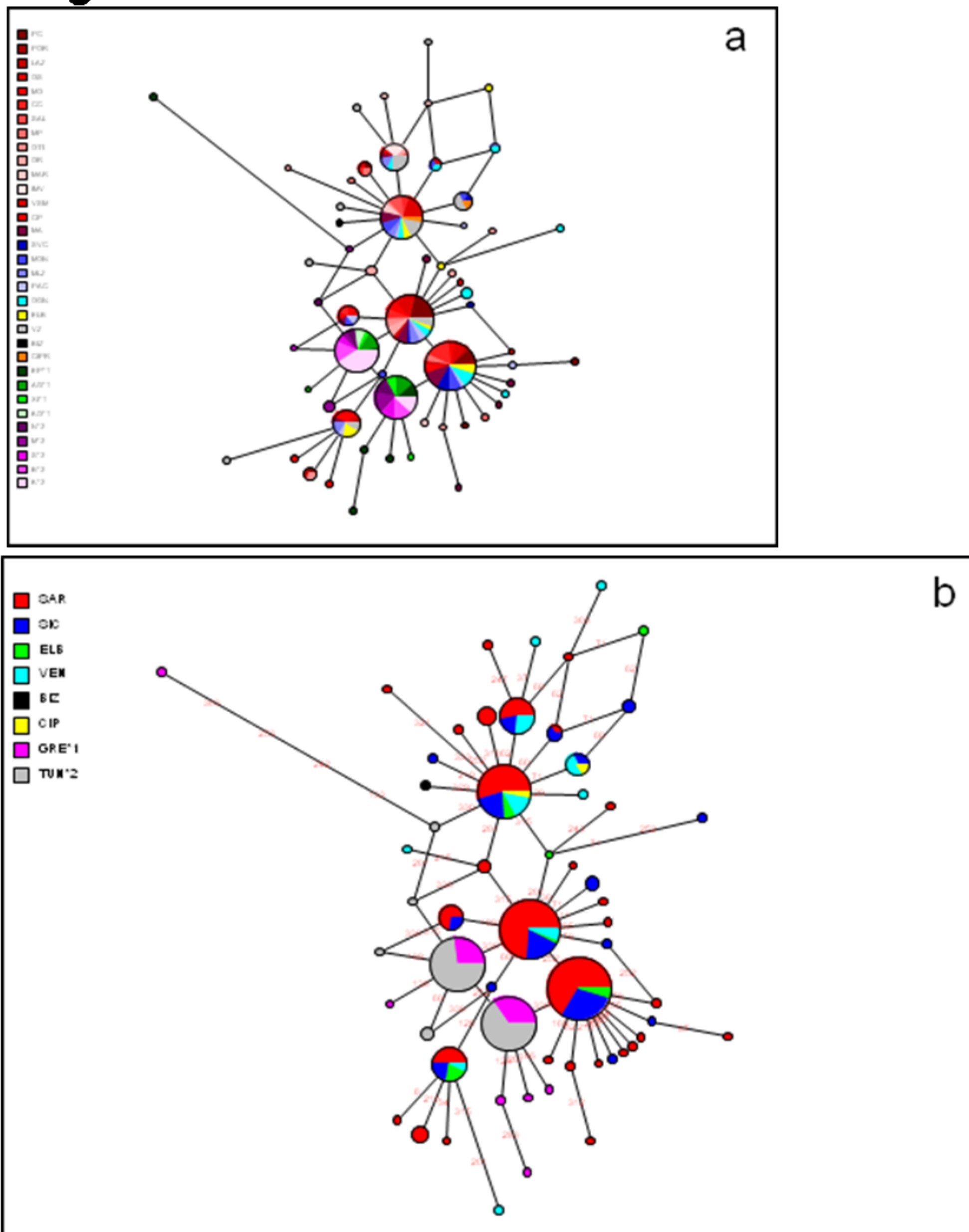


Fig. 3. Median-Joining network showing relationships among haplogroups identified by Bayesian analysis on the basis of COI region dataset.

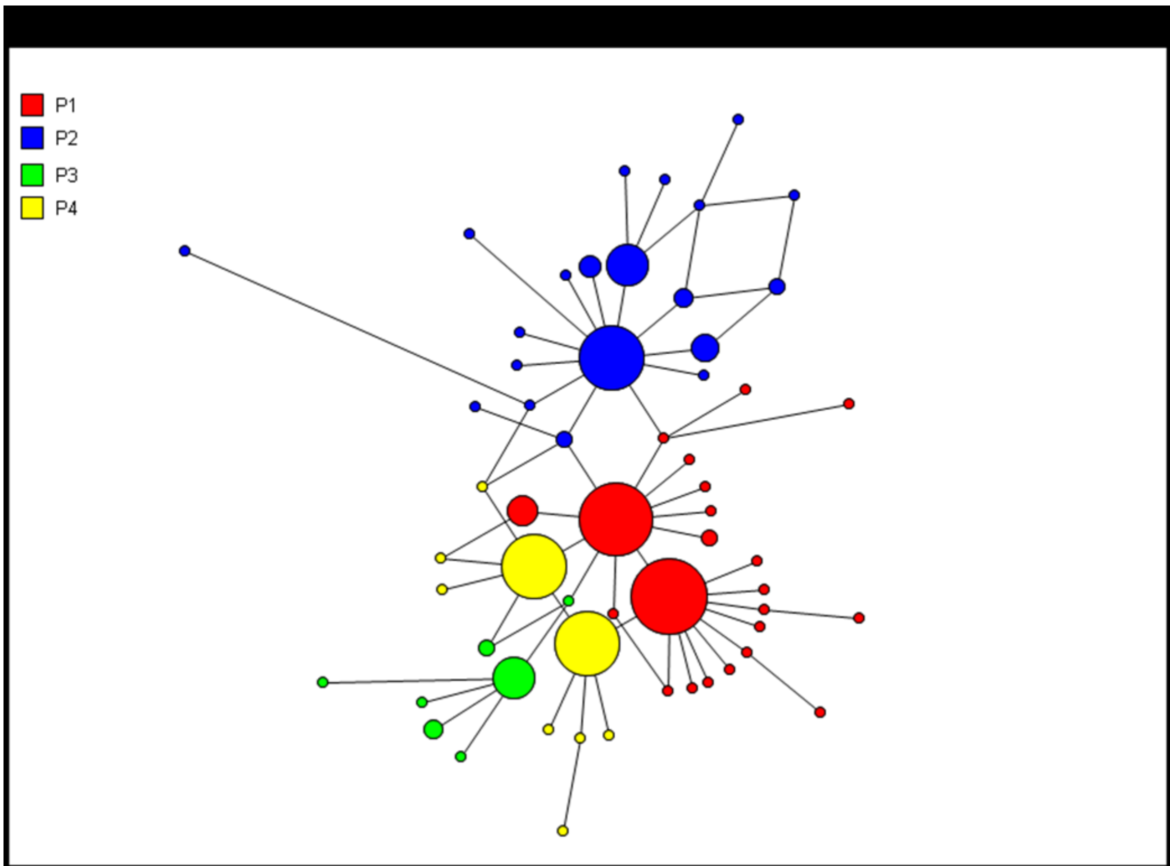

P1
P2
P3
P4

Fig. 4. interpolated maps of the frequency of haplogroups defined by bayesian clustering analyses. Intwerpolation was estimated by an inverse weight distance function. The frequency corresponding to each colour is depicted in the Scale bar on the right of each figure (a) P1; (b) P2; (c) P3; (d) P4.

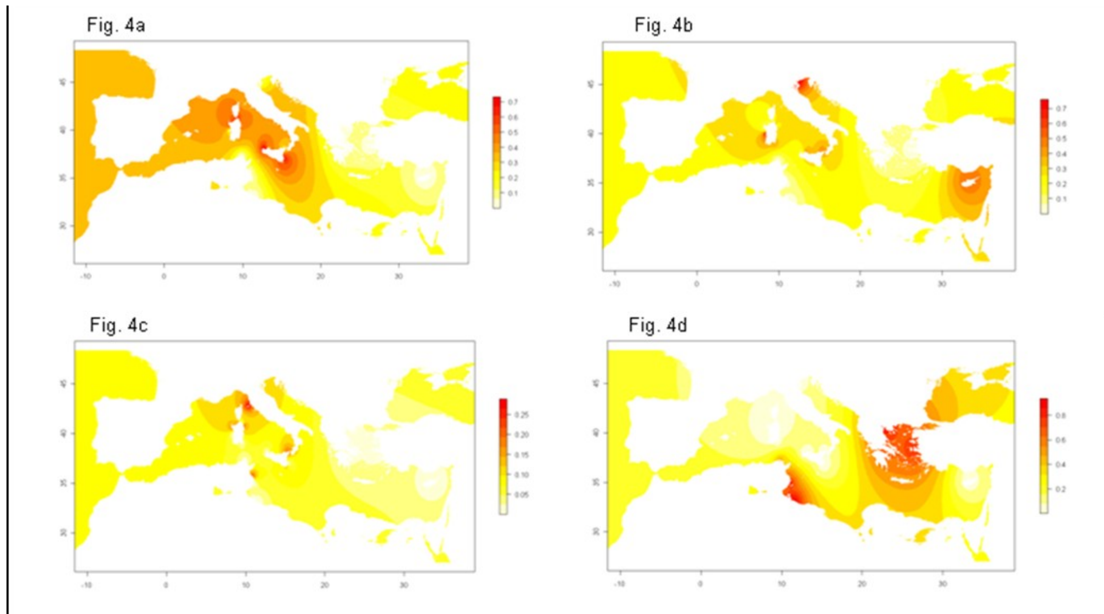


Fig 5. Graphic representation of PCA on the basis of COI region analysis; (a) first principal component which accounts for 47.28% of total variance; (b) second principal component which accounts for 21.01% of total variance; (c) third principal component which accounts for 18.30% of total variance.

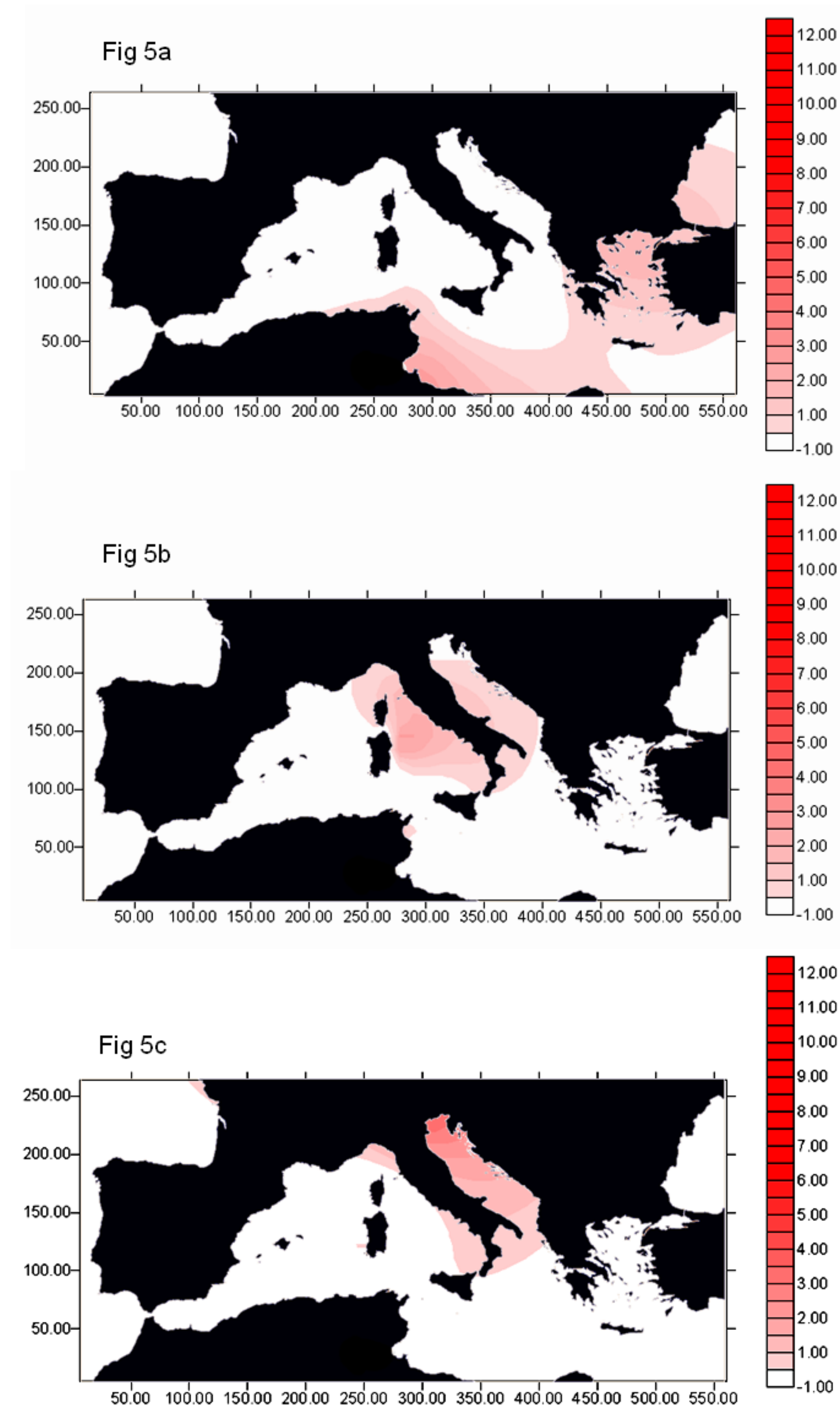


Fig 6. Graphs of mismatch distributions analysis carried out on COI region dataset; (a) whole sample; (b) SES (Sardinia, Elba Island and Sicily) group; (c) VL (Venetian Lagoon) group; (d) GT (Greece and Tunisia) group. On Y axis: frequencies; on X axis: observed distributions of pairwise nucleotide differences.

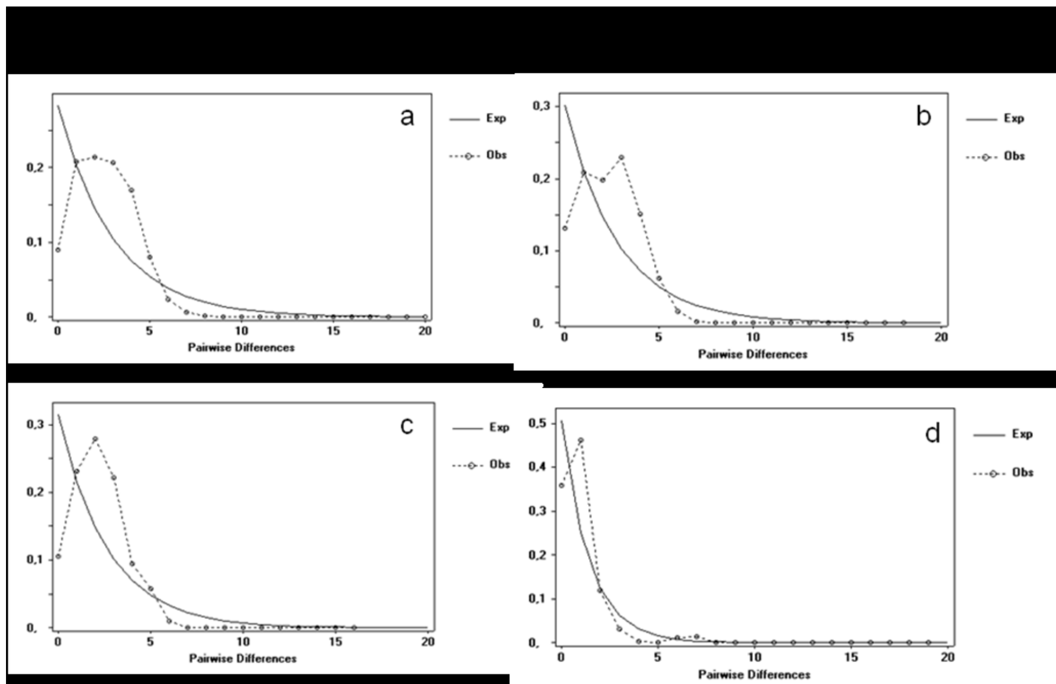


Fig 7. Fig. 2. a) Median-Joining network showing relationships among seven Mediterranean macro-areas on the basis of 16S region analysis. Asterisks (*)identify sequences taken from the GenBank database (Katsares et al. 2008). Macro-areas are labelled as in Fig. 2.

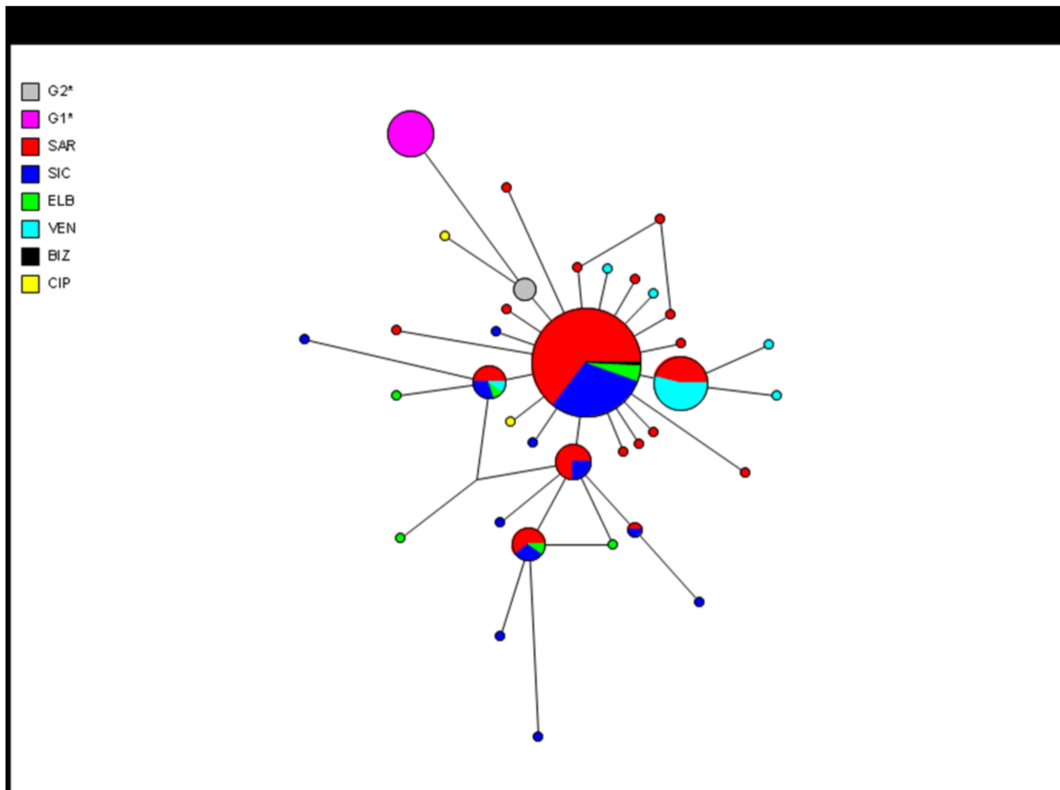


Fig 8. Fig 5. Plot of estimated genetic structure as inferred by Bayesian model-based clustering analysis on the basis of 16S region dataset. Black lines separate individuals from different sampling sites. The populations are labeled as in Table 1.

