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New mitochondrial and nuclear primers for the Mediterranean marine bivalve *Pinna nobilis*

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Abstract

Pinna nobilis is the largest endemic Mediterranean marine bivalve. During past centuries various human activities led to the regression of its populations. As a consequence of stringent protection policies (*P. nobilis* is included in Annex IV of the Habitat Directive and Annex II of the Barcelona Convention), demographic expansion to many sites is currently reported. The aim of this study was to test a set of four species-specific primers designed by the authors, which could prove useful for the assessment of the genetic variability in *P. nobilis*. The level of polymorphism of two mitochondrial (nad3 and 12S) and two nuclear (18S and 28S) primers was thus assessed in 136 specimens from 28 Mediterranean localities. Furthermore, a comparison with the level of polymorphism obtained using the mitochondrial specific primers pairs so far available (COI and 16S) was performed. The mitochondrial gene nad3 showed the highest level of variability, which was comparable to those obtained for COI and 16S. Very low levels were detected for the mitochondrial 12S and the nuclear 18S and 28S genes. This research also showed that the use of nad3, COI and 16S concatenated sequences represents a useful tool for future studies devoted to infer the genetic variability of *P. nobilis* populations, thus allowing the development of effective conservation plans.

Keywords: Fan mussel, Mediterranean endemic, mitochondrial and nuclear molecular markers, specific PCR-primers.

Introduction

The fan mussel *Pinna nobilis* Linnaeus, 1758 (Pteriomorphia: Pinnoidea) is a Mediterranean endemic flagship species (*sensu* Walpole & Leader-Williams, 2002; and Heywood, 1995). This bivalve is the largest mussel species in the Mediterranean and one of the largest bivalves in the world, reaching more than 1 m in total anterior-posterior length (Zavodnik *et al.*, 1991). This species lives partially buried, with the anterior part of the shell anchored through byssus filaments to the coastal soft-bottom areas of seagrass meadows (Zavodnik *et al*., 1991; Templado, 2004) and bare sandy bottoms at depths between 0.5 and 60 m (Zavodnik, 1967; Zavodnik *et al*., 1991; Katsanevakis, 2005).

In recent years, the populations of *P. nobilis* have strongly diminished as a result of recreational and commercial fishing, marine pollution, and the loss of the natural biotope (Vicente, 1990; Vicente & Moreteau, 1991; Richardson *et al*., 2004; Rabaoui *et al*., 2010). Thus, *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). The conservation value of *P. nobilis* has prompted several studies regarding the biology and ecology of this bivalve (*e.g*. Richardson *et al*., 1999; Šiletić & Peharda, 2003; Katsanevakis, 2005; Galinou-Mitsoudi *et al*., 2006; García-March *et al*., 2007; Rabaoui *et al*., 2007; Rabaoui *et al*., 2008; Rabaoui *et al*., 2009, 2010), but to date only three studies have investigated the population genetics of *P. nobilis* (Katsares *et al*., 2008; Rabaoui *et al*., 2011; Sanna *et al.*, 2013). In these studies the authors provided insights into the genetic variability of populations from different Mediterranean regions using the two DNA markers so far available for population genetics, the Cytochrome c Oxidase subunit I (COI) and the 16S ribosomal DNA (16S) mitochondrial genes. However, deeper levels of variability may be depicted using further specific primers for *P. nobilis*. This may allow to describe a more exhaustive scenario of Mediterranean genetic structuring, which would be useful in drafting focused conservation plans for this endangered species. In such a context, the use of a combined multiple-markers approach is advisable in order to obtain more complete and reliable results (see e.g. Pogson *et al.*, 1995; Neigel, 1997; Clements *et al.*, 2003; Sanna *et al.*, 2011).

