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) genes was thus assessed in 136 specimens from 28 Mediterranean localities. Furthermore, a comparison with the levels of variability 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 (Pteriomorpha: 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; Passamanecck *et al.*, 2004; Tsukuy *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 the authors.
the authors using Primer Premier 6.12 software (PRE-
MIER Biosoft International, Palo Alto, CA). The nad3
(L: CCTTATGARTGYYGGTTT; H: TCHATAAGYT-
ATARTAYARCCC), 12S (L: ACTACGAGCACTGC-
TTAAAACCT; H: ATGTTACGACTTTACCT), 18S(for:
GACATAAGAAACCCGGAAT; Rev: CGTTACCCG-
TTACCAACCAT), and 28S (for: CAAGGAGTCTACA-
TGCGG; Rev: CTATACCAGTTGAGCAGATC) prim-
ers were developed based on the conserved flanking
regions of the genes in the species belonging to the sub-
class Pteriomorpha, 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 Pinnioidea showed
the closest genetic relationship with the superfamilies
Pterioidea, Ostreoidea (Matsumoto, 2003; Plazzi & Pas-
samonti, 2010), and Mytiloidea (Sharma et al., 2012), all
the accessible sequences from the family Pinnidae and
some sequences from the families Pteriidae, Ostreoidea,
and Mytiloidea were used. Furthermore, COI and 16S
regions were amplified using the specific primers pre-
viously designed by Sanna et al. (2013), because those
provided by Katsares et al. (2008) for Aegean popula-
tions did not yield satisfactory results on our samples,
a possible consequence of high variability occurring in the
anealing region that led to mispriming in PCR. Indeed,
the occurrence of genetic divergence between individuals
from the Aegean Sea and those from the western Medi-
terranea and Adriatic was recently evidenced (Sadna
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 Eu-
roTaq DNA Polymerase (Euroclone), 1× reaction buffer,
and 200 μM of each dNTP. The MgCl₂ concentration was
set at 3 mM, and 12.5 μg of BSA were added to the reac-
tions. Both positive and negative controls were used to
assess the effectiveness of the PCR reagents. The PCR
amplifications were performed according to the follow-
ing steps: 1 cycle at 94°C for 2 min, followed by 35 cy-
cles 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,
thus excluding the possibility of multiple nuclear mi-
tochondrial 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
DNA SP 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 &
genetic distances with a gamma (±) correction according
to the best-fitting model of sequence evolution obtained
with jModeltest 2.1.1 (Posada, 2008) and the Akaike In-
fomation Criterion. Pairwise ΦST values among sampling
localities were calculated. A hierarchical analysis of mo-
lecular variance (AMOVA) (Excoffier et al., 1992) was
also performed excluding samples with less than five in-
dividuals, due to lack of statistical power. We firstly set
and tested five separate groups of populations: (1) Sardini-
an Sea (ORS, BPC, OSM, ORI, MAR, IMV, CPA, CPC),
Tyrrehenian 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). Fur-
ther tests were performed by combining these groups; in
particular, we tested the following hypotheses, grouping:
(2) a) Sardinian Sea; b) Tyrrehenian Sea; c) Strait of Messi-
na; and d) Ionian Sea, northern Adriatic Sea; (3) a) Sardini-
an Sea; b) Tyrrehenian Sea, Strait of Messina; c) Ionian
Sea; and d) northern Adriatic Sea; (4) a) Sardinian Sea;
b) Tyrrehenian Sea; c) Strait of Messina, Ionian Sea; and
d) northern Adriatic Sea; (5) a) Sardinian Sea; b) Tyrrehen-
ian Sea, Strait of Messina; and c) Ionian Sea, northern Adri-
atic Sea. (6) a) Sardinian Sea; b) Tyrrehenian Sea, Strait of
Messina, Ionian Sea; and c) northern Adriatic Sea. Signifi-
cance of both pairwise ΦST values and AMOVA ΦST
and ΦSC 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 correspond-
ed to the 16S gene (450 bp), while the smallest fragment

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corresponded to the nad3 gene (204 bp). Overall, the highest levels of genetic diversity estimates \((S = 37, H = 47, \pi = 0.084, h = 0.0114)\) 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 \((S = 4, H = 5, h = 0.058, \pi = 0.0002)\) (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 (BLZ) (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 \(\gamma = 0.7480\).

The pairwise \(\Phi_{st}\) 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 Mediterranean samples (OAL, SML) were significantly differentiated from the other samples in almost all comparisons, with the exception of the Venetian Lagoon sample from Otttagono 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) Tyrrenhenian Sea, Strait of Messina, Ionian Sea; and c) northern Adriatic Sea \((\Phi_{ct} = 0.077; P < 0.01)\). When alternative groupings of samples were tested, the AMOVA showed a decrease in the proportion of \(\Phi_{ct}\) variance (Table S5).

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; \(\pi\): nucleotide diversity. The haplotypes GenBank Accession Nos. are reported in the last column.

<table>
<thead>
<tr>
<th>Region</th>
<th>N</th>
<th>bp</th>
<th>S</th>
<th>H</th>
<th>(h)</th>
<th>(\pi)</th>
<th>GenBank</th>
</tr>
</thead>
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<tr>
<td>Mitochondrion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>COI</td>
<td>136</td>
<td>338</td>
<td>28</td>
<td>31</td>
<td>0.861</td>
<td>0.0071</td>
<td>KC811491-506, JX854835, JX854841, JX854863, JX854870, JX854873-874, JX854882, JX854891, JX854896, JX854916, JX854973-974, JX855005, JX855016, KF612603</td>
</tr>
<tr>
<td>nad3</td>
<td>136</td>
<td>204</td>
<td>37</td>
<td>47</td>
<td>0.805</td>
<td>0.0114</td>
<td>KC811507-526, KF612604-630</td>
</tr>
<tr>
<td>12S</td>
<td>136</td>
<td>339</td>
<td>3</td>
<td>4</td>
<td>0.058</td>
<td>0.0002</td>
<td>KC811465-486</td>
</tr>
<tr>
<td>16S</td>
<td>136</td>
<td>450</td>
<td>26</td>
<td>27</td>
<td>0.752</td>
<td>0.0022</td>
<td>KC811469-483, JX854566, JX854570, JX854636, JX854639, JX854651, JX854655, JX854687, JX854715, JX854768-769, KF612600-601</td>
</tr>
<tr>
<td>COI-16S-nad3</td>
<td>136</td>
<td>992</td>
<td>91</td>
<td>104</td>
<td>0.984</td>
<td>0.0058</td>
<td>-</td>
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<tr>
<td>Nucleus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>18S</td>
<td>136</td>
<td>256</td>
<td>3</td>
<td>3</td>
<td>0.029</td>
<td>0.0002</td>
<td>KC811484-485, KF612602</td>
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<tr>
<td>28S</td>
<td>136</td>
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<td>0.058</td>
<td>0.0002</td>
<td>KC811486-490</td>
</tr>
</tbody>
</table>

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), Rabanu *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) Tyrrenian 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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