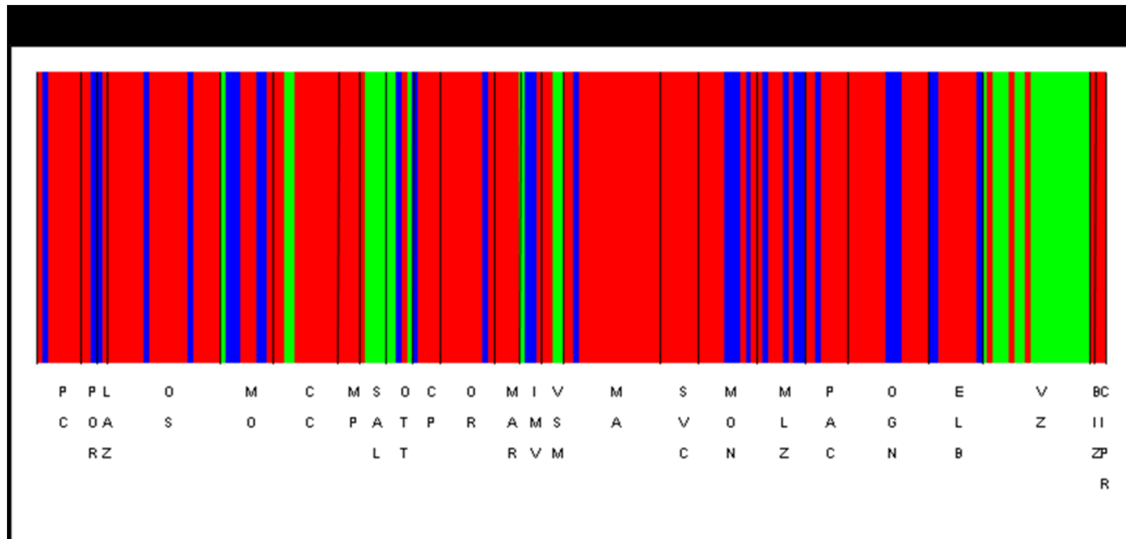
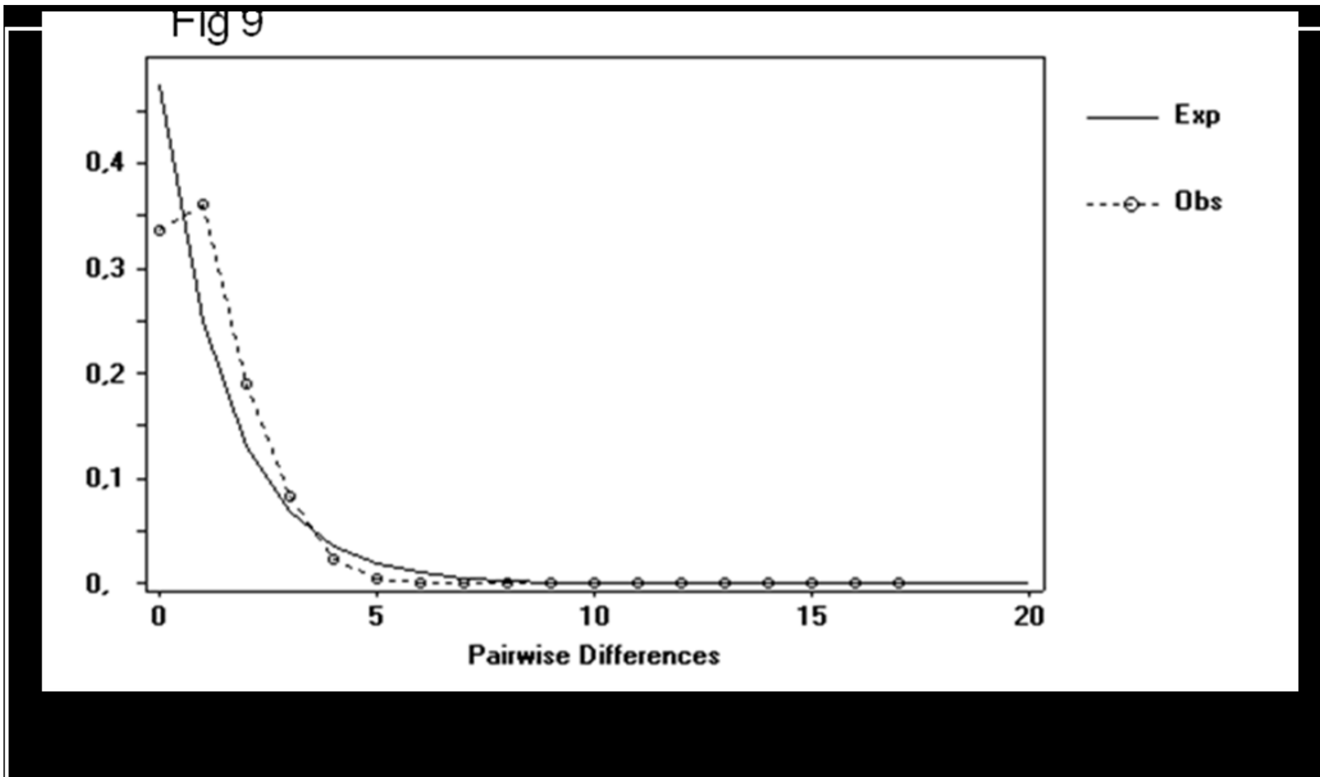


Fig 9. Graphs of mismatch distributions analysis carried out for the whole sample on the basis 16S region dataset.



TABLES

Table 1. Details on the populations of *P. nobilis* analysed. Asterisks (*) and numbers identify samples whose sequences were taken from the GenBank database: (1) Katsares et al. 2008; (2) Ramboui et al. 2011.

Region	*Site*	*Label*	*Sampling period*	*COI*	*16S*	*Coordinates*
Sardinia	Baia di Porto Conte	PC		18	8	40°36'28.56"N 8°12'40.32"E
	Torre del Porticciolo	POR		3	3	40°38'34.56"N 8°11'13.80"E
	Lazzaretto	LAZ		2	2	40°34'40.08"N 8°14'51.96"E
	Ospedale Marino	OS		21	21	40°34'44.90"N 8°18'26.81"E
	Molara	MO		11	10	40°51'46.26"N 9°43'21.96"E
	Capo Ceraso	CC		13	12	40°54'26.10"N 9°38'13.62"E

	Le Saline	SAL	5	5	40°54'48.18"N 9°35'0.61"E
	Monte Petrosu Sassi piatti and Isola Cava	MP	4	4	40°51'56.11"N 9°40'23.08"E
	Porto Ottiolu	OTT	5	5	40°44'16.50"N 9°42'54.22"E
	Oristano Sa Mardini and Torre Vecchia	OR	10	10	39°53'38.10"N 8°28'58.00"E
	Marceddì	MAR	5	5	39°44'43.00"N 8°29'54.30"E
	Isola di Mal di Ventre	IMV	4	4	39°59'14.10"N 8°18'27.50"E
	Villasimius Capo Caterina	VSM	4	4	39°5'6.78"N 9°29'8.46"E
	Costa Paradiso	CP	5	5	41° 0'43.43"N 8°52'22.45"E
	La Maddalena Cala Camiciotto	MA	18	18	41°12'41.41"N 9°25'42.10"E
Sicily	San Vito lo Capo Secca di Cala Rossa	SVC	7	7	38°9'36.10"N 12°46'17.71"E
	Palermo Mondello	MON	11	11	38°12'12.00"N 13°19'47.12"E
	Milazzo	MLZ	10	9	38°13'1.72"N 15°14'43.53"E
	Pachino (Marzamemi) Capo Passero	PAC	8	8	36°42'30.05"N 15°7'33.92"E
	Ognina Capo Murro di Porco	OGN	15	15	37°0'7.84"N 15°20'32.37"E
Elba Island	Capo Enfola	ELB	10	10	42°49'25.41"N 10°16'7.80"E
Venetian Lagoon	Ottagono Alberini and Santa Maria del Lago	VZ	20	20	45°21'48.21"N 12°19'17.17"E
Tunisia	Bizerta Lagoon	BIZ	1	1	37°14'55.15"N 9°51'37.49"E
Cyprus	Karaoglanoglu	CIPR	2	2	35°20'54.52"N 33°15'17.16"E
Corsica	Isola Piana	CE	15	15	41°22'33.90"N 9°13'47.73"E
	Cala Pesciu Cane	CW	13	13	41°26'47.25"N 9° 5'41.79"E
Greece*[1]	Epanomi	EP	9	8	40°23'13.00"N 22°54'02.00"E
	Aggeloxori	AG	9	9	40°29'12.00"N 22°49'01.00"E
	Xios Island	XI	5	5	38°29'09.00"N 26°08'13.00"E
	Korinthiakos Gulf	KO	3	3	38°00'10.00"N 22°52'45.00"E
Tunisia*[2]	Bizerta Lagoon	N	7		37°14'46.91"N 9°51'09.25"E
	Monastir Stah Jaber	M	9		35°45'20.50"N 10°50'03.05"E
	Kerkennah Island	S	7		35°01'40.67"N 11°00'44.24"E
	El Bibane lagoon	B	9		33°16'06.17"N 11°18'41.49"E
	El Ketef	K	17		33°10'52.21"N 11°29'35.89"E

Table 2. Sample sizes and genetic diversity estimates obtained for the mitochondrial COI region of Mediterranean populations of *P. nobilis*. N: sample sizes; S: number of polymorphic sites; H: number of haplotypes; *h*: haplotype diversity; *π*: nucleotide diversity; d: mean of pairwise nucleotide differences. Asterisks (*) and numbers identify samples whose sequences were taken from the GenBank database: (1) Katsares et al. 2008; (2) Ramboui et al. 2011. Populations are labelled as in Table 1.

Sample	*N*	*S*	*H*	h	π	*d*
PC	18	10	7	0.725	0.005	1.667
POR	3	3	2	0.667	0.006	2.000
LAZ	2	4	2	1.000	0.012	4.000
OS	21	9	8	0.829	0.006	1.886
MO	11	6	6	0.873	0.006	1.964
CC	13	5	5	0.705	0.005	1.615
SAL	5	3	3	0.700	0.003	1.200
MP	4	5	3	0.833	0.007	2.500
OTT	5	6	4	0.900	0.008	2.800
OR	10	8	7	0.911	0.007	2.356
MAR	5	7	4	0.900	0.009	3.000
IMV	4	0	1	0.000	0.000	0.000
VMS	4	5	4	1.000	0.008	2.833
CP	5	4	3	0.700	0.005	1.800
MA	18	11	10	0.895	0.007	2.242
SVC	7	5	4	0.714	0.005	1.714
MON	11	6	6	0.836	0.007	2.309
MLZ	10	6	5	0.867	0.007	2.533
PAC	8	8	7	0.964	0.007	2.536
OGN	15	7	9	0.886	0.007	2.362
ELB	10	7	6	0.889	0.008	2.822
VZ	20	10	10	0.895	0.006	2.184
BIZ	1	0	1	0.000	0.000	0.000
CIPR	2	1	2	1.000	0.003	1.000
EP*[1]	9	9	6	0.833	0.007	2.500
AG*[1]	9	2	3	0.667	0.002	0.778
XI*[1]	5	2	3	0.700	0.002	0.800
KO*[1]	3	0	1	0.000	0.000	0.000
N*[2]	7	2	3	0.667	0.002	0.857
M*[2]	9	4	4	0.694	0.004	1.389
S*[2]	7	2	3	0.667	0.002	0.857
B*[2]	9	1	2	0.556	0.002	0.556
K*[2]	17	1	2	0.382	0.001	0.382
TOT	287	42	58	0.911	0.007	2.543

Table 3. Frequencies' distribution COI region mitochondrial haplogroups identified by Bayesian analysis among Mediterranean populations of *P. nobilis*. n: absolute frequencie; %: relative frequencie. Asterisks (*) and numbers identify individuals whose sequences were taken from the GenBank database: (1) Katsares et al. 2008; (2) Ramboui et al. 2011. Populations are labelled as in Table 1.

Sample	***P1***		***P2***		***P3***		***P4***	
	n	%	n	%	n	%	n	%
PC	15	13.04	2	2.56	1	4.35	0	0.00
POR	2	1.74	0	0.00	1	4.35	0	0.00
LAZ	1	0.87	0	0.00	1	4.35	0	0.00
OS	13	11.30	7	8.97	1	4.35	0	0.00
MO	6	5.22	0	0.00	5	21.74	0	0.00
CC	10	8.70	3	3.85	0	0.00	0	0.00
SAL	1	0.87	4	5.13	0	0.00	0	0.00
MP	3	2.61	1	1.28	0	0.00	0	0.00
OTT	2	1.74	2	2.56	1	4.35	0	0.00
OR	6	5.22	4	5.13	0	0.00	0	0.00
MAR	1	0.87	4	5.13	0	0.00	0	0.00
IMV	0	0.00	4	5.13	0	0.00	0	0.00
VMS	2	1.74	2	2.56	0	0.00	0	0.00
CP	4	3.48	0	0.00	1	4.35	0	0.00
MA	13	11.30	4	5.13	1	4.35	0	0.00
SVC	6	5.22	1	1.28	0	0.00	0	0.00
MON	5	4.35	5	6.41	1	4.35	0	0.00
MLZ	3	2.61	5	6.41	2	8.70	0	0.00
PAC	5	4.35	2	2.56	1	4.35	0	0.00
OGN	11	9.57	4	5.13	0	0.00	0	0.00
ELB	4	3.48	3	3.85	3	13.04	0	0.00
VZ	2	1.74	16	20.51	2	8.70	0	0.00
BIZ	0	0.00	1	1.28	0	0.00	0	0.00
CIPR	0	0.00	2	2.56	0	0.00	0	0.00
EP*[1]	0	0.00	1	1.28	0	0.00	8	11.27
AG*[1]	0	0.00	0	0.00	0	0.00	9	12.68
XI*[1]	0	0.00	0	0.00	0	0.00	5	7.04
KO*[1]	0	0.00	0	0.00	0	0.00	3	4.23
N*[2]	0	0.00	0	0.00	0	0.00	7	9.86
M*[2]	0	0.00	1	1.28	2	8.70	6	8.45
S*[2]	0	0.00	0	0.00	0	0.00	7	9.86
B*[2]	0	0.00	0	0.00	0	0.00	9	12.68
K*[2]	0	0.00	0	0.00	0	0.00	17	23.94
TOT	115	40.07	78	27.18	23	8.01	71	24.74

Table 4. Population pairwise Φ_{ST} values between *P. nobilis* populations. Conventional Φ_{ST} values are shown below the diagonal and the significance of corresponding *P*-values (significance level ≤ 0.05) are shown above the diagonal. Asterisks (*) and numbers identify samples whose sequences were taken from the GenBank database: (1) Katsares et al. 2008; (2) Ramboui et al. 2011. Populations are labelled as in Table 1.

	Sardinia	***Sicily***	***Elba***	***Ven. Lag.***	***Greece***	***Tunisia***
Sardinia	-	0.24324	0.20721	0.00000**	0.00000**	0.00000**
Sicily	0.00165	-	0.40541	0.00000**	0.00000**	0.00000**
Elba	0.01174	-0.00178	-	0.01802*	0.00000**	0.00000**
Ven. Lag.	0.24103	0.20246	0.17111	-	0.00000**	0.00000**
Greece*[1]	0.35564	0.37846	0.44073	0.60191	-	0.15315
Tunisia*[2]	0.38328	0.44044	0.57112	0.69421	0.01345	-

Table 5. Tajima's D and Fu's F_S statistics in *P. nobilis* populations. N: sample sizes; *P*: statistical significance. Asterisks (*) identify samples whose sequences were taken from the GenBank database: Katsares et al. 2008 (Greece) and Ramboui et al. 2011 (Tunisia) respectively.

Region	***Zone***	***N***	***D***	**P**	***F_S***	**P**
SES	Sardinia, Elba Island, Sicily	189	-1.565	0.026	-26.896	0.000
VL	Venetian Lagoon	20	-0.787	0.247	-4.167	0.008
GT*	Greece, Tunisia	75	-1.774	0.014	-7.246	0.001
Tot	SES, VL,GT,CIP,BIZ	287	-2.186	0.001	-29.342	0.000

Table 6. Sample sizes and genetic diversity estimates obtained for the mitochondrial 16S region of Mediterranean populations of *P. nobilis*. N: sample sizes; S: number of polymorphic sites; H: number of haplotypes; *h*: haplotype diversity; π: nucleotide diversity; d: mean of pairwise nucleotide differences. Populations are labelled as in Table 1.

Sample	***N***	***S***	***H***	**h**	π	***d***
PC	8	3	4	0.643	0.001	0.750
POR	3	2	2	0.667	0.003	1.333
LAZ	2	3	2	1.000	0.006	3.000
OS	21	3	4	0.271	0.001	0.371
MO	10	3	4	0.778	0.002	1.111
CC	12	2	3	0.439	0.001	0.470
SAL	5	1	2	0.400	0.001	0.400
MP	4	2	2	0.500	0.002	1.000
OTT	5	2	3	0.700	0.002	1.000
OR	10	3	3	0.378	0.001	0.600
MAR	5	1	2	0.400	0.001	0.400
IMV	4	3	3	0.833	0.003	1.667
VMS	4	2	3	0.833	0.002	1.167
CP	5	4	4	0.900	0.003	1.600
MA	18	6	5	0.549	0.002	0.850
SVC	7	0	1	0.000	0.000	0.000
MON	11	8	8	0.891	0.004	1.782
MLZ	9	2	3	0.667	0.002	0.944
PAC	8	3	3	0.607	0.002	0.929
OGN	15	7	4	0.371	0.003	1.286
ELB	10	5	6	0.778	0.003	1.711
VZ	20	7	6	0.447	0.002	0.958
BIZ	1	0	1	0.000	0.000	0.000
CIPR	2	0	2	1.000	0.004	2.000
TOT	199	29	34	0.674	0.002	1.141

PCR-RFLP: a practical method for the identification of specimens of *Patella ulyssiponensis s.l.* (Gastropoda: Patellidae)

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1. Introduction

The species belonging to the genus *Patella* (Gastropoda: Patellidae) are known to have an important influence on the structure and function of the rocky shore communities, and therefore are considered as crucial “keystone” species (Raffaelli & Hawkins 1996). However, many of *Patella* species have long been recognised as taxonomically challenging (Fisher-Piette 1938). Furthermore, the application of molecular methods has recently contributed to reveal the occurrence of complexes of cryptic species even in taxa assumed easy to identify in the field, such as *Patella rustica* Linnaeus, 1758 (Sá-Pinto et al. 2010). Similarly, in *Patella ulyssiponensis* Gmelin, 1791, one of the most widely distributed limpet, the finding of high level of genetic differentiation evidenced by the allozymes between the populations from the Mediterranean and the north-eastern continental shoreline of the Atlantic, and the populations from the Macaronesian islands, led Weber & Hawkins (2005) to hypothesise the presence of two distinct species: *P. ulyssiponensis* from the European continent, and *Patella aspera* Röding, 1798 restricted to the Macaronesia. However, no consensus emerged on the taxonomic status of these populations, *e.g.* Sá-Pinto et al. (2005, 2008) referred to the populations as continental and Macaronesian “forms” of *P. ulyssiponensis*, respectively.