Therefore, the aim of the present study was to provide a set of specific primers for mitochondrial and nuclear markers, while evaluating their polymorphism rates in *P. nobilis*. The knowledge of these values is crucial for making a well-addressed choice of the most suitable combination of molecular markers for population genetics in this species. Primers for the mitochondrial NADH dehydrogenase subunit 3 (nad3), and 12S ribosomal DNA (12S) genes and the nuclear 18S ribosomal DNA (18S) and 28S ribosomal DNA (28S) genes were thus designed for this scope and proved for their effectiveness in *P. nobilis* specimens. The four regions (nad3, 12S, 18S and 28S) were selected according to their potential to depict different levels of genetic variability. In particular, since according to Plazzi *et al.* (2011) mitochondrial genes can be highly informative markers in bivalves, the nad3 gene was chosen because it may potentially unravel finer levels of genetic substructuring in Mediterranean areas. The mitochondrial 12S gene and the two nuclear 18S and 28S genes, being conservative regions usually, in bivalves included (see Giribet *et al.*, 2001; Plazzi *et al.*, 2011, for 12S; Adamkewicz *et al.*, 1997; Passamaneck *et al.*, 2004; Tsubaky *et al.,* 2011; Salvi & Mariottini, 2012, for 18S and 28S), were chosen to identify potential specific nucleotide polymorphisms associated to discrete groups of populations in Mediterranean subregions.

Estimates of genetic variability for the new markers were calculated and compared with estimates obtained for COI and 16S genes, sequenced by the specific primers previously provided by Sanna *et al.* (2013).

Materials and Methods

Sampling

A total of 136 specimens of *P. nobilis* were sampled from 28 Mediterranean localities (Fig. 1), with an average number of five individuals per site. No field study involving impacting manipulation, dislocation, or removal of *P. nobilis* individuals was performed. For each protected area, all necessary permits were obtained for the sampling activities from the competent authority. In order to avoid lethal effects, we developed a specific low-impact sampling method, performed by SCUBA divers, which does not cause significant damage to the shell and soft tissues of *P. nobilis* (see Sanna *et al.*, 2013 for details). A 20-50 mg tissue sample from each individual was collected and stored in 75% ethanol until analysis.

DNA extraction and PCR

DNA was isolated using the Qiagen DNeasy tissue kit, and the concentration estimated using a fluorimeter (average value per sample: 100 ng/µl). Since specific primers were not yet available in the literature, two mitochondrial (nad3 and 12S) and two nuclear (18S and 28S) regions were amplified with specific primers designed by