Among the European limpets, *P. ulyssiponensis sensu lato* is one of the most difficult to identify, as it shows a broadly overlapping morphological variability with the other *Patella* species living in the lower part of intertidal zone. This fact made the identification of specimens with “intermediate” morphology impossible even for skilled observers (Fisher-Piette 1938; Evans 1953; Sella et al. 1993). For instance, in the Mediterranean the distinction between *P. ulyssiponensis s.l.* and the endemic *Patella caerulea* Linnaeus, 1758 is particularly troublesome. In several localities, neither the shell and foot colour pattern nor the shell shape may allow to unequivocally discriminate between the two species

(Cretella et al. 1990; Sella et al. 1993; Mauro et al. 2003; Casu et al. 2010). Similar problems may also arise in the distinction between *P. ulyssiponensis s.l.* and *Patella vulgata* Linnaeus, 1758, distributed on the rocky shore from Portugal to Norway, and *P. ulssiponensis s.l.* and *Patella candei* (d'Orbigny, 1839), endemic to the Macaronesian islands (*i.a.*, Cabral 2003; Pagarete 2005).

Since the specimens of *P. ulyssiponensis s.l.* often share the same habitat (lower part of the intertidal rocky shores) with the above-mentioned three species (Fretter & Graham 1976; Cretella et al. 1990; Sella et al. 1993; Weber et al. 1998; Pagarete 2005; Petraccioli et al. 2010), the ecologists are often compelled to classify them as "*Patella* spp." (*i.a.*, Maggi et al. 2009).

Specific constant genetic patterns ("DNA barcoding") have been helpful in species attribution of individuals whose morphological features cannot be used confidently at this scope (see Hebert et al. 2003; but also Ebach & Holdrege 2005). In particular, different studies (Mauro et al. 2003; Sá-Pinto et al. 2005, 2008; Casu et al. 2010) have proved that the "traditional" molecular taxonomy, based on the sequences of the mitochondrial gene Cytochrome c Oxidase subunit I (COI), the so-called "Folmer region" (Folmer et al. 1994), unequivocally discriminates *P. ulyssiponensis s.l.* from *P. caerulea*, *P. vulgata* and *P. candei*.

Among the analytical methods based on the identification of specific mutations in a nucleotide sequence, the Restriction Fragment Length Polymorphisms PCR-based (PCR-RFLP) (Wolf et al. 1999) technique represents an alternative, useful tool for the identification of species, such as those belonging to Mollusca (*e.g.*, Boudry et al. 1998; Fernández et al. 2000; Chapela et al. 2003; Zhang et al. 2005). Since PCR-RFLP does not require nucleotide sequencing procedures, it may be routinely applicable in surveys devoted to rapid taxonomic identifications of numerous samples.

Consequently, the aim of the present study was to analyse the mitochondrial COI sequence of *P. ulyssiponensis s.l.*, *P. caerulea*, *P. vulgata* and *P. candei*, in order to identify a species-specific restriction site. This site can be used as diagnostic RFLP markers to facilitate unequivocal distinction of *P. ulyssiponensis s.l.* from the other three species, whose correct taxonomic attribution on morphological bases alone may raise concerns.

2. Materials and Methods

2.1. Designing of specific COI primers and RFLPs

A new pair of COI primers was designed, since universal primers for this gene (Folmer et al. 1994) did not always give satisfactory results for *P. ulyssiponensis s.l.* from Mediterranean. Specific primers (L: 5'-TTAATTCGAGCTGAACTTGG-3'; H: 5'-ATTCGTTCAAGTTGCATAGC-3') were designed after the analysis of COI sequences - aligned by means of the software Clustal W Multiple alignment implemented in BioEdit 7.0.5.2 (Hall 1999) - on a total of 131 individuals of *P. ulyssiponensis s.l.* (51 Mediterranean and Atlantic specimens), *P. caerulea* (24), *P. vulgata* (11) and *P. candei* (45) (GenBank Accession No. are given in Table I), choosing two conserved internal regions suitable for the annealing (see Sanna et al. 2009 for the methodology). These primers allowed amplifications of an internal portion of the "Folmer region" (Folmer et al. 1994) which was 437 base pair (bp) long.

Forty COI sequences of the following congenerics: *Patella depressa* Pennant, 1777 (GenBank Accession Nos.: DQ089613-15; EF462972-75; EU073890-95), *Patella lugubris* Gmelin, 1791 (DQ089576-77; EU073889), *Patella ferruginea* Gmelin, 1791 (AY996038-43; GQ469870-76), *Patella rustica* Linnaeus, 1758 (AB238579; EF462954-56; GQ469878-82), and *Patella pellucida* Linnaeus, 1758 (DQ089620-21) were also added to the above-mentioned 131 sequences in order to find a restriction site able to unequivocally

distinguish *P. ulyssiponensis s.l.* not only from *P. caerulea*, *P. vulgata* and *P. candei*, but also from an enlarged number of *Patella* species. We expected optimal results by the use of the TCGA restriction sites, cut by the endonuclease *Taq*I (Figures 1 and 2).

2.2 Sampling

A total of 65 specimens of *Patella* spp. were collected from the lower intertidal zone of different localities in the Mediterranean (Sardinia, Sicily, Tuscany, northern Adriatic Sea, 32 specimens), north-eastern Atlantic (Brittany, 18 specimens), and Canaries (Tenerife, 15 specimens) (see Table II for details). Sampling efforts mainly focused on the collection of individuals whose morphology was not conclusive for the taxonomic attribution (Table II). The shells of the specimens analysed were deposited in the Zoological Museum of the Dipartimento di Zoologia e Genetica Evoluzionistica (University of Sassari, Italy).

2.3. COI analysis

All 65 *Patella* specimens collected were identified by a molecular taxonomy approach, using the COI Folmer region. DNA was extracted using the DNeasy® Tissue Kit (QIAGEN Inc.®). The PCR, carried out in a total volume of 25 µl, contained about 10 ng/µl of total genomic DNA on average, 2.5 U of Taq DNA polymerase (Eurotaq® by Euroclone®), 1× reaction buffer, 1.25 mM of $MgCl_2$, 0.4 µM of both primers, and 200 µM of dNTPs mix. PCR amplification was performed in a MJ PTC-100® Thermal Cycler (MJ research®) programmed as follows: a preheating of 2 min at 94° C, 35 cycles of 40 s at 94° C, 45 s at 55° C and 1 min and 40 s at 72° C. At the end, a post-treatment of 5 min at 72° C and a final cooling at 4° C were performed. Both positive and negative controls were used to test the effectiveness of the PCR reagents. Electrophoresis was carried out on a 2% agarose

gels in a 0.5× TBE buffer ethidium bromide stained (10 mg/ml), at 4 V/cm for 20 min. Purification and sequencing were performed as reported in Casu et al. (2010).

The 65 new COI sequences (GenBank Accession Nos. are in Table I) were aligned using Clustal W with the 131 sequences of *P. ulyssiponensis s.l.*, *P. caerulea*, *P. vulgata* and *P. candei* as in Section 2.1 (Table I). *Cymbula nigra* (da Costa, 1771) (GenBank Accession No.: AB445098) was used as confamilial outgroup.

Molecular taxonomic attribution was done by cluster analysis applying the Maximum Likelihood (ML) and the Bayesian Inference (BI) methods. For ML, the best of SPR and NNI branch rearrangements were applied on a starting tree obtained using the BioNJ. Following Sá-Pinto et al. (2005), the general time reversible (GTR) model was used, allowing the estimation of proportion of invariable sites and the gamma distribution. Branch support for the nodes with the highest likelihood score was assessed by a non-parametric bootstrap (1,000 replicates). For BI, two independent runs each consisting of seven Metropolis-coupled MCMC chains were run simultaneously. Parameters were specified according to GTR model by setting N_{ST} = 6, rates = invgamma, assuming a Dirichlet distribution. Each analysis was run for 5,000,000 generations sampling trees every 500 generations in each of the two runs. The first 25% of sampled trees were discarded. Nodes of the ML and BI trees with bootstrap higher than 70% and posterior probability higher than 90% respectively are considered highly supported. All the above-mentioned bioinformatic analyses were carried out on the freely available website Bioportal (www.bioportal.uio.no). Intraspecific and interspecific average number of pairwise differences were calculated by means of Arlequin 3.0 (Excoffier et al. 2005) using the Kimura 2-parameter (K2P) model (Kimura 1980).

2.4. RFLPs analysis

The effectiveness of the restriction enzyme *Taq*I in distinguishing *P. ulissyponensis s.l.* was tested on COI gene amplification products obtained by the newly designed primers on the 65 specimens of *Patella* sampled. To carry out the enzymatic digestions, an aliquot of 10 µl of each PCR product was mixed with a reaction solution containing 1 µl of enzyme (*Taq*I, FastDigest® by Fermentas®) and 0.67× Green Buffer® in a final volume of 30 µl. Samples were incubated at the temperature of 65° C for 5 min, and then separated on the 2% agarose gel for 90 min. In Figure 2, all obtainable *Taq*I digestion fragments on a 437 bp long COI sequence of *P. ulyssiponesis s.l.*, *P. caerulea*, *P. vulgata* and *P. candei* are reported. Note that, for the analysis, we did not take into consideration fragments lower than 100 bp, since we chose to rely only on data unequivocally scorable. As well, differences in gel migrations among fragments smaller than 50 bp were considered with caution.

3. Results

3.1. COI results

Based on a total of 197 sequences (65 of which were obtained in this research) 437 bp long, ML retrieved three main clusters (Figure 3): A) a basal clade which groups together *P. vulgata* specimens; B) a clade grouping all *P. ulyssiponensis s.l.* specimens, which further splits in two distinct clusters, “continental” and “Macaronesian” *P. ulyssiponensis* (B1 and B2, respectively); and C) a monophyletic clade which encompasses *P. caerulea* and *P. candei* specimens, with the former species (C1) nested within two distinct clusters of the second that include *P. candei* from Canaries (C2) and *P. candei* from Azores (C3). BI was consistent with ML in showing the same tree topology, with the exception of the cluster which grouped *P. ulyssiponensis s.l.*, in which individuals of the “continental” form did not form a monophyletic clade supported by an high posterior probability value (Figure 3).

Sixty-five *Patella* specimens sampled were thus identified (Figure 3): 33 individuals belonged to *P. ulyssiponensis s.l.*, 16 to *P. caerulea*, six to *P. candei*, and ten to *P. vulgata* (see Table II for details). In particular, our *P. ulyssiponensis s.l.* specimens grouped into the cluster B1 or B2 of the ML tree (Figure 3), depending on their “continental” or “Macaronesian” provenience. As well, our *P. candei* specimens from Tenerife clustered within the C2 branch.

The K2P average distances showed the existence of barcoding gaps, since interspecific distances, comprised between 36.079 (*P. caerulea* vs. *P. candei*) and 82.521 (*P. vulgata* vs. *P. candei*) were much higher than the intraspecific ones, comprised between 1.350 (*P. vulgata*) and 18.358 (*P. candei*) (Table III).

3.2. RFLP results

The electrophoretic run of the enzymatic digestion products by *Taq*I evidenced easily scorable fragments 228 and 174 bp long in *P. ulyssiponensis s.l.*, 431 bp long in *P. caerulea*, 358 bp long in *P. vulgata*, and 422 bp long in *P. candei* (Figure 4a). The occurrence of such species-specific fragments in *P. ulyssiponensis s.l.* allowed to attribute to this species 33 out of 65 individuals analysed by RFLPs, consistently with COI sequencing analysis.

4. Discussion

The problem of a correct attribution of *Patella* individuals may be relevant in the ecological and biodiversity studies. Indeed, it regards not only single puzzling specimens within a population, but entire populations as well. For instance, during a study on *P. caerulea* and *P. ulyssiponensis* along the Sicilian coasts, Mauro et al. (2003) noted that, at Capo Gallo, it was not possible to identify any specimens of the two *Patella* species, based on the morphological features.

Accordingly, we observed that some populations from our Mediterranean sampling sites (as in Insel Cres, Molarotto Island, and Ceuta harbour; see Table II for details) are composed almost entirely by specimens of *P. ulyssiponensis* and *P. caerulea* with overlapping morphology.

Similar difficulties occurred for all the *Patella* specimens from Las Galletas (Tenerife) as well as for some specimens from Fort du Dellec (Brittany). Noteworthy, the preliminary tentative attribution of sampled individuals to *P. ulyssiponensis s.l.*, based on shell morphology and external foot colour, led to deceptive results (Table II).

An instance of the high morphological similarity between the shells of *P. ulyssiponesis s.l.* and those of the sympatric species *P. caerulea* (in Mediterranean), *P. vulgata* (in the Atlantic continental coasts) and *P. candei* species complex (in Macaronesia) is reported in Figure 4b.

It should be noted that *P. ulyssiponensis s.l.* individuals are more similar to the congenerics from the same geographical area (pairs 1-2, 3-4, 5-6) in comparison to individuals from different localities (specimens 1-3-5).

The wide morphological variation, with the overlapping morphology and colour displayed by *P. ulyssiponensis s.l.* and some congenerics occurring in the intertidal, is presumably promoted by the habitat heterogeneity which characterises the zone, with differences in

wave exposures, and variability of chemical-physic parameters over short vertical distances (Beaumont & Wei 1991; Nolan 1991; Corte-Real et al. 1996; Mauro et al. 2003). Indeed, these authors pointed to the existence of a high morphological plasticity in the genus *Patella*, leading to ecophenotypes largely determined by environmental conditions (De Wolf et al. 2000; Titselaar 1998; Johannesson 2003; Pagarete 2005). The result of such ecological plasticity is that, at each locality, the coexisting species under the influence of environmental selection tend to converge morphologically, till they become hardly distinguishable. Conversely, individuals of the most widespread species, *P. ulyssiponensis s.l.* from different geographical areas, may be extremely dissimilar.

All these facts led different authors (*i.a.*, Mauro et al. 2003; Sá-Pinto et al. 2005; Casu et al. 2010) to suggest the necessity of a genetic approach, in order to distinguish the four *Patella* species. Therefore, the PCR-RFLP method provided in the present study offers a rapid and alternative molecular tool that could be routinely used for an unambiguous taxonomic distinction of *P. ulyssiponensis s.l.* from *P. caerulea*, *P. vulgata* and *P. candei*, when the morphology offers discordant or incomplete information. In this context it may be highlighted that, although so far no study showed hybridisation between *P. ulyssiponesis* and the other three species, further molecular analysis using multilocus nuclear markers should be advisable to shed more light on this possibility.

Sequences used to design restriction sites (Table I) belong to individuals from locations that almost completely cover the geographical distribution of the species analysed. Thus, we may expect that *Taq*I produces an identical banding pattern throughout the whole distribution range.

The strength of the RFLP analysis for the taxonomic attribution of *P. ulyssiponesis s.l.* has been proven by the consistency with the COI sequencing results. In fact it was observed that both the methods converged in attribute the same individuals to the species

P.ulyssiponensis s.l. (Table II).

It should be noted also that ML (Figure 3) was able to distinguish between continental and Macaronesian "forms" of *P. ulyssiponensis*. On the other hand, the enzymatic digestion performed by *Taq*I did not provide differential fragments for the two "forms". However, this fact does not represent an impediment for the application of the proposed method, as this study was aimed at the distinction of *P. ulyssiponensis s.l.* from other similar sympatric species, and the ranges of distribution of continental and Macaronesian "forms" reflect a disjunct pattern).

In conclusion, the possibility of identifying large numbers of individuals using a non-lethal protocol, such as that adopted for the endangered *P. ferruginea* (Casu et al. 2006), opens a new perspective for ecological surveys, which may take into account specimens of *P. ulyssiponensis s.l.* separately from individuals of *P. caerulea*, *P. vulgata* and *P. candei*. Indeed, it should be evidenced that insufficient and/or wrong taxonomic analysis may negatively affect the statistic used and, consequently, may lead to misleading ecological conclusions. Thus, as suggested by Valentini et al. (2008), molecular techniques should progressively be more used as additional tools by ecologists, who have the need to "not only identify a single species from a specimen or an organism's remains, but also determine the species composition of environmental samples".

Finally, the increasing decline evidenced for different *Patella* species, in particular for the Macaronesian *P. ulyssiponensis*, which underwent a severe decline due to overexploitation (Cardigos et al. 2004), reinforces the importance of reliable identifications, and promotes the use of non-lethal sampling methods, which avoid the sacrifice of the animals.

Acknowledgments

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Tables

Table I. GenBank Accession numbers of 196 *Patella* COI sequences. Sequences obtained in the present study are in bold. The asterisks (*) indicate sequences deposited in Genbank as belonging to *P. aspera*.

Species	GenBank Accession Nos.
P. ulyssiponensis s.l.	DQ089588 to DQ089601; EF462964* to EF462971*; EU073914 to EU073935; GQ469883 to GQ469889; **JN105803 to JN105835**
P. caerulea	AB201519 to AB201525; AJ291549 to AJ291552; DQ089583 to DQ089587; GQ469862 to GQ469869; **JN105781 to JN105796**
P. vulgata	AB238580; DQ089616 to DQ089619; EF462957 to EF462962; **JN105836 to JN105835**
P. candei	DQ089564 to DQ089575; DQ089578 to DQ089582; DQ089625, DQ089626; EU073864 to EU073888; EU073896; **JN105797 to JN105845**

Table II. Details on 65 *Patella* specimens analysed. In bold are reported specimens which were wrongly classified on morphological basis.

No.	Individual code	Specimen voucher	Sampling site	Coordinates	Genetic attribution
1	PuBO	CZM 352	Bosa, W Sardinia	40°17’22’’N 8°28’28’’E	*P. ulyssiponensis s.l.*
2	PuBO	CZM 353	Bosa, W Sardinia	40°17’22’’N 8°28’28’’E	*P. ulyssiponensis s.l.*
3	PuPF	CZM 354	Punta Falcone, NW Sardinia	40°58’18’’N 8°12’12’’E	*P. ulyssiponensis s.l.*
4	PuPF	CZM 355	Punta Falcone, NW Sardinia	40°58’18’’N 8°12’12’’E	*P. ulyssiponensis s.l.*
5	PuPA	CZM 356	Punta Ala, Tuscany	42°48’33’’N 10°44’20’’E	*P. ulyssiponensis s.l.*
6	PuPA	CZM 357	Punta Ala, Tuscany	42°48’33’’N 10°44’20’’E	*P. ulyssiponensis s.l.*
7	PuAR	CZM 358	Argentiera, NW Sardinia	40°43’30’’N 8°8’50’’E	*P. ulyssiponensis s.l.*
8	PuAR	CZM 359	Argentiera, NW Sardinia	40°43’30’’N 8°8’50’’E	*P. ulyssiponensis s.l.*
9	PuLA	CZM 360	Lampedusa Island	35°30’13’’N 12°35’4’’E	*P. ulyssiponensis s.l.*
10	**PsLA**	CZM 361	Lampedusa Island	35°30’13’’N 12°35’4’’E	*P. caerulea*
11	**PsCR**	CZM 362	Insel Cres, Croatia	44°57’22’’N 14°24’38’’E	*P. caerulea*
12	**PsCR**	CZM 363	Insel Cres, Croatia	44°57’22’’N 14°24’38’’E	*P. caerulea*
13	**PsMO**	CZM 364	Molarotto Island, NE Sardinia	40°52’27’’N 9°46’43’’E	*P. ulyssiponensis s.l.*
14	**PsMO**	CZM 365	Molarotto Island, NE Sardinia	40°52’27’’N 9°46’43’’E	*P. caerulea*
15	**PsAL**	CZM 366	Alghero, W Sardinia	40°32’22’’N 8°19’21’’E	*P. caerulea*
16	PuAL	CZM 367	Alghero, W Sardinia	40°32’22’’N 8°19’21’’E	*P. ulyssiponensis s.l.*
17	PcAL	CZM 368	Alghero, W Sardinia	40°32’22’’N 8°19’21’’E	*P. caerulea*
18	PcAL	CZM 369	Alghero, W Sardinia	40°32’22’’N 8°19’21’’E	*P. caerulea*
19	PcPF	CZM 370	Punta Falcone, NW Sardinia	40°58’18’’N 8°12’12’’E	*P. caerulea*
20	**PsPF**	CZM 371	Punta Falcone, NW Sardinia	40°58’18’’N 8°12’12’’E	*P. caerulea*
21	PuPA	CZM 372	Punta Ala, Tuscany	42°48’33’’N 10°44’20’’E	*P. ulyssiponensis s.l.*
22	PcPA	CZM 373	Punta Ala, Tuscany	42°48’33’’N 10°44’20’’E	*P. ulyssiponensis s.l.*
23	PcAR	CZM 374	Argentiera, NW Sardinia	40°43’30’’N 8°8’50’’E	*P. caerulea*
24	PcAR	CZM	Argentiera, NW Sardinia	40°43’30’’N 8°8’50’’E	*P. caerulea*

		375			
25	PcLA	CZM 376	Lampedusa Island	35°30’13’’N 12°35’4’’E	*P. caerulea*
26	PcLA	CZM 377	Lampedusa Island	35°30’13’’N 12°35’4’’E	*P. caerulea*
27	PcTR	CZM 378	Trieste, Northern Adriatic	45°42’23’’N 13°42’41’’E	*P. caerulea*
28	PcTR	CZM 379	Trieste, Northern Adriatic	45°42’23’’N 13°42’41’’E	*P. caerulea*
29	PuMO	CZM 380	Molarotto Island, NE Sardinia	40°52’27’’N 9°46’43’’E	*P. ulyssiponensis s.l.*
30	PuMO	CZM 381	Molarotto Island, NE Sardinia	40°52’27’’N 9°46’43’’E	*P. ulyssiponensis s.l.*
31	**PsCA**	CZM 382	Ceuta harbour, Southern Spain	35º53’54’’N 5º19’06’’W	*P. caerulea*
32	**PsCA**	CZM 383	Ceuta harbour, Southern Spain	35º53’54’’N 5º19’06’’W	*P. ulyssiponensis s.l.*
33	PuLG	CZM 384	Las Galletas, Tenerife	28°00’30’’N 16°39’44’’W	*P. ulyssiponensis s.l.*
34	PuLG	CZM 385	Las Galletas, Tenerife	28°00’30’’N 16°39’44’’W	*P. ulyssiponensis s.l.*
35	PcdLG	CZM 386	Las Galletas, Tenerife	28°00’30’’N 16°39’44’’W	*P. candei*
36	**PsLG**	CZM 387	Las Galletas, Tenerife	28°00’30’’N 16°39’44’’W	*P. ulyssiponensis s.l.*
37	**PsLG**	CZM 388	Las Galletas, Tenerife	28°00’30’’N 16°39’44’’W	*P. candei*
38	**PsLG**	CZM 389	Las Galletas, Tenerife	28°00’30’’N 16°39’44’’W	*P. ulyssiponensis s.l.*
No.	**Individual code**	Specimen voucher	Sampling site	Coordinates	*Genetic attribution*
39	**PuBR**	CZM 390	Fort du Dellec	48°21’43’’N 4°32’46’’E	*P. ulyssiponensis s.l.*
40	**PuBR**	CZM 391	Fort du Dellec	48°21’43’’N 4°32’46’’E	*P. ulyssiponensis s.l.*
41	**PvuBR**	CZM 392	Fort du Dellec	48°21’43’’N 4°32’46’’E	*P. vulgata*
42	**PuBR**	CZM 393	Fort du Dellec	48°21’43’’N 4°32’46’’E	*P. ulyssiponensis s.l.*
43	**PuBR**	CZM 394	Fort du Dellec	48°21’43’’N 4°32’46’’E	*P. ulyssiponensis s.l.*
44	**PvuBR**	CZM 395	Fort du Dellec	48°21’43’’N 4°32’46’’E	*P. vulgata*
45	**PuBR**	CZM 396	Fort du Dellec	48°21’43’’N 4°32’46’’E	*P. ulyssiponensis s.l.*
46	**PuBR**	CZM 397	Fort du Dellec	48°21’43’’N 4°32’46’’E	*P. ulyssiponensis s.l.*
47	**PuBR**	CZM 398	Fort du Dellec	48°21’43’’N 4°32’46’’E	*P. ulyssiponensis s.l.*
48	**PvuBR**	CZM 399	Fort du Dellec	48°21’43’’N 4°32’46’’E	*P. vulgata*
49	**PvuBR**	CZM 400	Fort du Dellec	48°21’43’’N 4°32’46’’E	*P. vulgata*
50	**PvuBR**	CZM 401	Fort du Dellec	48°21’43’’N 4°32’46’’E	*P. vulgata*
51	**PvuBR**	CZM 402	Fort du Dellec	48°21’43’’N 4°32’46’’E	*P. vulgata*
52	**PsBR**	CZM	Fort du Dellec	48°21’43’’N	*P. ulyssiponensis*

		403		4°32’46’’E	*s.l.*
53	**PvuBR**	CZM 404	Fort du Dellec	48°21’43’’N 4°32’46’’E	*P. vulgata*
54	**PvuBR**	CZM 405	Fort du Dellec	48°21’43’’N 4°32’46’’E	*P. vulgata*
55	**PvuBR**	CZM 406	Fort du Dellec	48°21’43’’N 4°32’46’’E	*P. vulgata*
56	**PvuBR**	CZM 407	Fort du Dellec	48°21’43’’N 4°32’46’’E	*P. vulgata*
57	**PuLG**	CZM 448	Las Galletas, Tenerife	28°00’30’’N 16°39’44’’W	*P. ulyssiponensis s.l.*
58	**PuLG**	CZM 449	Las Galletas, Tenerife	28°00’30’’N 16°39’44’’W	*P. ulyssiponensis s.l.*
59	**PsLG**	CZM 450	Las Galletas, Tenerife	28°00’30’’N 16°39’44’’W	*P. candei*
60	**PsLG**	CZM 451	Las Galletas, Tenerife	28°00’30’’N 16°39’44’’W	*P. candei*
61	**PsLG**	CZM 452	Las Galletas, Tenerife	28°00’30’’N 16°39’44’’W	*P. candei*
62	**PsLG**	CZM 453	Las Galletas, Tenerife	28°00’30’’N 16°39’44’’W	*P. ulyssiponensis s.l.*
63	**PsLG**	CZM 454	Las Galletas, Tenerife	28°00’30’’N 16°39’44’’W	*P. candei*
64	**PsLG**	CZM 455	Las Galletas, Tenerife	28°00’30’’N 16°39’44’’W	*P. ulyssiponensis s.l.*
65	**PsLG**	CZM 456	Las Galletas, Tenerife	28°00’30’’N 16°39’44’’W	*P. ulyssiponensis s.l.*

Table III. Average number of pairwise differences at intraspecific and interspcific level. Diagonal elements: values within species; below diagonal: values between species. *P. ulyssiponensis s.l.* (Pu); *P. caerulea* (Pc); *P. candei* (Pcd); *P. vulgata* (Pvu).

	Pu	Pc	Pcd	Pvu
Pu	7.64949			
Pc	67.01509	1.97708		
Pcd	61.03291	36.07944	18.35829	
Pvu	68.72343	81.85784	82.52152	1.35012

Legends to figures

Figure 1. Alignment of DNA sequences of a 437 bp fragment of the mitochondrial COI gene in nine species of the genus *Patella*. Restriction site cut by *Taq*I is evidenced in the boxes.

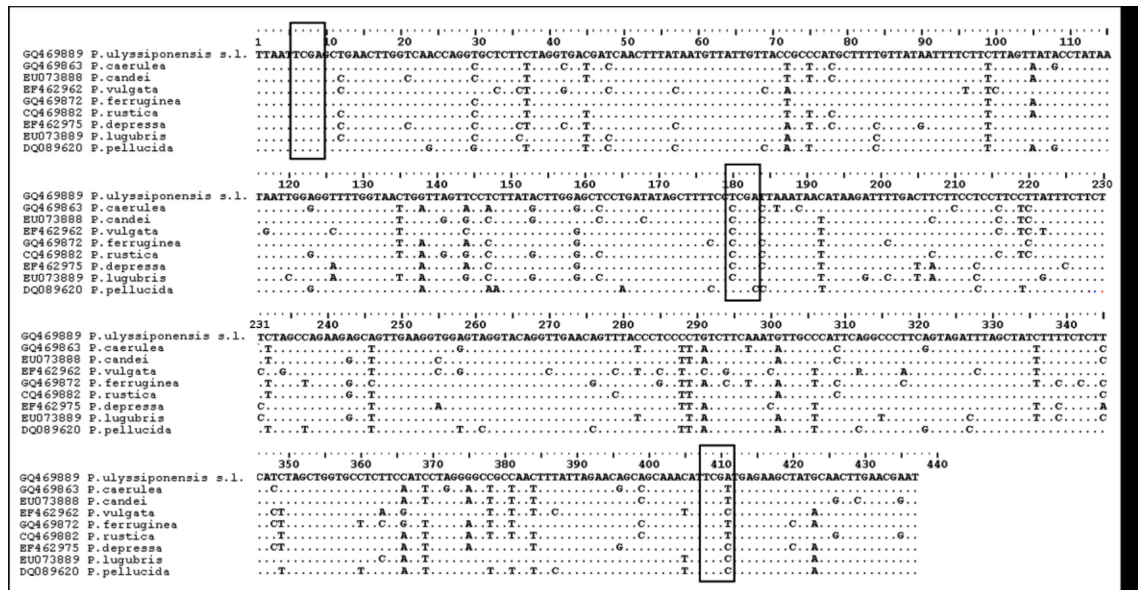


Figure 2. Sketch of the *Taq*I digestion fragments on a 437 bp long COI sequence in nine species of the genus *Patella*: *P. ulyssiponensis s.l.* (Pu); *P. caerulea* (Pc); *P. candei* (Pcd); *P. vulgata* (Pvu); *P. ferruginea* (Pf); *P. rustica* (Pr); *P. depressa* (Pd); *P. lugubris* (Pl); and *P. pellucida* (Pp). Bands not unequivocally scorable on 2% agarose gel are in grey (see text for details).

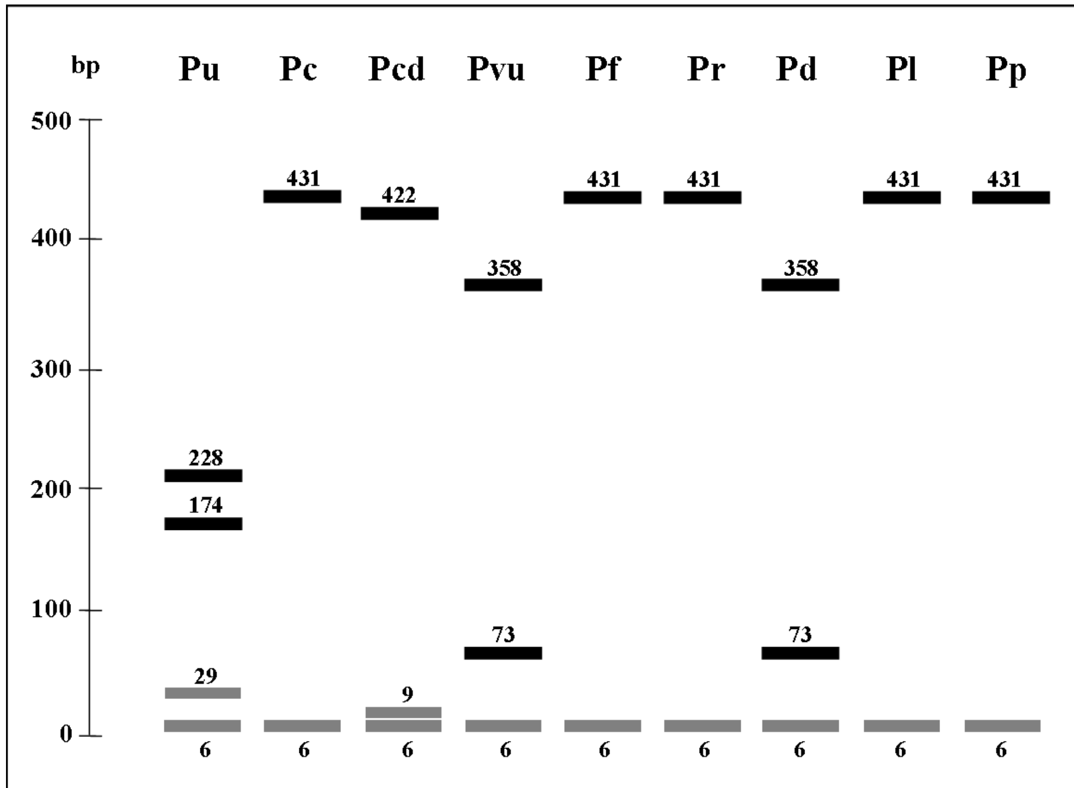


Figure 3. Tree obtained by ML. The scale bar refers to the number of substitutions per site. Supports of main nodes are indicated above for ML (bootstrap) and below for BI (posterior probability) only when higher than 50%. Individuals analysed in the present study are in grey. *Cymbula nigra* (Cysaf) was used as outgroup. A: *P. vulgata*. B: *P. ulyssiponensis* (B1. continental “form”; B2. Macaronesian “form”. C: *P. caerulea* (C1) + *P. candei* (C2. Canaries and Selvagens; C3. Azores and Madeira). Individual codes as in Table II.

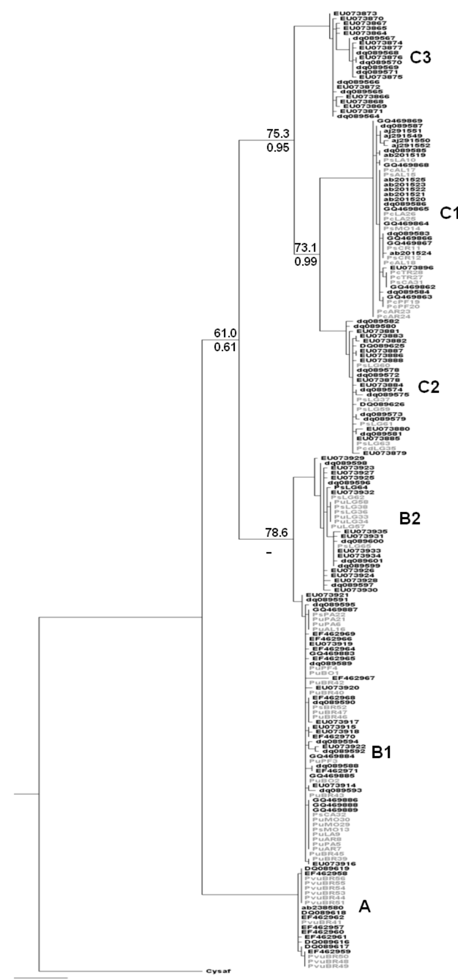
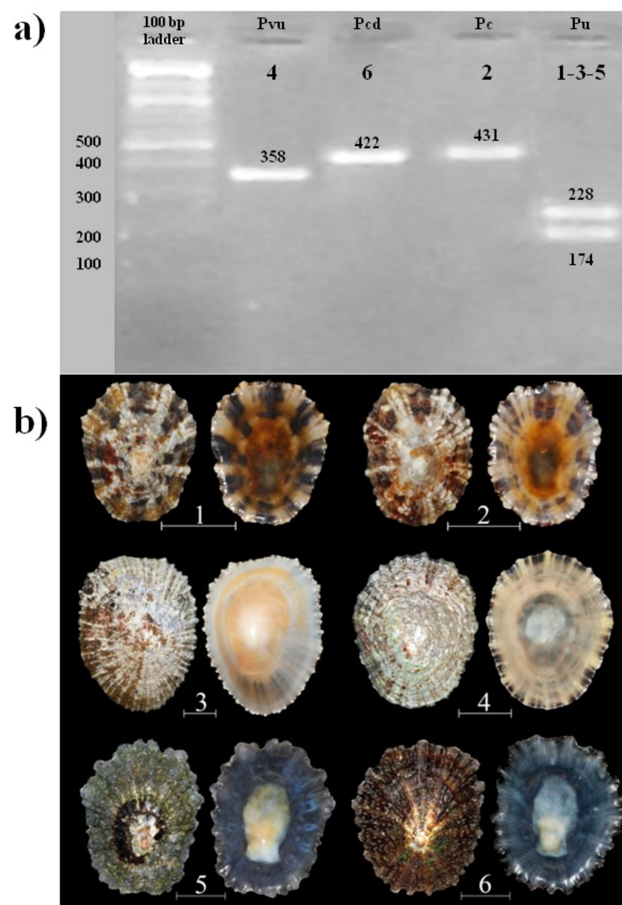


Figure 4. a) An example of *Taq*I electrophoresis banding patterns for *P. ulyssiponensis* (1-3-5), *P. caerulea* (2), *P. vulgata* (4), and *P. candei* (6). **b**) Corresponding shell: 1. *P. ulyssiponensis* (PsMO13), and 2. *P. caerulea* (PsMO14), from the Mediterranean; 3. *P. ulyssiponensis* (PsBR52) and 4. *P. vulgata* (PvuBR44), from the north-eastern Atlantic; 5. *P. ulyssiponensis* (PsLG36) and 6. *P. candei* (PsLG37) from Tenerife. Individual codes as in Table II. Scale bar: 1 cm.