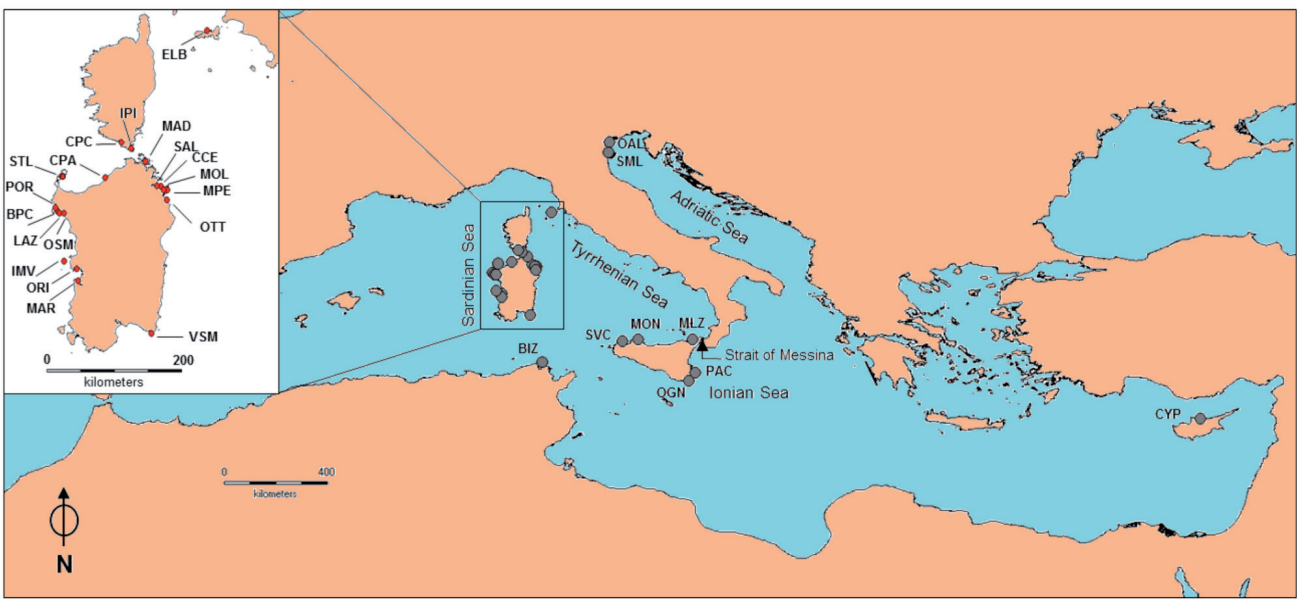

Fig. 1: Map of the Mediterranean indicating the sampling sites. The location of the Sardinian, Corsican, and Elba sites are magnified in the inbox in the upper left of the figure. The Mediterranean biogeographic sectors (Bianchi, 2007) tested in this study are indicated on the map. Numbers of individuals per site are indicated in parentheses.
Sardinia - STL (6): Stagno Lungo; BPC (6): Baia di Porto Conte, POR (2): Torre del Porticciolo, LAZ (2): Lazzaretto, OSM (6): Ospedale Marino, MOL (6): Molara, CCE (5): Capo Ceraso, SAL (5): Le Saline, MPE (5): Monte Petrosu, OTT (4): Porto Ottiolu, ORI (7): Oristano, MAR (6): Marceddì, IMV (5): Isola di Mal di Ventre, VSM (4): Villasimius, CPA (5): Costa Paradiso, MAD (5): Isola di La Maddalena. **Corsica** - IPI (6): Isola Piana, CPC (6): Cala Pesciu Cane. **Elba Island** - ELB (5): Capo Enfola. **Sicily** - SVC (5): San Vito lo Capo, MON (5): Mondello, MLZ (5): Milazzo, PAC (5): Pachino, OGN (5): Ognina. **Venetian Lagoon** - OAL (6): Ottagono Alberoni, SML (6): Santa Maria del Lago. **Tunisia** - BIZ (1): Bizerte Lagoon. **Cyprus** - CYP (2): Karaoglanoglu.

the authors using Primer Premier 6.12 software (PREMIER Biosoft International, Palo Alto, CA). The nad3 (L: CCTTATGARTGYGGBTTT; H: TCHATAAGYTCATARTAYARCCC), 12S (L: ACTACGAGCAACTGCTTAAAACTC; H: ATGTTACGACTTACCTC), 18S (For: GACATAGTGAAACCGCGAAT; Rev: CGTTACCCGTTACAACCAT), and 28S (For: CAAGGAGTCTAACATGTGCG; Rev: CTATACCCAAGTTTGACGATC) primers were developed based on the conserved flanking regions of the genes in the species belonging to the subclass Pteriomorphia, whose sequences were available in the NCBI gene database (see Table S1 for details on the species used Supplementary Files in electronic edition). In particular, because the superfamily Pinnoidea showed the closest genetic relationship with the superfamilies Pterioidea, Ostreoidea (Matsumoto, 2003; Plazzi & Passamonti, 2010), and Mytiloidea (Sharma *et al*., 2012), all the accessible sequences from the family Pinnidae and some sequences from the families Pteriidae, Ostreoidae, and Mytiloidae were used. Furthermore, COI and 16S regions were amplified using the specific primers previously designed by Sanna *et al*. (2013), because those provided by Katsares *et al*. (2008) for Aegean populations did not yield satisfactory results on our samples, a possible consequence of high variability occurring in the annealing region that led to mispriming in PCR. Indeed, the occurrence of genetic divergence between individuals from the Aegean Sea and those from the western Mediterranean and Adriatic was recently evidenced (Sanna *et al.,* 2013).