Analysis of the ISSR genetic variability of *Patella ulyssiponensis* (Mollusca: Gastropoda) from Western Mediterranean

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Introduction

The limpet *Patella ulyssiponensis* Gmelin, 1791 (Mollusca: Gastropoda) is distributed in the shallow subtidal and low intertidal zones where it can be found on the more exposed shores as well as in mid-shore rock pools (Guerra and Gaudêncio 1986; Hawkins et al. 1990). The species is one of the most widely distributed limpets, though the assessment of its distribution range has been hampered by two factors. First, the identification of *P. ulyssiponensis* is difficult because of the broadly overlapping morphological variability with the other *Patella* species living in the lower part of intertidal zone. For instance, in several Mediterranean localities *P. ulyssiponensis* may not be safely distinguished by the endemic *Patella caerulea* Linnaeus, 1758. Indeed, the shell and foot colour pattern as well as the shell shape may not allow to unequivocally discriminate between the two species (Cretella et al. 1990; Sella et al. 1993; Mauro et al. 2003; Casu et al. 2010). Similarly, along the Atlantic European coasts the distinction between *P. ulyssiponensis s.l.* and *Patella vulgata* Linnaeus, 1758, may be equally troublesome (*i.a.*, Cabral 2003; Pagarete 2005). Furthermore, the picture has been complicated by the fact that, based upon the extremely variable shell sculpture and colour *P. ulyssiponensis* was described as a complex of subspecies (Christiaens 1973). Only recently, the use of molecular tools allowed both a better resolution of the relationships within the *P. ulyssiponensis* complex (Weber and Hawkins 2005; Sá Pinto et al. 2005, 2008) and a more accurate identification of the species with respect to its congeners (Sanna et al. 2011 *in press*). The high level of genetic differentiation evidenced by allozymes between populations from the Mediterranean and the European continental shoreline of the Atlantic, and those from the Macaronesian islands, led Weber and Hawkins (2005) to hypothesise the presence of two distinct species: *P. ulyssiponensis* from the European continental coasts, and *Patella aspera* Röding, 1798

restricted to the Macaronesia. On the other hand, Sá-Pinto et al. (2005, 2008) based upon mtDNA data referred to continental and Macaronesian "forms" of *P. ulyssiponensis*, respectively. Hence, the species range should be restricted to the Mediterranean and the Atlantic coasts of Europe up to South Norway according to Weber and Hawkins (2005), whereas it should be extended to Macaronesian islands according to Sa Pinto et al. (2005, 2008). Furthermore, both the sequencing mitochondrial DNA (COI) and a PCR-RFLP assay on the same region allowed to distinguish without uncertainty *P. ulyssiponensis* from the congeneric species *P. ulyssiponensis s.l.* from *P. caerulea*, *P. vulgata* and *P. candei* (Sá Pinto et al. 2005, 2008; Sanna et al *in press*).

In this work we present a survey of genetic variation in the species at large geographical scale. This study differs from former ones that were more targeted on the assessment of species status (e.g. Weber and Hawkins 2005) or the phylogeographic patterns across Macaronesian islands and the continent (e.g. Sa Pinto et al. 2008). Here we focus the aim on deciphering the patterns of spatial genetic structure in the species/form spread along the European Mediterranean and Atlantic coasts. Such information may be useful in addressing issues related to the ecology and conservation of a species (Garrick et al. 2010). For instance, the assessment of genetic differentiation among populations may be used to infer species dispersal capabilities and genetic connectivity among populations (Kinland and Gaines 2003). Moreover, the knowledge of the amount of genetic variability and distribution in space and time is critical for a correct diagnosis of the status, threats and viability of populations (Frankham 1995; Escudero et al. 2003). To reach these goals, we used nuclear ISSR markers (Zietckiewicz et al. 1994) because this technique is affordable and less laborious compared with other fingerprinting methods. Simulations indicate that dominant markers, such as ISSRs, may be as

efficient as the codominant ones in estimating genetic differentiation among populations (Mariette et al. 2002). Furthermore, ISSRs generate highly reproducible bands, and detect high levels of genetic variability in populations of marine organisms, even at a small spatial scale (*e.g.*, Casu et al. 2005, 2006; Hassan et al. 2003; Lai et al. 2008; Machkour et al. 2009).

Materials and methods

Sampling and DNA extraction

A total of 478 specimens of *Patella ulyssiponensis* were collected from the intertidal zones of 40 localities over most of the species distribution range (see Table 1, Fig. 1 for details). Individuals were collected from each sampling site using the following a non lethal protocol: the individual was gently removed from the substrate by means of a wood chisel, and a 30-60 mg sample of foot muscle was excised using a sterilised surgical forceps. The individual was then repositioned in its so-called 'home scar', a depression in the rock formed by abrasion by the shell, resulting in a tighter fit to the rock and reduced risk of desiccation. Genomic DNA was extracted from the tissue using the Macherey-Nagel NucleoSpin® Tissue.

ISSR amplifications

A set of 22 primers was preliminarily assayed on a limited number of individuals identify the primers that produced scorable and reproducible bands (Table 1). The PCR reaction mixture (25 µl volume) contained 0.5 units of Taq DNA Polymerase from Thermus aquaticus with 10x reaction buffer without $MgCl_2$ (Sigma-aldrich®), 1× reaction buffer (Sigma aldrich®), 2.5 mM $MgCl_2$, 0.2 µM primer, 200 µM of each dNTP (Sigma-aldrich®), and up to 30 ng of genomic DNA. PCR amplification was performed in a i-cycler Thermal Cycler (Biorad®) programmed for 1 cycle of 3 min at 94° C, 35 cycles of 40 s at 94° C, 45 s at 50° C, and 1 min and 40 s at 72° C. At the end of these cycles a post-treatment at 72° C for 5 min to complete partial amplification and a final

cooling at 4° C were performed. For each primer, negative controls were included and amplifications were repeated on 20% of samples in order to verify the repeatability of results.

Electrophoresis and visualisation of amplification products

The PCR products were analysed by electrophoresis using a 2% agarose gel in 1× SBA buffer (Sodium boric acid Ph. 8.2) stained with 1 µl/20 ml ethidium bromide solution. Gels were run at 90 V (4,5 V/cm) for 2 h and ISSR banding patterns on gels were visualized using a photo-UV transilluminator system and recorded by digital photography. One hundred base pair ladder (GeneRuler 100bp Plus DNA Ladder, Fermentas®) were run for reference with each primer.

ISSR data analysis

ISSRs are dominant diallelic markers; the presence of a given band, the dominant phenotype, is scored as 1, whereas its absence (the recessive phenotype) is scored as 0. Polymorphisms (i.e. presence/absence of a band) are due to primer divergence, insertions, deletions, or chromosomal rearrangement (Wolfe & Liston 1998).

Genetic structure within the ISSR data set was assessed by means of an individual based approach, which may avoid the artefacts due to the assumption of predefined populations (Mank & Avise 2004). The population structure was inferred by the Bayesian model-based clustering algorithm implemented in the software STRUCTURE 2.3.1 (Pritchard et al. 2000, Falush et al. 2007). This method assigns individuals to clusters according to the multilocus genotype, without prior knowledge of their geographical origin. The number of clusters (K) is chosen in advance, and the posterior

probability of data, lnP(D), is estimated for each model with a given number of clusters. We applied the admixture model with correlated allelic frequencies (Falush et al. 2003) as this is the more general and flexible model (Francois et al. 2010). For each value of *K*, ranging from one to the maximum number of populations plus three, 10 independent runs were performed, each consisting of 100000 iterations after a burn-in period of 100000, in order to assess consistency of results across runs. All analyses were performed on the Bioportal server at the University of Oslo (Kumar and Skjaeveland 2009). To identify clusters of individuals which might reflect sharp discontinuities in gene frequencies (Garnier et al. 2004), we considered: 1) the average value of lnP(D), 2) the consistency of results across replicated runs for a given *K*, and 3) the method described in Evanno et al. (2005) which retrieves the highest hierarchical structure present in the data.

The software CLUMPP 1.1.2 (Jakobsson & Rosenberg 2007) was used to check the consistency of results across replicate runs. The average pairwise similarity (*H*) among replicate runs for a given *K* was estimated by a Greedy search algorithm with 10'000 random input orders. Replicate runs with similarity values above 0.85, which corresponds to a similar population structure (Rosenberg et al. 2002), were then merged to obtain the average membership of all individuals to each cluster. The results were displayed by the software DISTRUCT 1.1 (Rosenberg 2004).

Genetic structuring was also investigated by a hierarchical analysis of molecular variance (AMOVA) using the approach described in Excoffier et al. (1992) and implemented in the software package ARLEQUIN 3.52 (Excoffier & Lischer 2010). The method partition the total variance into covariance components corresponding to differences within populations, between populations, between populations within groups and among groups. First, we performed a two level AMOVA analysis to assess

the overall level of genetic differentiation among populations; then we applied a three level AMOVA analysis to assess differences among groups of populations. Several grouping schemes that may reflect geographical features such as biogeographical boundaries, different basins, marine circulation patterns, and/or the presence of islands were tested (Table 3). The significance of the fixation indices associated with the different groupings was assessed by a non parametric permutation test with 10'000 replicates (Excoffier et al. 1992).

Results

We scored a total of 66 bands over the five primers that give the more reproducible and reliable banding patterns (Table 2). The percentage of polymorphic loci averaged over all samples was 50.6% (± 2.60%), which corresponded to an average of 35.47 bands per population. Of these bands only 7.15, on average, were locally common in 50% or less of populations, and 2.25 in 25%. Remarkably, only one private band was found over 40 locations (Isole delle Formiche, IFO).

Bayesian clustering results evidenced an increasing lnP(D) value from K =1 to 12, after which the curve reached a plateau (Fig. 2a). The presence of a more or less plateau is due to the way in which the lnP(D) is estimated by approximation in an *ad hoc* way (Pritchard et al. 2000), and occurs for values of K that are equal or superior to the actual number of populations in the sample (Garnier et al. 2004). Furthermore, the increase of the lnP(D) began to flatten for $K > 4$, after which the gain of information exhibited gradually decreasing values (Fig. 2a). This result means that the information brought by the fifth and the following clusters is much less important than the information brought by the former ones. Accordingly, the most consistent results across replicate runs were

found in models considering two, three and four clusters. Here, the algorithm found the same clustering solution across all runs, with an average pairwise similarity of $H = 0.98$ that was far beyond the threshold value of 0.85 for highly similar clustering results (Rosenberg et al. 2004). Conversely, several clustering solutions appeared for $K > 4$; these results may occur when trying to identify more gene pools than there are sets of allele frequencies in the model (Garnier et al. 2004).

The uppermost hierarchical structure present in the data, the ΔK statistics (Evanno et al. 2005) evidenced a weak peak occurring at $K = 2$ (Fig. 2b); nevertheless, the two clusters (A and B) did not identify groups of geographically isolated populations (Fig. 3). At $K = 3$ we found a cluster that was almost restricted to the Mediterranean sea (cluster C), whereas the other showed a patchy distribution in the Atlantic, western Mediterranean and eastern Mediterranean basins. However cluster C did not result from the split of one cluster found at K = 2; rather individuals and/or populations assigned to clusters A and B were merged in this cluster (Fig. 3). Similarly, the fourth cluster (D) found at $K = 4$ resulted from the re-allocation of individuals previously assigned to clusters A, B, C. Using an individual membership coefficient threshold of Q ≥ 0.51 56 out of 478 individuals (12 %) were re-allocated to cluster D; this fraction decreased to 9% (42 individuals) according to a threshold of Q ≥ 0.80 which denote pure ancestry to a cluster (Vaha et al. 2007).

The two-level AMOVA evidenced a significant genetic structure among populations which accounted for 27% of total genetic variation ($\Phi_{ST} = 0.271$, $P < 0.001$). The three-level AMOVA did not show a significant genetic differentiation between Tenerife (Macaronesia) and the continental samples, as well as between Atlantic and Mediterranean regions (Table 4). Conversely, we found a significant, albeit slight genetic differentiation between western and eastern Mediterranean basins,

accounting for 3.90% of total variation (Φ_{CT} = 0.039, P < 0.01). Interestingly, a further partitioning of the two Mediterranean basins into smaller groups increased the portion of total variance explained by differences among groups (Table 4). Among these groupings, the fixation index was maximised when populations were grouped according to the following scheme: Alboran sea, Balearic sea and Gulf of Lions (group 1); Sardinian-Corsican region and western Tyrrhenian sea (BDF, IFO and CGA); south-eastern Sicily (MRS, TOR, PSE, APE and PLM); Lampedusa island; Cyprus. Genetic differentiation among these groups accounted for about 6% of total variation (Φ_{CT} = 0.059, P < 0.001).

Discussion

Studies conducted so far analysed the pattern of genetic variation among Macaronesian and continental populations of *Patella ulyssiponensis*. The congruent patterns found with different marker systems such as alloenzymes (Weber and Hawkins 2005) and mtDNA (Sa Pinto et al. 2008, Sanna et al. *in press*) evidenced 1) a clear genetic differentiation between Macaronesian and continental populations and 2) lack of spatial genetic structure throughout continental Europe. Conversely, results obtained with ISSR markers, did not support the genetic differentiation between samples from Tenerife (Macaronesia) and those from Europe (Fig. 3, Table 4). This result must be interpreted with caution as samples from Macaronesia were collected at a single point location; we cannot rule out that a more thorough sampling of Macaronesia would give a different picture. Assuming this pattern is true the lack of genetic differentiation between Tenerife and continental samples may be due to a high degree of homoplasy (i.e. co-migrating bands are identical by state rather than by descent) or to a scant signal of differentiation in ISSR banding patterns. Both hypotheses cannot be excluded *a priori*;

Gian Luca Dedola: ANALISI DELLA VARIABILITÀ GENETICA DI *PATELLA FERRUGINEA, PATELLA ULYSSIPONENSIS* (MOLLUSCA: GASTROPODA) E *PINNA NOBILIS* (MOLLUSCA: BIVALVIA): IL CONTRIBUTO DEI DATI MOLECOLARI ALLA CONSERVAZIONE DI SPECIE MINACCIATE - Tesi di dottorato in Scienze della natura e delle sue risorse - indirizzo Biologia Ambientale, Università degli Studi di Sassari

of the 45 bands scored at Tenerife none was private to this population. Furthermore, 31 out 45 bands were scored in more than 50% of sampling localities. Nonetheless, homoplasy is unlikely to distort analysis restricted to intraspecific level or to groups of closely related species, as it has been shown that most of co-migrating fragments in these cases are identical by descent (Nybom 2004). Similarly, we may discard the hypothesis of a weak signal, as indeed we found a significant genetic differentiation among populations ($\Phi_{ST} = 0.271$, $P < 0.001$). This pattern, suggesting population differentiation without geographical structuring is congruent with the results obtained with alloenzymes in continental populations of P. ulyssiponensis (Weber and Hawkins 2005).

Another result of this work is the lack of genetic differentiation observed between Atlantic and Mediterranean samples, across well known physical barriers to dispersal such as the Gibraltar strait and the Oran-Almeria front (a jet of water between these localities that may prevent along-shore dispersal). Interestingly, in this case the pattern retrieved by ISSRs is congruent with mtDNA data for *P. ulyssiponensis* which did not evidenced the occurrence of separated Atlantic and Mediterranean lineages (Sa Pinto et al. 2008, Sanna et al. *in press*). Very similar patterns have been observed in other species of the genus; for instance, one of the three mtDNA lineages found in *P. rustica* is spread across the Atlantic and western Mediterranean, whereas the others were found in the eastern Mediterranean (Sa Pinto et al. 2005). Though information on Patellidae dispersal abilities is contradictory and little is known about their pelagic larval duration (Espinosa and Ozawa 2006), several authors suggested that *P. ulyssiponensis* and *P. rustica* may have a high potential for dispersal (Sella et al. 1993, Weber and Hawkins 2005). On the other hand, both ISSR and mtDNA data indicated that *P. ferruginea*, which is believed to have poor dispersal capabilities, is not geographically structured

across the OAF as well as along the north-African shores (Espinosa and Ozawa 2006, Casu et al. *accepted*). These results agree with the growing evidence that potential for dispersal alone may be a poor predictor of population differentiation in coastal invertebrates, as marine currents, habitat preferences and historical processes play an important role (Ayre and Hughes 2009, Kelly and Palumbi 2010). These processes are not uncommon in intertidal limpets; for instance, in three hawayan species of the genus *Cellana* with similar life history traits contrasting phylogeographic patterns were mainly related to biogeographical range and habitat specificity (Bird et al. 2007). As well, species with different dispersal capabilities may show similar patterns of genetic variation, albeit the causal factors may be different as shown in *Syphonaria* concinna and *S. nigerrima* (Teske et al. 2011).

Similarly, the lack of divergence between Atlantic and Mediterranean populations of many species has been related to a recent expansion and /or (re-)colonisation of one area rather than differences in the dispersal potential (Patarnello et al. 2007). Though ISSR markers do not allow inferences on population demographic history, the decreasing average membership to cluster A for $K = 2$ in a eastward direction, from the Atlantic to eastern Mediterranean may cope with this scenario (Fig. 2c). A similar pattern but decreasing westwards, from eastern Mediterranean to the Atlantic, was observed in cluster B. These patterns reflected in each region the relative proportion of individuals assigned to cluster A or B, according to a membership coefficient ≥ 0.8 (Vähä et al. 2007). Most of individuals from the Atlantic were assigned to cluster A (66%), whereas in the eastern Mediterranean 81% of individuals were assigned to cluster B (Fig. 4). The similar numbers of individuals assigned to either cluster A or B in the western Mediterranean (40 and 39 %), suggest this region may be actually a contact zone (Fig. 4). Noteworthy, similar clinal patterns have been reported in *P.*

rustica, where three lineages (one from Atlantic and western Mediterranean, the others from eastern Mediterranean) formed a contact zone along southern shores of Italian peninsula (Sa Pinto et al. 2010). Interestingly, the presence of clines and a contact zone may fit the results observed at $K = 3$ and 4 in which clusters C and D resulted from the re-allocation of individuals as well as populations belonging to clusters B and A rather than to a further split of one group. In fact, bayesian clustering of data simulating a secondary contact zone between two divergent gene pools may result in a further cluster, grouping admixed individuals/populations of the secondary contact zone (Francois et al. 2010).

The different frequency with which individuals belonging to clusters A and B are distributed in the western and eastern Mediterranean may explain the slight, albeit significant genetic differentiation found between and within these basins and (Table 4). The genetic homogeneity found between Atlantic and Mediterranean, but not at regional and sub-regional spatial scales (within and between the Mediterranean basins) may reflect complex patterns of connectivity at smaller geographical scale, where mesoscale and coastal circulation as well as coastal geometry may affect dispersal (Ribeiro et al. 2010). Nonetheless, the genetic differentiation among groups found within western Mediterranean was much less than observed in *P. ferruginea* where at least one fifth of total variation was due to differences among groups (Casu et al. accepted). Given the scarce knowledge on the biology of these species we could only hypothesise the cause for such differences. *P. ulyssiponensis* is found above all over the mid-littoral zone, whereas *P. ferruginea* occupies the supralittoral and the high-midlittoral rocky shores, where the two species' distribution overlaps. The partially different vertical distribution of the species may affect the degree of genetic structure, as it may imply, for instance, different selective regimes as shown in many species of coastal invertebrates (Kelly and

Palumbi 2010). Different habitat requirements have been invoked to explain the different genetic structure in co-distributed limpets belonging to *Syphonaria* (Teske te al. 2011) and *Cellana* genera (Bird et al. 2007). Other potential explanations may rely on the larval life history (e.g. Riginos and Victor 2001). Though both species may have similar larval duration, the larger size and higher density of oocytes may reduce dispersal in *P. ferruginea* (Templado 2001). Indeed, Teske et al. (2011) suggested that a larger size of eggs may reduce the potential for long distance dispersal as larvae are likely to settle more quickly. Another hindrance to dispersal in this species may be the lower number of released larvae which may affect the recruitement in this species (Laborel-deguen and Laborel 1991).