Each 25 µl-PCR mixture contained 100 ng of total genomic DNA, 0.32 µM of each primer, 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 12.5 µg of BSA were added to the reaction. Both positive and negative controls were used to assess the effectiveness of the PCR reagents. The PCR amplifications were performed according to the following steps: 1 cycle at 94° C for 2 min, followed by 35 cycles at 94° C for 1 min, at 46° C for 1 min (except for the nad3 gene whose annealing temperature was set at 44° C), and at 72° C for 1 min 30 s. A final elongation step at 72° C for 5 min was also applied. A visual checking was carried out by electrophoresis on 2% agarose/1× SBA gel stained with ethidium bromide (10 mg/mL) at 4 V/cm for 20 min. Both PCR product purification (exonuclease I - shrimp alkaline phosphatase clean up) and sequencing were carried out by an external sequencing core service (Macrogen Inc., Europe). Each region was sequenced in both forward and reverse direction. Cross-amplifications in the species *Pinna rudis* Linnaeus, 1758 were also performed on two individuals from Gran Canaria Island (Atlantic Ocean).

Dual peaks of similar height, interpreted as evidence of mitochondrial pseudogenes in the nucleus (Numts) (Bensasson *et al.*, 2001) or heteroplasmy (see Passamonti, 2007 for details), were not observed in the electropherograms of mitochondrial markers. In addition, no evidence of non-specific DNA bands was detected on the agarose gel, thus excluding the possibility of multiple nuclear mitochondrial DNA-like sequences (Bensasson *et al.*, 2001).

Statistical analysis

The sequences were aligned using the ClustalW programme (Thompson *et al*., 1994), as implemented in BioEdit 7.1.3.0 software (Hall, 1999) and the identified haplotypes were deposited in GenBank (see Table 1 for Accession Nos). Estimates of number of polymorphic sites (S), number of haplotypes (H), haplotype diversity (h), and nucleotide diversity (π) were obtained using the DnaSP 5.10 software (Librado & Rozas, 1999). Gaps were considered for the analysis.

Patterns of genetic differentiation at the population level were assessed by Arlequin 3.5.1.3 (Excoffier & Lischer, 2010) using a matrix of Tamura and Nei (1993) genetic distances with a gamma (γ) correction according to the best-fitting model of sequence evolution obtained with jModeltest 2.1.1 (Posada, 2008) and the Akaike Information Criterion. Pairwise Φ_{ST} values among sampling localities were calculated. A hierarchical analysis of molecular variance (AMOVA) (Excoffier *et al.*, 1992) was also performed excluding samples with less than five individuals, due to lack of statistical power. We firstly set and tested five separate groups of populations: (1) Sardinian Sea (ORS, BPC, OSM, ORI, MAR, IMV, CPA, CPC), Tyrrhenian Sea (MOL, CCE, SAL, MPE, IPI, ELB, SVC, MON), Strait of Messina (MLZ), Ionian Sea (PAC, OGN), and northern Adriatic Sea (OAL, SML), according to the biogeographic subdivision of the Mediterranean suggested by Bianchi (2007) (see Fig. 1 for population codes). Further tests were performed by combining these groups; in particular, we tested the following hypotheses, grouping: (2) a) Sardinian Sea; b) Tyrrhenian Sea; c) Strait of Messina; and d) Ionian Sea, northern Adriatic Sea; (3) a) Sardinian Sea; b) Tyrrehenian Sea, Strait of Messina; c) Ionian Sea; and d) northern Adriatic Sea; (4) a) Sardinian Sea; b) Tyrrhenian Sea; c) Strait of Messina, Ionian Sea; and d) northern Adriatic Sea; (5) a) Sardinian Sea; b) Tyrrhenian Sea, Strait of Messina; and c) Ionian Sea, northern Adriatic Sea; (6) a) Sardinian Sea; b) Tyrrhenian Sea, Strait of Messina, Ionian Sea; and c) northern Adriatic Sea. Significance of both pairwise Φ_{ST} values and AMOVA Φ_{CT} Φ_{ST} and Φ_{SC} values was assessed by a permutation test (with 10,000 random replicates).