Results of the present work highlight that ISSR markers may be a useful tool in analysing patterns of genetic variation in *P. ulyssiponensis*. At continental scale genetic patterns were congruent with those found in other studies, whereas the lack of differentiation between Macaronesian and European samples may reflect a bias due to the poor sampling of this area. A more thorough sampling of the western and partly the eastern Mediterranean sea allow us to evidence a shallow genetic structuring between and within Mediterranean basins. In addition, our results suggest that western Mediterranean may represent the contact zone of two genetically divergent clusters showing clinal variation in opposite directions. Noteworthy these results are similar to those reported in several Mediterranean populations of *P. rustica* (Sa Pinto et al. 2010), albeit *P. ulyssiponensis* showed a shallower degree of genetic differentiation. Sequencing of mitochondrial DNA markers, should allow a better understanding of the present-day and historical patterns of connectivity of *P. ulyssiponensis* across the Mediterranean, and ensure an even more detailed picture of the phylogeographical patterns in this area.

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Figure legends

Fig. 1 Sampling locations of *P. ulyssiponensis* from Canary islands (Macaronesia), eastern Atlantic and Mediterranean sea. Sampling locations from Sardinian_Corsican region are also displayed in the inset with greater detail. See Table 1 for labels identification.

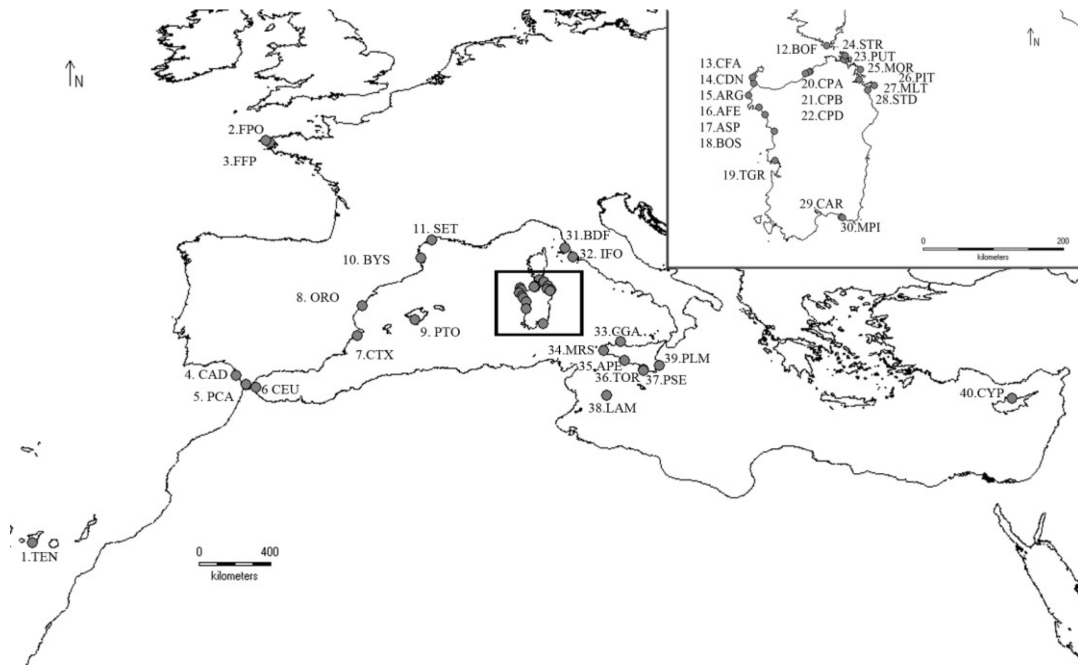
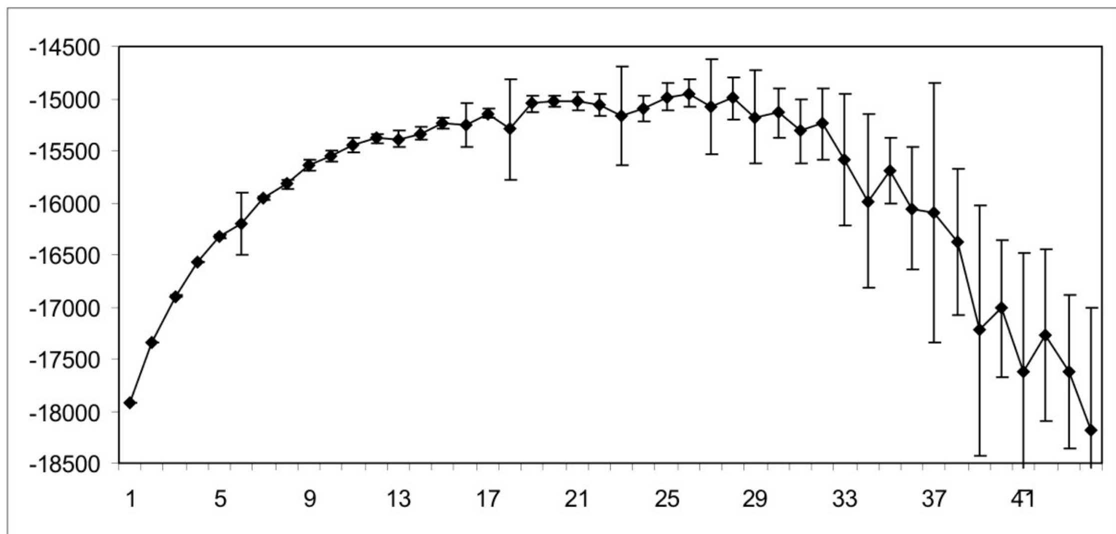


Fig. 2 *P. ulyssiponensis*: a) curve of lnP(D) values averaged over tne replicate runs (y-axis) versus the number of clusters (K) plotted in the x-axis. Error bars represent the standard deviation of lnP(D) values; b) curve of ΔK statistics (y axis) plotted against the number of K (x-axis), estimated according to Evanno et al. (2005). The peak value ($K = 2$) corresponds to the uppermost hierachical strrucutre found in the data.

a



b

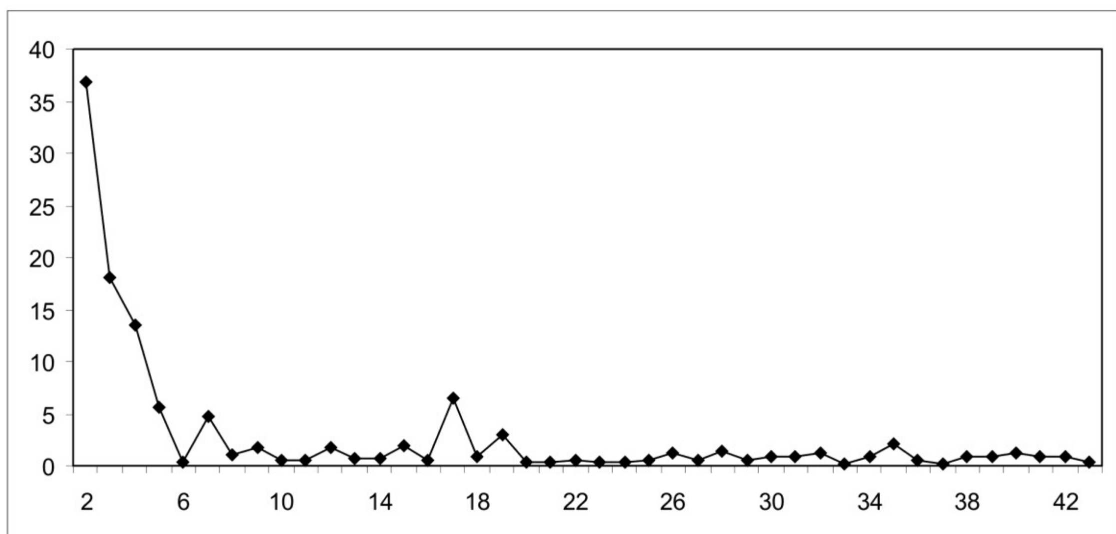


Fig. 3 *P. ulyssiponensis*. Each individual is represented by a thin horizontal line, which is partitioned into *K*-coloured segments (*K* = number of clusters). The height of each segment is proportional to the individual estimated membership in the corresponding cluster. Black lines separate individuals from different sampling sites ordered as in Table 1. Only the clustering solutions with a Similarity coefficient (*H*) greater than 0.85 are displayed. These values correspond to highly consistent results across replicate runs (Rosenberg 2004). Colour legend is as follows: green = cluster A; white = cluster B; red = cluster C; blue = cluster D.

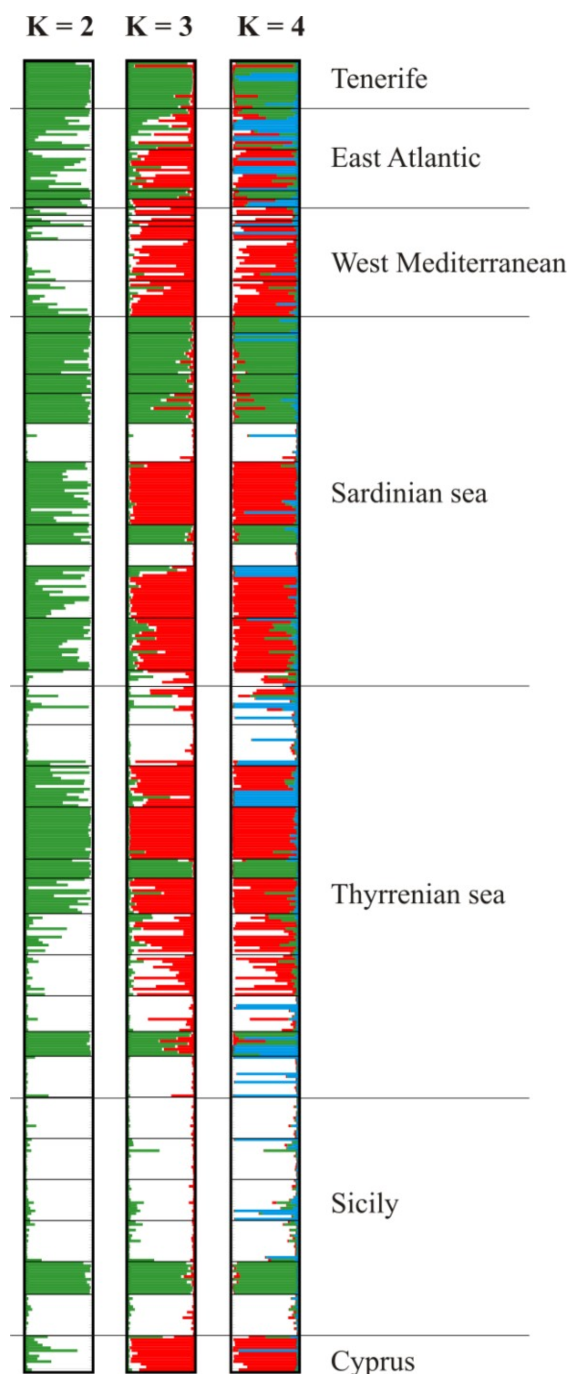


Fig. 4 *P. ulyssiponensis*: plot of the average membership of individuals to clusters A and B, and of the proportion of individuals assigned to cluster A and B with $Q \geq 0.8$ (threshold of membership coefficient for individuals of pure ancestry) and $0.2 \leq Q < 0.8$ (individuals showing admixture between cluster A and B). Q_A = average membership to cluster A; Q_B = average membership to cluster B; Q_{A80} = proportion of individuals assigned to cluster A with $Q \geq 0.8$; Q_{B80} = proportio of individuals assigned to cluster A with $Q \geq 0.8$; Q_{ADMIX} = proportion of admixed individuals

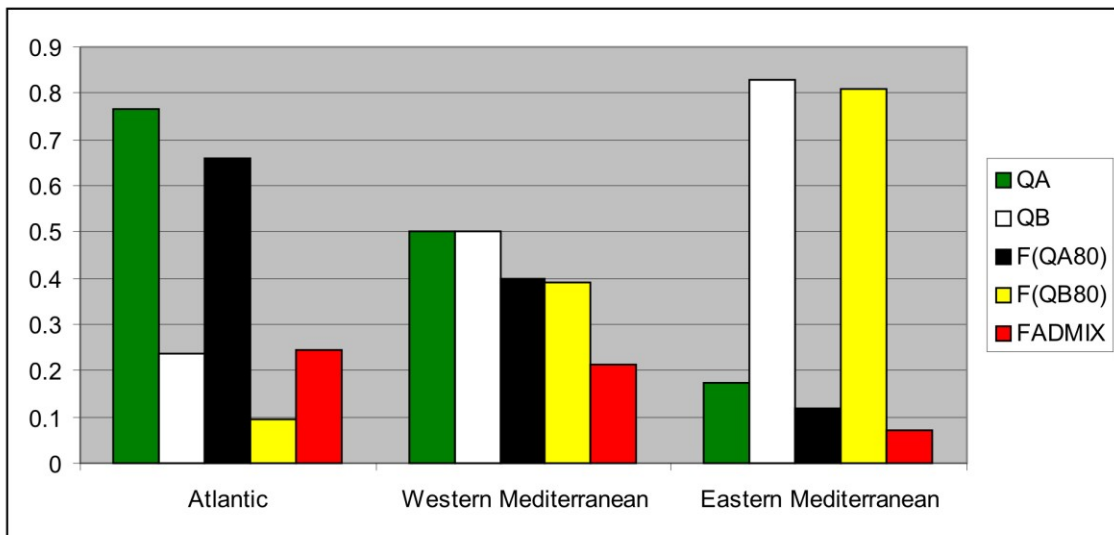


Table 1 Sampling details of *P. ulyssiponensis*.

Sampling station	Code	Geographical region of sampling station	Latitude	Longitude
Tenerife	TEN	Canary islands	-16,6636	28,00819
Fort Dudellec	FFD	East Atlantic	-4,56804	48,35169
Porspoder	FPO	East Atlantic	-4,77953	48,49845
Cadiz	CAD	East Atlantic	-6,30824	36,53046
Punta Camarinal	PCA	East Atlantic	-5,79589	36,07795
Ceuta	CEU	Alboran sea	-5,28678	35,90488
Cala Tio Ximo	CTX	Balearic sea	-0,10398	38,52785
Oropesa port	ORO	Balearic sea	0,132744	40,07296
Torrent de Cala Pi	PTO	Balearic sea	2,830811	41,02724
Banyuls sur mer	BYS	Gulf of Lion	3,137131	42,48221
Sete	SET	Gulf of Lion	3,700717	43,39484
Bonifacio	BOF	Bonifacio strait	9,161667	41,38667
Capo Falcone	CFA	Sardinian sea	8,203333	40,97167
Coscia di Donna	CDN	Sardinian sea	8,22	40,9
Argentiera	ARG	Sardinian sea	8,146667	40,74167
Alghero-Fertilia	AFE	Sardinian sea	8,283628	40,5911
Alghero-La Speranza	ASP	Sardinian sea	8,366458	40,49571
Bosa	BOS	Sardinian sea	8,483333	40,285
Torregrande	TGR	Sardinian sea	8,490689	39,90231
Costa Paradiso A	CPA	Bonifacio strait	8,946617	41,05681
Costa Paradiso B	CPB	Bonifacio strait	8,932825	41,0479
Costa Paradiso C	CPD	Bonifacio strait	8,886844	41,02724
Punta tegge	PUT	Bonifacio strait	9,381206	41,21134
Lo Strangolato	STR	Bonifacio strait	9,399231	41,25876
isola Mortorio	MOR	Thyrrenian sea	9,596872	41,07667
Pittulongu	PIT	Thyrrenian sea	9,577992	40,94767
Molarotto	MLT	Thyrrenian sea	9,778333	40,875
Punta Aldia	STD	Thyrrenian sea	9,691592	40,81328
Cala Regina	CAR	Sardinian channel	9,351942	39,17779
Mari Pintau	MPI	Sardinian channel	9,371214	39,17126
Buca delle fate	BDF	Ligurian sea	10,48407	42,98684
Punta Ala	IFO	Thyrrenian sea	10,89167	42,56667
Capo Gallo	CGA	Thyrrenian sea	13,31463	38,22442
Marsala	MRS	Siculo-tunisian strait	12,42441	37,80213
Porto Empedocle	APE	Siculo-tunisian strait	13,49708	37,28821
Torre di mezzo	TOR	Siculo-tunisian strait	14,47841	36,80496
Punta secca	PSE	Siculo-tunisian strait	14,49193	36,78911
Lampedusa	LAM	Siculo-tunisian strait	12,58528	35,5
Plemmirio	PLM	Ionian sea	15,30678	37,04106
Karaoglonoglu	CYP	Levantine sea	33,25477	35,34848

Table 2 ISSR dataset: primer names and sequences, number of polymorphic bands *per* primer and range of molecular weight in base pairs (bp)

Primer	Sequence (5'-3')	No. of bands	Size range of bands (bp)
IT1	$(CA)_8GT$	16	350-1500
SAS3	$(GAG)_4GC$	14	300-1500
UBC81	$(GA)_8C$	13	300-1400
+GAC	WB(GACA)	11	200-800
+CA	$RY(CA)_7$	10	400-1200
IT1	$(CA)_8GT$	16	350-1500
SAS3	$(GAG)_4GC$	14	300-1500

Table 3 ISSR dataset: Grouping schemes used in the analysis of molecular variance (AMOVA)

Groups	Sampling locations
TEN	TEN
ATL	FFD, FFP, CAD, PCA
WMED	CEU, CTX, ORO, PTO, BYS, SET
SCR	BOF, CFA, CDN, ARG, AFE, ASP, BOS, TGR, CPA, CPB, CPC, PUT, STR, MOR, PIT, MLT, STD, CAR, MPI
TUS	BDF, IFO
WTHYR	BDF, IFO, CGA
SES	MRS, APE, TOR, PSE, PLM
Sicily	CGA, MRS, APE, TOR, PSE, PLM
Sardinian sea	BOF, CFA, CDN, ARG, AFE, ASP, BOS, TGR, CPA, CPB, CPC
Thyrrenian sea	PUT, STR, MOR, PIT, MLT, STD, CAR, MPI , BDF, IFO, CGA
LAM	LAM
CYP	CYP

Table 4: ISSR dataset: Two-level and thre-level Analysis of molecular variance (AMOVA). Groups for three-level AMOVA were defined *a priori* according to geographical features (see Table 3 for details). d.f.: degrees of freedom, SS: sum of squares, var. comp.: variance component, %var: percentage of variation. NS not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Source of variation	d.f.	SS	Var. comp.	%var	Φ-*statistics*
		Two-level AMOVA			
Among populations	39	1440.831	2.53457	27.14	$\Phi_{ST} = 0.271$***
		Three-level AMOVA with Atlantic samples			
Macaronesia vs Continent	1	54.455	0.15223	1.61	$\Phi_{CT} = 0.0161^{NS}$
Atlantic vs Mediterranean	1	62.471	0.21323	2.24	$\Phi_{CT} = 0.022^{NS}$
Atlantic, western and eastern Mediterranean	2	161.194	0.34745	3.65	$\Phi_{CT} = 0.036$***
TEN, ATL, WMED, SCR, WTHYR, SES, LAM, CYP	6	367.202	0.47048	4.96	$\Phi_{CT} = 0.050$***
TEN, ATL, WMED, SCR, TUS, Sicily, LAM, CYP	7	382.725	0.38403	4.06	$\Phi_{CT} = 0.041$***
TEN, ATL, WMED, SCR+TUS, Sicily, LAM, CYP	6	351.917	0.42282	4.46	$\Phi_{CT} = 0.045$***
TEN, ATL, WMED, SCR+WTHYR, SES, LAM, CYP	5	327.390	0.51690	5.42	$\Phi_{CT} = 0.054$***
		Three-level AMOVA without Atlantic samples			
Western and eastern Mediterranean	1	98.723	0.37441	3.90	$\Phi_{CT} = 0.039$**
WMED, SCR, WTHYR, SES, LAM, CYP	5	303.441	0.47882	5.02	$\Phi_{CT} = 0.050$***
WMED, SCR, TUS, Sicily, LAM, CYP	5	286.128	0.42119	4.42	$\Phi_{CT} = 0.044$***
WMED, SCR+TUS, Sicily, LAM, CYP	4	255.319	0.48183	5.03	$\Phi_{CT} = 0.050$***
WMED, SCR+WTHYR, SES, LAM, CYP	4	264.592	0.56601	5.87	$\Phi_{CT} = 0.059$***
WMED, Sardinian sea, Thyrrenian sea, SES, LAM, CYP	5	309.369	0.40773	4.31	$\Phi_{CT} = 0.043$***

GENETIC VARIABILITY IN THE SARDINIAN POPULATION OF THE MANILA CLAM, *RUDITAPES PHILIPPINARUM*

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1. INTRODUCTION

The introduction of allochthonous species in marine habitats is a traditional practice in aquaculture, particularly when fisheries are depleted due to the overexploitation of native species (see e.g. Breber, 1985; Pellizzato et al., 1989). However, this method can often pose serious risks (e.g., local extinction, hybridization) to indigenous species, particularly when farming is performed without separation of the farmed area from natural biotopes (see Wonham and Carlton, 2005). The introduction of the Manila clam *Ruditapes philippinarum* (Adams and Reeve, 1850) (Bivalvia: Veneridae) to Europe in the 1970s and 1980s (Gosling, 2003) from its native habitat on the West coast of the Pacific from Russia to the Philippines serves as a paradigm for such a phenomenon. Since then, the Manila clam has rapidly spread along European coastlines, becoming the main contributor to local clam fisheries. The Manila clam was released in the North-East Adriatic in the 1980s (Breber, 1985) to counter the dramatic depletion of several native species due to overexploitation and/or pollution (Savini et al., 2010). In the Adriatic, the Manila clam progressively occupied the ecological niche of the indigenous European grooved carpet shell, *Ruditapes decussatus* L., 1758, which led to the extinction of local populations (Jensen et al., 2004).

In 2008, several specimens of *R. philippinarum* were found in the Gulf of Olbia (North-East Sardinia). This species has now become abundant (Cannas et al., 2009) and has established the first recorded population of Manila clams in Sardinia. The introduction of this species was likely the result of the 'accidental' release of adult individuals a few years earlier due to careless management by fishery operators that were conducting legal stabulation processes in the town of Olbia (Cannas, pers. comm.; Cristo, pers. comm.).

The amount of initial genetic variability, as well as the potential for dispersal and a high growth rate, may play an important role in determining the spread of an invasive species by favoring its adaptation to new environments (Roman and Darling, 2007; Estoup and Guillemaud, 2010). Furthermore, a high genetic variability may overcome the deleterious effects of inbreeding on population growth (Bakker et al., 2009; Eales et al., 2010). Notably, the evolutionary potential of an invasive species relies more on the initial amount of genetic variability than the number of introduction events (Eales et al., 2010).

Despite the importance of following the evolution of genetic variability of allochthonous species, few studies on the genetic structure of the Manila clam have been performed to date (Kijima et al., 1987; Oniwa et al., 1988; Yokogawa, 1998; Sekine et al., 2006; Vargas et al., 2008; Chiesa et al., 2011). To our knowledge, only two of these studies focused on Mediterranean clam populations: Hurtado et al. (2011) uncovered evidence for hybridization between *R. philippinarum* and *R. decussatus* along North-West Spain, and Chiesa et al. (2011) suggest that patterns of genetic variation in clam populations from the North Adriatic sea may have been shaped by multiple introduction events.

Therefore, our study was designed to analyze the genetic variation of microsatellites among *R. philippinarum* specimens collected at different sites in the Gulf of Olbia, with the twofold aim of detecting whether i) genetic substructuring is present within this area and whether ii) a recent founder effect occurred in the Gulf of Olbia. To this end, we compared estimates of within-population genetic variability of Sardinian samples with those of a formerly established, farmed population from the North Adriatic.

2. MATERIALS AND METHODS

A total of 195 specimens were collected from tidal flats at six different sites within the Gulf of Olbia at an average depth of 5-6 m. The sediment structure at the six sites was mostly characterized by coarse sand (0.5-1.0 mm grain size). Furthermore, 99 specimens were sampled within a farming area in the Lagoon of Sacca di Goro (SGR). For each individual, the total length, width, and height were measured (Table 1, Fig. 1). Genomic DNA was isolated either from the muscle or from the mantle tissue using a modified alkaline/CTAB/NaCl lysis method and subsequent silicon dioxide treatment (Neudecker and Grimm, 2000). Approximately 50 mg of frozen tissue was incubated for 30 min at 65°C in 200 µl of extraction buffer (NaOH 0.02 M, NaCl 2.5 M, CTAB 1%). The extract was then neutralized with the addition of 0.02 M Tris-HCl buffer (0.01 M Tris pH 8.0, 0.02 M HCl).

Six microsatellite loci (*Asari16*, *Asari24*, *Asari43, Asari55, Asari62*, and *Asari64*) (Yasuda et al., 2007) were used in this study. PCR was performed in a 25 µl solution containing 20-40 ng of genomic DNA, 1× reaction buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 1U of EuroTaq (Euroclone), multiplexed fluorescent dye-labeled primers at different concentrations, and deionized water. PCR was performed in a MJ DNA Engine PTC-100 thermal cycler under the following conditions: an initial cycle at 95°C for 5 min, followed by 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s. Microsatellite loci were visualized on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems) and analyzed using GENEMAPPER 4.0 software (Applied Biosystems).

Genetic relationships among the sampling sites were visualized by means of a principal coordinate analysis (PCoA). The ordination plot was built using GENALAEX 6.3 software (Peakall and Smouse, 2006) on a matrix of genotypic distances.

Genetic structure was also investigated with an individual-based approach (Mank and Avise, 2004). Genetic substructuring was inferred by the Bayesian model-based clustering algorithm that is included in the STRUCTURE 2.2.3 software (Pritchard et al., 2000), applying the admixture model with correlated allelic frequencies (Falush et al., 2003). The most likely population structure was inferred from multilocus individual genotypes by estimating the posterior probability of the data (PPD) according to Pritchard and Wen (2004) and, if necessary, the consistency of results across runs. For each value of K (the number of assumed genetic clusters), 10 independent runs were performed, each consisting of 100,000 iterations after a burn-in period of 100,000. Furthermore, the partitioning of genetic variation within and among sampling sites was estimated by AMOVA (Analysis of Molecular Variance) using ARLEQUIN 3.52 software (Excoffier and Lischer, 2010). The significance of genetic differentiation was tested by a permutation test (10,000 replicates). Overall $\square_{ST}$ was calculated followed by a pairwise computation of R_{ST} values (Slatkin, 1995) between all pairs of sampling sites within the Gulf of Olbia and the Adriatic sample. The significance of the random distribution of individuals among sampling sites was evaluated with exact tests that are analogous to Fisher's contingency tables (Raymond and Rousset, 1995). We defined two populations from subsequent genetic analyses based upon the results of genetic structuring: one population was obtained by pooling the samples from the Gulf of Olbia (SPOOL), and the other population represented the sample from the Lagoon of Sacca di Goro (SGR).

Conformity to Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were tested with the Markov chain randomization test (Guo and Thompson, 1992) using GENEPOP 4.0 (Rousset, 2008). Whenever necessary, the sequential Bonferroni

correction was applied to *P* values to avoid type I errors that can occur due to multiple testing (Hochberg, 1988).

Estimates of genetic variability were computed using the GENALEX software. The number and size range of alleles, allelic frequencies, observed (H_o) and expected (H_e) heterozygosity, and inbreeding coefficient (F_{IS}) values were estimated at each microsatellite locus. Moreover, the mean number of alleles (Na), the number of effective alleles (Ne), and the observed (H_o) and expected (H_e) heterozygosity were also calculated for the loci of each inferred population (SPOOL and SGR).

The statistical significance for differences in the expected heterozygosity was tested with a Wilcoxon rank-sum test, using the R 2.11.1 package (R Development Core Team, 2010).

The possibility of a founder effect due to recent colonization or expansions in SPOOL and SGR was tested using the software BOTTLENECK 1.2.02 (Cornuet and Luikart, 1996~~7~~; Piry et al., 1999). Indeed, after genetic bottlenecks, the number of alleles reduced faster than heterozygosity, leading to excess of gene diversity when compared to a mutation-drift equilibrium. Conversely, an expanding population accumulates new alleles at a high rate, which contribute little to heterozygosity because of their low frequency, resulting in a gene diversity that was lower than expected at mutation-drift equilibrium (Luikart et al., 1998). Because the expected heterozygosity is highly dependent on the selected model of microsatellite evolution (Hawley et al., 2006), the analysis was performed using both the strict stepwise mutation model (SMM) and the two-phase mutation model (TPM) (see Cornuet and Luikart, 1996). Here, the reported results are based on simulations with a TPM model set at 95% and the infinite allele model at 5%. The significance of the tests was assessed using Wilcoxon rank-sum test,

which is the most appropriate test when fewer than 20 microsatellite loci are used (Piry et al., 1999).

3. RESULTS

Screening 294 specimens of *R. philippinarum* yielded a total of 87 alleles (see the Appendix), resulting in 286 unique individual multilocus genotypes (representing approximately 97% of the specimens).

A PCoA plot of the first two principal coordinates (77 % of total genetic variation) did not show a clear separation between the Adriatic and Sardinian samples (Fig. 2). However, the ordination of samples from the Gulf of Olbia along the first principal coordinate appeared to confirm their geographic position (Fig. 1).

Nevertheless, the highest posterior probability of the data was obtained using the model with $K = 1$, which was retrieved by STRUCTURE (Table 2), supporting the lack of genetic structuring both within the Gulf of Olbia and between samples from the Gulf of Olbia and the Lagoon of Sacca di Goro. Similar results were obtained using AMOVA ($\square_{ST} = 0.004$, $P > 0.05$). Exact tests of population differentiation did not demonstrate divergence i) among sampling sites within the Gulf of Olbia, and ii) between these samples and the Adriatic sample (pairwise R_{ST} values are given in Table 3).

After pooling the Sardinian samples (SPOOL), we found that the allele frequencies were very similar to the Adriatic population (SGR) (Appendix, Fig. 3). Although population-specific alleles (private alleles) were present in both populations (18 and 10 in SPOOL and SGR, respectively), they occurred at very low frequencies, and 20 out of 28 alleles were found in a single individual. Alleles 165 and 177 at *Asari16*, the private alleles with the highest frequency, were found in seven and six specimens in SPOOL, respectively (Appendix, Fig. 3).

Significant deviations from HWE ($P < 0.05$ after sequential Bonferroni correction for multiple tests) were found at locus *Asari55* in SPOOL and at loci *Asari24* and *Asari55*

in SGR. In both cases, the deviations from HWE are caused by a deficit of heteozygotes, as evidenced by the positive F_{IS} values at these loci (Table 4).

Only 2 out of the 30 pairs of loci showed a significant LD after the sequential Bonferroni correction ($P < 0.05$). Because pairwise comparisons at one or two loci are expected to show genotypic disequilibrium over 30 pairs by chance (specifically 1.5 out of 30, corresponding to the cut-off of 5%), the loci were considered to be unlinked. Moreover, two pairs of loci with a significant LD (*Asari16*-*Asari55* and *Asari16*-*Asari62*) were found only in SPOOL.

The mean number and the effective number of alleles were comparable for SPOOL (Na = 12.833 ±1.797; Ne = 4.074 ±0.892) and SGR (Na = 11.500 ±1.607; Ne = 4.151 ±0.814). In SPOOL, we found H_o = 0.621 ±0.077, and H_e = 0.662 ±0.091. Comparable values were obtained in SGR (H_o = 0.626 ±0.090; H_e = 0.680 ±0.087). Indeed, the expected heterozygosity of SPOOL was not significantly lower than SGR (P = 1; Wilcoxon rank-sum test). Table 5 reports the Wilcoxon rank-sum test results for mutation-drift equilibrium under the SMM and TPM models.

4. DISCUSSION AND CONCLUSION

This study demonstrates a high degree of genetic variability of *R. philippinarum* at six microsatellite loci. At each locus, the size range of the alleles, the number of alleles, and the number of effective alleles were higher than those reported by Yasuda et al. (2007). The observed and expected heterozygosity displayed values that were comparable in magnitude to those reported by Yasuda et al. (2007) and Chiesa et al. (2011). In particular, although the values of expected heterozygosity were lower in our samples at *Asari24*, *Asari43* and *Asari55* (Table 4), the differences in our values and in those detected by Yasuda et al. (2007) were not significant (Wilcoxon rank-sum test, P = 0.394). Two out of six loci, *Asari24* (in SGR) and *Asari55* (in SPOOL and SGR), showed an overall deficit of heterozygosity, a phenomenon that is common in other bivalves (i.e. Hare et al., 1996; Raymond et al., 1997; Vargas et al., 2008).

Chiesa et al. (2011) found moderate levels of genetic differentiation in microsatellites among clam populations from the North Adriatic sea, hypothesizing that such genetic differentiation may be the result of multiple introductions from different gene pools. In contrast, we detected a lack of genetic substructuring within the Gulf of Olbia (Tables 2 and 3). Our results are suggestive of i) a single introduction event or ii) multiple introductions either from the same source population or from genetically undifferentiated populations.

The results provided by model-based clustering for the whole dataset (Table 2) also identified a common origin for the Sardinian and Adriatic samples. Although private alleles were found in SPOOL, the two populations share the same gene pool, likely because not enough time has elapsed to achieve a significant amount of divergence (Hutchison and Templeton 1999).

Notably, while SPOOL showed numerous loci departing from HWE that are lower than SGR, only two pairs of loci displaying LD were found in the Sardinian population. Assuming that LD was not due to chance, the physical linkage of the loci can be ruled out because the same pairs did not display LD in SGR. Furthermore, non-random associations of loci may occur in populations departing from HWE (Liu et al., 2006) and determine the so-called zygotic disequilibrium (Weir, 1996). A substantial LD may also be found in expanding populations that were initially bottlenecked as well as in recently introduced populations (Hansson et al., 2004, and references therein). The latter hypothesis fits with the historical data that point to a recent introduction of the Sardinian population.

A further outcome of the present study was the great similarity in the level of within-population genetic diversity among the Sardinian samples from the Gulf of Olbia, regardless of whether they were pooled, and also in the sample from the Lagoon of Sacca di Goro, as evidenced by Wilcoxon rank-sum test, which was applied to the expected heterozygosity (P = 1). In fact, there was little evidence of a significant loss of genetic variability in SPOOL and SGR under the SSM and TPM mutation models (Table 5). Instead, they exhibited a significant deficit of heterozygozity with respect to genetic diversity.

This finding suggests that the 'wild' population of the Gulf of Olbia is currently expanding (see Ribeiro et al., 2010). This hypothesis is supported by the presence of several alleles that occur at low frequencies; for example, 25 out of 77 alleles (~32 %) in SPOOL and 29 out of 69 alleles (~28 %) in SGR were found at frequencies lower than 1 % (Appendix). Thus, we can conclude that the two populations are either derived from the same source, or that the Sardinian population is derived from individuals that were farmed in the Adriatic and released *in situ*.

Regardless of the source of Sardinian samples, the number of individuals of *R. philippinarum* that were scattered in the Gulf of Olbia may have been high enough to avoid the consequence of a founder effect. Indeed, the higher fecundity rates that invasive or introduced species exhibit in new environments (i.a. Grosholz and Ruiz, 2003) often overcome the loss of genetic diversity that is caused by genetic bottlenecks or the founder effect (Andreakis et al., 2009). In this context, *R. philippinarum* may be considered a 'good invader' species due to its effective larval dispersal and high rate of reproduction. Cannas et al. (2009) reported the occurrence of *R. philippinarum* recruits one year after their first recorded release in the Gulf of Olbia.

Interestingly, Delgado and Perez-Camacho (2007) have reported that under the same experimental conditions, *R. philippinarum* demonstrates superior reproductive performance when compared to *R. decussatus*. This trait, coupled with high levels of genetic diversity, may allow *R. philippinarum* to quickly adapt to the new environmental conditions (see Estoup and Guillemaud, 2010, and the references therein).

The previously recorded hybridization between *R. philippinarum* and *R. decussatus* (Hurtado et al., 2011) represents a further threat, because hybridization may enhance the invasiveness of an allochthonous species by producing novel genotypes that better adapt to local conditions (see Henry et al., 2009, and the references therein).

These factors raise concerns about the conservation and management of the autochthonous *R. decussatus*, whose populations may suffer from competition with *R. philippinarum* (Chessa et al., 2005) as well as the maladaptation that occurs as a result of hybridization between the two species (Hurtado et al., 2011). Finally, the recent introduction of *R. philippinarum* in Sardinia presents the opportunity to follow the

evolution of genetic variability in a geographic area where a newly introduced invasive species has begun to spread.

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FIGURES LEGENDS

Figure 1. Sampling sites of *Ruditapes philippinarum*. The samples from the Gulf of Olbia are reported in the inset. For the sample codes, see Table 1.

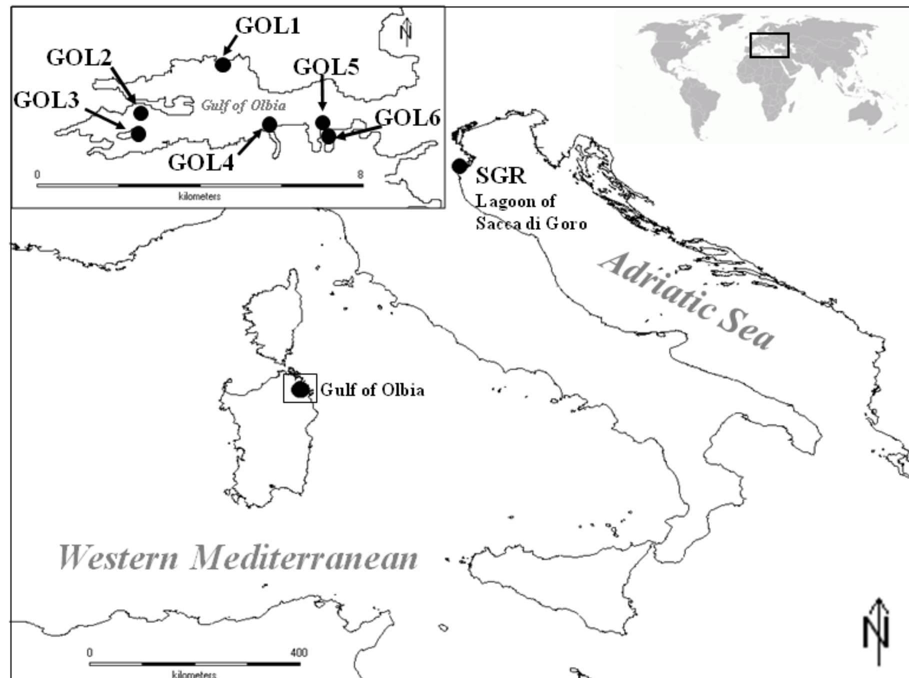


Figure 2. Principal Coordinate Ordination (PCoA). The plot depicts the genetic relationships among all sampling sites. For the sample codes, see Table 1.