Results

Positive results were obtained for all the primer pairs tested on *P. nobilis* (Table S1). Among the mitochondrial markers tested, the largest amplified fragment corresponded to the 16S gene (450 bp), while the smallest fragment

Table 1. Sample sizes and genetic diversity estimates obtained for the mitochondrial and nuclear regions. N: sample size; bp: base pairs; S: number of polymorphic sites; H: number of haplotypes; *h*: haplotype diversity; π: nucleotide diversity. The haplotypes GenBank Accession Nos. are reported in the last column.

Region	N	bp	S	H	*h*	π	GenBank
Mitochondrion							
COI	136	338	28	31	0.861	0.0071	KC811491-506, JX854835, JX854841, JX854863, JX854870, JX854873-874, JX854882, JX854891, JX854896, JX854916, JX854973-974, JX855005, JX855016, KF612603
nad3	136	204	37	47	0.805	0.0114	KC811507-526, KF612604-630
12S	136	339	3	4	0.058	0.0002	KC811465-468
16S	136	450	26	27	0.752	0.0022	KC811469-483, JX854566, JX854570, JX854636, JX854639, JX854651, JX854655, JX854687, JX854715, JX854768-769, KF612600-601
COI-16S-nad3	136	992	91	104	0.984	0.0058	-
Nucleus							
18S	136	256	3	3	0.029	0.0002	KC811484-485, KF612602
28S	136	241	4	5	0.058	0.0002	KC811486-490

corresponded to the nad3 gene (204 bp). Overall, the highest levels of genetic diversity estimates (S = 37, H = 47, h = 0.804, π = 0.0114) were identified for the nad3 gene; the COI gene, however, showed the highest haplotype diversity (h = 0.861) (Table 1). The nuclear markers exhibited comparable amplification fragment lengths: 256 and 241 bp for the 18S and 28S genes, respectively. Among them, the 28S gene showed the highest estimates of genetic diversity (S = 4, H = 5, h = 0.058, π = 0.0002) (Table 1). Considering both mitochondrial and nuclear markers, the most variable were in decreasing order; the nad3, COI, and 16S mitochondrial genes showing a percentage of different haplotypes equal to 35%, 23%, and 19%, respectively. Conversely, the ribosomal 12S, 18S, and 28S genes showed a percentage of different haplotypes equal to 3%, 2%, and 4%, respectively. A new COI haplotype was detected in the Tunisian sample (BIZ) (GenBank Accession No: KF612603); moreover, two new 16S haplotypes (GenBank Accession Nos: KF612600-601) were detected in two individuals from the northern Sardinian sample of Stagno Lungo (STL). The set of primers tested here also allowed to obtain high-quality amplifications in the congeneric species *P. rudis*. The corresponding haplotypes were deposited in GenBank (Accession Nos: COI: KC965063; nad3: KC965064; 12S: KC965058; 16S: KC965059, KC965060; 18S: KC965061; 28S: KC965062).

The estimates of genetic divergence for 12S, 18S, and 28S showed very low levels of variation (Table 1), with few private haplotypes that were not associated with any discrete group of samples or geographic areas; therefore, no further statistic elaborations were carried out. Conversely, nad3, COI, and 16S, genes showed high levels of genetic variability (Table 1). Thus, further analyses were performed on the concatenated COI, 16S and nad3 dataset (see **Table S2** for the DNA alignment). Estimates of genetic divergence for the 992 bp long combined dataset are reported in Table 1. A higher number of polymorphic sites (S = 91) and haplotypes (H = 104), and the highest level of haplotype diversity (h = 0.984) were found (see Table S3 for the frequencies distribution of haplotypes). jModelTest software calculated the TPM2uf+I+G 6-rates custom model as the appropriate model of nucleotide substitution with a gamma correction of γ = 0.7480.

The pairwise Φ_{ST} estimates (Table S4) showed a large number of significant comparisons. In particular, both the Venetian Lagoon samples (OAL, SML) were significantly differentiated from the other Mediterranean sites in all comparisons, the only two exceptions being those of Sardinian individuals from Le Saline (SAL) and Isola di Mal di Ventre (IMV); in addition, the Isola di Mal di Ventre (IMV) sample was significantly differentiated from the other samples in almost all comparisons, with the exception of the Venetian Lagoon sample from Ottagono Alberoni (OAL). The Sardinian sample from the Asinara Island (STL) is genetically differentiated from all other samples, with the exception of the two Corsican sites, Isola Piana (IPI) and Cala Pesciu Cane (CPC).