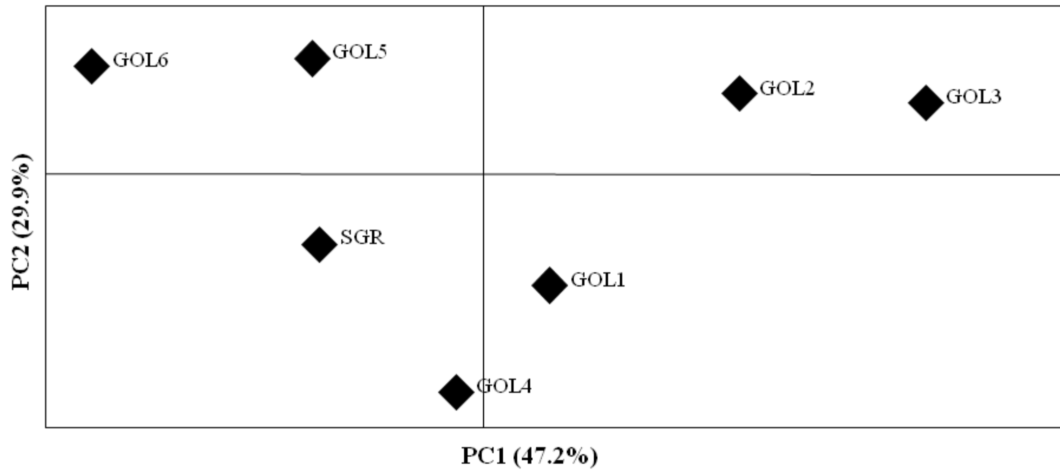
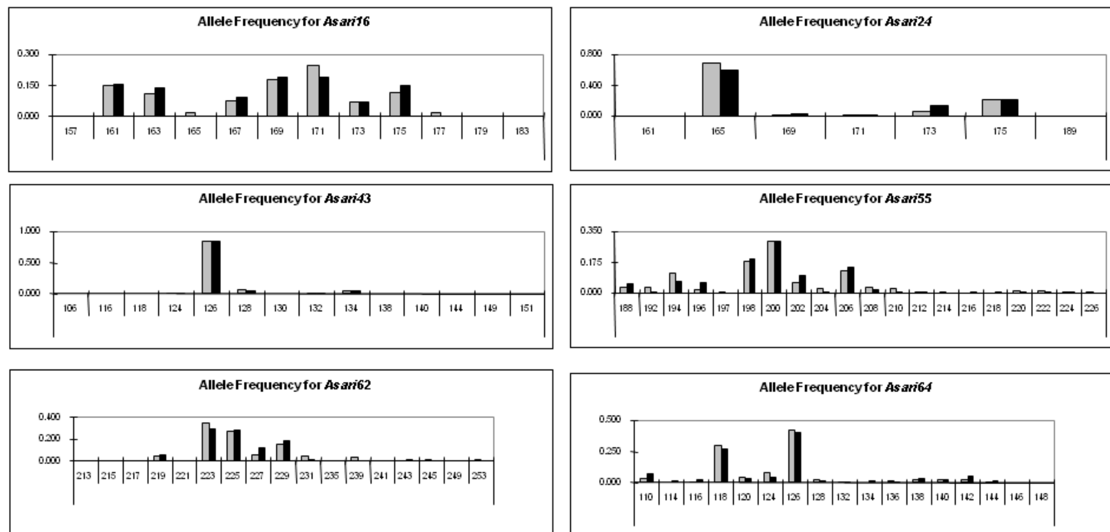


Figure 3. Histogram depicting the allelic frequencies of each microsatellite locus in the Gulf of Olbia and the Lagoon of Sacca di Goro. Alleles for each locus are reported on the x-axis and their frequencies on the y-axis. Grey bar: SPOOL (Sardinian pooled sample); black bar: SGR (Adriatic sample).



TABLES

Table 1. Details of the sampling plan. The codes of *Ruditapes philippinarum*, sampling sites, coordinates, number of individuals, and shell size vary at each locality. TL: total length; TW: total width; TH: total height.

Code	Locality	Coordinates	# of individuals	Size range (mm)		
				TL	TW	TH
GOL1	Gulf of Olbia, NE Sardinia	40°55'55.67"N ; 09°31'54.08"E	50	13.6-40.0	8.9-27.7	4.9-16.8
GOL2	Gulf of Olbia, NE Sardinia	40°55'16.54"N ; 09°30'47.63"E	50	15.1-33.0	10.7-23.0	6.0-16.0
GOL3	Gulf of Olbia, NE Sardinia	40°55'00.59"N ; 09°30'46.48"E	25	13.8-42.2	9.4-28.9	5.3-19.7
GOL4	Gulf of Olbia, NE Sardinia	40°55'09.26"N ; 09°32'31.60"E	25	10.3-35.0	7.3-25.8	4.2-16.7
GOL5	Gulf of Olbia, NE Sardinia	40°55'09.19"N ; 09°33'16.13"E	48	13.2-32.1	8.6-26.6	4.7-14.4
GOL6	Gulf of Olbia, NE Sardinia	40°55'00.23"N ; 09°33'19.76"E	25	13.9-38.6	9.4-29.4	5.0-21.0
SGR	Lagoon of Sacca di Goro, N Adriatic	44°48'49.78"N ; 12°16'50.63"E	99	31.6 - 41.5	24-30.1	17.5-22.4

Table 2. Estimation of the best-fit model of population structure. The number of assumed genetic clusters (*K*), the likelihood of the posterior probability of the model given the data (LnP(D)), and the posterior probability of the data (PPD) are reported.

K	LnP(D)	PPD
1	-5575.84	1
2	-5757.19	~0
3	-6253.89	~0
4	-7391.75	~0
5	-6212.24	~0

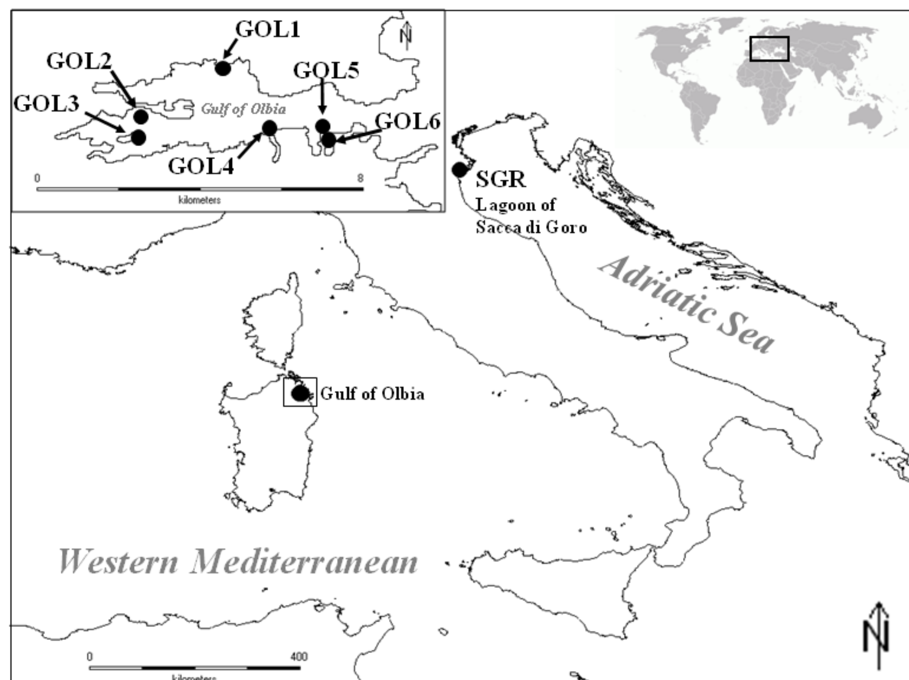


Table 3. Pairwise R_{ST} values. The R_{ST} values are shown below the diagonal. The probability values based on an exact test of population differentiation based on a Markov chain with 100,000 steps and 10,000 dememorization steps are shown above the diagonal. For the sample codes, see Table 1.

R_{ST}	GOL1	GOL2	GOL3	GOL4	GOL5	GOL6	SGR
GOL1	-	0.103	0.116	0.110	0.058	0.231	0.107
GOL2	-0.0015	-	1.000	1.000	1.000	1.000	1.000
GOL3	-0.0175	0.0280	-	1.000	1.000	1.000	1.000
GOL4	-0.0035	0.0325	0.0054	-	1.000	1.000	1.000
GOL5	-0.0036	0.0256	-0.0144	-0.0068	-	1.000	1.000
GOL6	0.0047	0.0024	0.0296	0.0597	0.0354	-	1.000
SGR	-0.0049	0.0221	-0.0060	-0.0065	-0.0272	0.0232	-

* = $P<0.05$; ** = $P<0.01$; *** $P<0.001$.

Table 4. Summary of the microsatellite-based estimates of intrapopulation genetic variability. The locus name, number of alleles, size range, observed heterozygosity (H_O), expected heterozygosity (H_E) and their respective standard errors (SE), and inbreeding coefficient (F_{IS}) are reported.

Locus	# of alleles	Size range (bp)	H_O (±SE)	H_E (±SE)	F_{IS}
Asari16	12	157-183	0.821 ±0.022	0.836 ±0.006	0.019
Asari24	7	161-189	0.448 ±0.025	0.503 ±0.020	0.109
Asari43	14	106-151	0.319 ±0.039	0.284 ±0.031	-0.121
Asari55	20	188-226	0.617 ±0.049	0.816 ±0.015	0.245
Asari62	17	213-253	0.746 ±0.034	0.759 ±0.013	0.018
Asari64	17	110-148	0.737 ±0.027	0.708 ±0.014	-0.041

Table 5. Wilcoxon rank-sum test of the mutation-drift equilibrium. The stepwise mutation model (SMM) and a two-phase model (TPM) were estimated at six microsatellite loci in SGR (Adriatic sample) and SPOOL (Sardinian pooled sample).

	Heterozygosity deficiency		Heterozygosity excess	
	SMM	TPM	SMM	TPM
SGR	0.016	0.016	0.992	0.992
SPOOL	0.008	0.016	1.000	0.992

Appendix. Allele frequencies. Private alleles in SGR (Adriatic sample) and SPOOL (Sardinian pooled population) are highlighted in bold. For the sample codes, see Table 1.

Locus	Allele	GOL1	GOL2	GOL3	GOL4	GOL5	GOL6	SGR	SPOOL
Asari16	N	22	50	25	25	48	25	96	195
	157	0.000	0.000	0.020	0.000	0.000	0.000	0.005	0.001
	161	0.091	0.170	0.180	0.180	0.135	0.160	0.156	0.154
	163	0.091	0.100	0.120	0.120	0.125	0.120	0.135	0.113
	165	0.068	0.000	0.020	0.080	0.000	0.000	0.000	**0.021**
	167	0.114	0.060	0.060	0.020	0.104	0.100	0.094	0.077
	169	0.227	0.250	0.080	0.140	0.156	0.160	0.193	0.177
	171	0.273	0.210	0.340	0.240	0.271	0.140	0.193	0.244
	173	0.000	0.070	0.040	0.060	0.073	0.180	0.073	0.072
	175	0.136	0.090	0.120	0.140	0.115	0.120	0.151	0.115
	177	0.000	0.050	0.000	0.000	0.011	0.020	0.000	**0.018**
	179	0.000	0.000	0.020	0.020	0.000	0.000	0.000	**0.005**
	183	0.000	0.000	0.000	0.000	0.010	0.000	0.000	**0.003**
Asari24	N	21	50	25	22	48	25	97	191
	161	0.000	0.000	0.000	0.000	0.000	0.000	**0.005**	0.000
	165	0.690	0.690	0.640	0.636	0.688	0.700	0.598	0.678
	169	0.000	0.020	0.040	0.046	0.031	0.020	0.031	0.026
	171	0.000	0.000	0.000	0.046	0.010	0.000	0.015	0.008
	173	0.048	0.020	0.080	0.136	0.094	0.060	0.134	0.068
	175	0.262	0.270	0.200	0.136	0.177	0.220	0.211	0.215
	189	0.000	0.000	0.040	0.000	0.000	0.000	0.006	0.005
Asari43	N	22	50	25	25	45	25	96	192
	106	0.023	0.000	0.000	0.000	0.000	0.000	0.000	**0.003**
	116	0.000	0.000	0.000	0.000	0.011	0.000	0.000	**0.003**
	118	0.000	0.000	0.000	0.000	0.000	0.000	**0.005**	0.000
	124	0.000	0.000	0.000	0.000	0.022	0.100	0.016	0.018
	126	0.818	0.880	0.800	0.920	0.767	0.840	0.839	0.836
	128	0.136	0.050	0.060	0.000	0.089	0.040	0.047	0.063
	130	0.000	0.000	0.020	0.000	0.000	0.000	0.000	**0.003**
	132	0.000	0.010	0.000	0.000	0.033	0.020	0.016	0.013
	134	0.023	0.050	0.120	0.080	0.067	0.000	0.057	0.057
	138	0.000	0.000	0.000	0.000	0.011	0.000	0.005	0.001
	140	0.000	0.010	0.000	0.000	0.000	0.000	0.000	**0.003**
	144	0.000	0.000	0.000	0.000	0.000	0.000	**0.005**	0.000
	149	0.000	0.000	0.000	0.000	0.000	0.000	**0.005**	0.000
	151	0.000	0.000	0.000	0.000	0.000	0.000	**0.005**	0.000
Asari55	N	20	50	24	24	46	25	97	189
	188	0.000	0.060	0.019	0.000	0.054	0.000	0.057	0.032
	192	0.100	0.020	0.021	0.000	0.065	0.000	0.010	0.034
	194	0.100	0.110	0.042	0.042	0.196	0.100	0.072	0.111
	196	0.000	0.010	0.063	0.000	0.033	0.000	0.062	0.019
	197	0.000	0.000	0.021	0.000	0.000	0.000	0.000	**0.003**
	198	0.200	0.130	0.292	0.125	0.141	0.260	0.191	0.177
	200	0.350	0.330	0.396	0.333	0.228	0.160	0.294	0.294
	202	0.075	0.090	0.000	0.104	0.054	0.020	0.098	0.061
	204	0.050	0.000	0.021	0.063	0.043	0.000	0.010	0.026
	206	0.075	0.080	0.104	0.188	0.109	0.240	0.144	0.124
	208	0.025	0.070	0.021	0.021	0.000	0.040	0.021	0.032
	210	0.025	0.010	0.000	0.021	0.011	0.120	0.010	0.026
	212	0.000	0.020	0.000	0.021	0.000	0.000	0.006	0.007
	214	0.000	0.010	0.000	0.021	0.022	0.000	0.000	**0.011**
	216	0.000	0.000	0.000	0.000	0.000	0.000	**0.005**	0.000
	218	0.000	0.000	0.000	0.000	0.000	0.000	**0.005**	0.000
	220	0.000	0.010	0.000	0.042	0.011	0.020	0.005	0.013

	222	0.000	0.020	0.000	0.019	0.011	0.040	0.005	0.016
	224	0.000	0.030	0.000	0.000	0.011	0.000	0.005	0.011
	226	0.000	0.000	0.000	0.000	0.011	0.000	0.000	**0.003**

Appendix: continued.

Locus	Allele	GOL1	GOL2	GOL3	GOL4	GOL5	GOL6	SGR	SPOOL
Asari62	N	21	50	25	25	48	25	96	194
	213	0.000	0.020	0.000	0.000	0.000	0.000	0.005	0.005
	215	0.000	0.010	0.000	0.020	0.000	0.000	0.005	0.005
	217	0.000	0.010	0.000	0.000	0.011	0.000	0.000	**0.005**
	219	0.000	0.060	0.060	0.020	0.042	0.080	0.063	0.046
	221	0.000	0.000	0.000	0.000	0.021	0.000	0.000	**0.005**
	223	0.333	0.380	0.500	0.300	0.292	0.300	0.297	0.348
	225	0.357	0.190	0.200	0.320	0.292	0.320	0.276	0.268
	227	0.048	0.060	0.040	0.040	0.073	0.040	0.120	0.054
	229	0.119	0.140	0.100	0.180	0.177	0.180	0.182	0.152
	231	0.024	0.050	0.040	0.080	0.031	0.040	0.016	0.044
	235	0.000	0.010	0.000	0.000	0.000	0.000	0.000	**0.003**
	239	0.095	0.010	0.020	0.020	0.031	0.040	0.005	0.031
	241	0.000	0.000	0.000	0.000	0.010	0.000	0.000	**0.003**
	243	0.000	0.000	0.000	0.000	0.010	0.000	0.016	0.003
	245	0.024	0.010	0.040	0.020	0.000	0.000	0.000	**0.013**
	249	0.000	0.000	0.000	0.000	0.000	0.000	**0.005**	0.000
	253	0.000	0.050	0.000	0.000	0.010	0.000	0.010	0.015
Asari64	N	22	50	25	24	48	25	98	194
	110	0.023	0.040	0.020	0.000	0.052	0.020	0.066	0.031
	114	0.000	0.000	0.000	0.000	0.000	0.000	**0.010**	0.000
	116	0.000	0.010	0.000	0.000	0.000	0.040	0.020	0.008
	118	0.205	0.280	0.360	0.208	0.354	0.360	0.276	0.302
	120	0.136	0.040	0.040	0.021	0.012	0.040	0.031	0.041
	124	0.023	0.140	0.120	0.063	0.052	0.040	0.041	0.080
	126	0.477	0.370	0.400	0.542	0.396	0.420	0.403	0.420
	128	0.000	0.020	0.020	0.042	0.031	0.000	0.010	0.021
	132	0.000	0.010	0.000	0.000	0.000	0.020	0.005	0.005
	134	0.000	0.000	0.000	0.000	0.000	0.000	**0.010**	0.000
	136	0.045	0.000	0.000	0.019	0.031	0.000	0.005	0.015
	138	0.045	0.050	0.020	0.021	0.000	0.000	0.036	0.023
	140	0.023	0.040	0.020	0.021	0.031	0.000	0.026	0.026
	142	0.023	0.000	0.000	0.042	0.021	0.040	0.046	0.018
	144	0.000	0.000	0.000	0.021	0.010	0.000	0.015	0.004
	146	0.000	0.000	0.000	0.000	0.010	0.000	0.000	**0.003**
	148	0.000	0.000	0.000	0.000	0.000	0.020	0.000	**0.003**