The AMOVA failed to unravel significant genetic structuring when the five groups of populations were considered separately (Table S5). However, the AMOVA maximised the largest differences among groups when we tested the combination of three groups: a) Sardinian Sea; b) Tyrrhenian Sea, Strait of Messina, Ionian Sea; and c) northern Adriatic Sea (Φ_{CT} = 0.077; $P < 0.01$). When alternative groupings of samples were tested, the AMOVA showed a decrease in the proportion of Φ_{CT} variance (Table S5).

Discussion

In this study, we test a set of four mitochondrial and two nuclear-specific pairs of primers yielding different levels of polymorphism in *P. nobilis*. The most variable molecular markers identified were the mitochondrial nad3, COI, and 16S genes, respectively. A remarkable result of this study is that the nucleotide diversity observed for the portion of the nad3 gene analysed is about one and a half higher than the rate found for the COI fragment and quintuple the rate found for 16S. For this reason, the combined use of a greater number of mitochondrial regions (COI-16S-nad3) proposed here allowed to detect a high number of haplotypes, which may lead to infer the levels of genetic variability of *P. nobilis* in greater detail.

The high variability obtained for COI and 16S was expected, considering that they were already used by Katsares *et al*. (2008), Rabaoui *et al*. (2011), and Sanna *et al.* (2013) for population genetics purposes. The first two studies, although highlighting high levels of variability, showed a lack of genetic structuring among populations from two relatively restricted Mediterranean areas (Aegean Sea and Tunisian coastlines respectively). The third study (Sanna *et al.*, 2013), based on a larger number of individuals and sites, pointing out higher levels of variability, also showed the occurrence of three well-defined groups of populations, corresponding to three large Mediterranean main biogeographic sectors (western Mediterranean, Adriatic, and eastern Mediterranean). However, a further genetic substructuring within Mediterranean main sectors might be revealed if more variable molecular markers were available. In this context, our study has provided the primers for a mitochondrial coding-gene fragment, the nad3, whose high levels of variability (which exceed those reported for COI and 16S) could represent an interesting starting point for deeper insight into the genetic structure of *P. nobilis*. It is noteworthy that the reduced size of the nad3 segment used here (204 bp) may not represent a bias, since it has been demonstrated that sequences of about or less than two hundred bp, even when singularly used, can correctly depict the phylogenetic/phylogeographic traits of the species (Tillier *et al*., 1992; Kirby & Reid, 2001; Bucklin & Allen, 2004; Hajibabaei *et al*., 2006; Sanna *et al*., 2009). Results obtained by our study, coupling nad3 with other mitochondrial markers (COI and 16S), although based on a lower number of individuals/sites, not only support the occurrence of the genetic structuring between the western Mediterranean and the Adriatic, as reported by Sanna *et al.* (2013), but also unravel a deeper level of genetic structuring within the western Mediterranean, where two distinct groups - 1) Sardinian Sea and 2) Tyrrhenian Sea, Strait of Messina, Ionian Sea - were found. In the future, the combination of the molecular markers used in this study could be applied to a larger number of samples, to clarify the mechanisms underlying the influence of environmental parameters and anthropogenic stress on the genetic variability of *P. nobilis*, with the further aim to support conservation plans.

Our study also provided specific primers for the 12S, 18S, and 28S ribosomal genes, whose low levels of genetic variability suggest an elevated conservation rate for *P. nobilis*. In the future, these markers may be useful for studies devoted to shedding light on the phylogenetic relationships within the entire genus *Pinna*. In such a context, the satisfactory results achieved by the 12S, 18S, and 28S cross-amplification in *P. rudis* represent the first step to verify whether these primers can be used for other species belonging to the genus. Nonetheless, it would be desirable to essay the potential of the whole set of markers in revealing the genetic variability of *P. rudis* on a larger number of individuals/populations.

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