VERSITA

Biologia **63**/3: 412—417, 2008
Section Zoology
DOI: 10.2478/s11756-008-0061-8

Genetic structure of the endangered species *Pinna nobilis* (Mollusca: Bivalvia) inferred from mtDNA sequences

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Abstract: This study examines the population genetic structure of the endangered bivalve *Pinna nobilis* (Mollusca: Bivalvia), based on novel mtDNA sequences (partial COI and 16S rDNA mtDNA genes). The analyzed nucleotide sequences of COI were 729 bp in size, coding for a 243 amino acid peptide, while the analyzed nucleotide sequences of 16S rDNA were 489 bp in size. These sequences of *P. nobilis* were the first DNA sequences of the species submitted to any Genetic Data Base. Population samples from four geographic regions from Greece, as well as a population sample of *Atrina fragilis* (as an outgroup) were used. High values of haplotypic diversity were found in the population samples of *P. nobilis*, based on the COI sequences. A single base in the analyzed 16S rDNA sequences was different in all analyzed individuals from a single population sample (Chios island) differentiating it from the other ones. These mtDNA sequences could be informative for further genetic analyses of the endangered species, contributing in conservation plans for its protection and/or aquaculture investigations.

Key words: *Pinna nobilis*; mtDNA; genetic structure; sequencing; COI; 16S rDNA; Bivalvia

Introduction

Pinna nobilis L., 1758 (endemic in the Mediterranean Sea) is an endangered and/or protected bivalve mollusc species (Directive 92/43/EEC – Treaty of Accession 2003) from human activities (i.e., trawling, anchoring and fishing). It is the largest Mediterranean bivalve [max length: 108 cm (FAO 1987)] and lives in depths up to 60 m with a muddy and/or sandy substrate and in phanerogams meadows (i.e., *Posidonia oceanica)* with low values of hydrodynamism. It is a successive hermaphrodite protandric organism with an asynchronous maturation (de Gaulejac 1995). The *P. nobilis* life span is affected by environmental conditions. The pinnids age seems to be fluctuating due to environmental and biological conditions and may depend on the method used, observed and calculated/estimated. In all cases, the individuals with a length of 67–68 cm were mentioned as 10–27 years old (Morteau & Vicente 1982; Richardson et al. 2004; Katsanevakis 2005; Galinou-Mitsoudi et al. 2006).

Although there are several publications referring to population studies of *P. nobilis* (Richardson et al. 1999; Šiletić & Peharda 2003; Katsanevakis 2005; Galinou-Mitsoudi et al. 2006; Garcia-March et al. 2007), only a single study deals with a partial COI mtDNA sequence in the Pteriomorphia subclass in which *P. nobilis* belongs (Matsumoto 2003), but it does not include *P. nobilis*. There is neither nuclear nor mtDNA sequence, as well as no genetic analysis of the species published. Furthermore, only few mtDNA sequences from Pinnidae bivalves have been characterized, despite the occurrence of endangered and of economic importance species.

Mitochondrial DNA (mtDNA) has been considered as a potential genetic marker in phylogeny, population and evolutionary studies for a wide variety of animal taxa (Saccone 1999), including the bivalves, due to its maternal inheritance, its haploid nature and the fact that its effective population size is the one fourth of that of the nuclear DNA. The characterization of mitochondrial genes has contributed to the identification of informative sequences that have improved our understanding of organism evolution and the diversity of the mitochondrial genome.

Cytochrome oxidase subunit I (COI) gene of the mitochondrial DNA shows varying degree of conservation throughout its sequence, and a range of nucleotide substitution rates that can be used for different evolutionary analyse. It has been extensively used for evolutionary studies in bivalves (Kojima et al. 1995, 1997; Matsumoto 2003; Cho et al. 2007). 16S rDNA mtDNA gene is generally useful as an interspecific taxonomic marker and was used to resolve taxonomic problems in the family Mytilidae (Rawson & Hilbish 1995), Veneridae (Canapa et al. 1996) and Pectinidae (Canapa et al. 2000; Saavedra & Pena 2003).

In this study, a major part of the COI mtDNA gene, as well as a part of the 16S rDNA mtDNA gene of two Pinnidae species, *P. nobilis* and *Atrina fragilis* (Pennant, 1777), were genetically characterized and

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Fig. 1. Sampling sites of the analyzed population samples of *P. nobilis* and *A. fragilis*.

used for a preliminary analysis of the population genetic structure of *P. nobilis*. The characterization of specific mtDNA regions in these species could provide informative markers for screening genetic variability and population dynamics, as well as for conservation plans for their protection and/or aquaculture investigations.

Material and methods

Sampling and DNA extraction

Pinna nobilis and *A. fragilis* individuals were collected from four Greek geographic regions (Fig. 1). In total, 30 adult individuals were collected (25 *P. nobilis* and 5 *A. fragilis* individuals) (Table 1). Total genomic DNA was extracted from the anterior adductor muscle according to Grew et al. (1993), using CTAB.

New primers and PCR conditions

As far as the COI mtDNA gene is concerned, a novel primer set that enables the successful and repetitive amplification of a major part of the COI mtDNA gene in both species, was designed. The primers were based on a COI sequence from *Pinna muricata* L., 1785 (GenBank Accession Number: AB076929; Matsumoto 2003), a congeneric species of *P. nobilis* and they were designed using the computer software Primer3 (Rozen & Skaletsky 1998). The sequences of the sense and the antisense primers were: 5'-TGATAGGGGTTCCGGATATG-3' and 5'-GAAAGTGCCCGGTAACAAAA-3', respectively.

The reaction mixture contained template DNA (approximately 100 ng), 1× PCR buffer, 2.25 mM $MgCl_2$ in total (polymerase buffer contains 1.5 mM), 20 pmol of each primer, 0.25 mM of each dNTP and 0.5 U of Qiagen polymerase. Amplification was started by 94 ℃ for 3 min, followed by 33 cycles with 94 ℃ for 1 min, 51 ℃ for 1 min and 72 ℃ for 1 min. PCR products (approximately 860 bp) were electrophorised and visualized under UV in EtBr stained 2% (w/v) agarose gels. The desired bands of both species were extracted and purified using an agarose gel extraction kit according to manufacturer's specifications. A sequencing analysis on a 3730×l DNA Analyzer (Applied Biosystems) was followed using both primers for crosscheck. These sequences were submitted to GenBank under the accession numbers DQ448217 (*P. nobilis*) and DQ448215 (*A. fragilis*).

Species-specific COI primers

The new sequences of both species were aligned using Clustal X software (Thompson et al. 1997) and the BioEdit software (Hall 1999), set to default parameters and corrected by eye. Based on the aligned sequences for COI gene, new species-specific *P. nobilis* primers were designed using again the computer software Primer3 (Rozen & Skaletsky 1998). The sequences of the sense and the antisense novel species-specific primers were: 5'-TTTTGGCTTTTGCCTTCTTC-3' and 5'-CCCTGCCAAATTACACCAGT-3'. The sequences of both primers were chosen based on common nucleotide sequences on both species, allowing the successful amplification of the same region to *A. fragilis*, as well. The reaction mixture, as well as the PCR conditions, was the same as mentioned above. These PCR products (approximately 770 bp), after being sequenced as mentioned above, were used for the further population analysis. The new sequences (729 bp) were submitted to GenBank under the accession numbers DQ448216–7, EF536827–49 (*P. nobilis*) and EF536850–54 (*A. fragilis*). They were aligned as above.

16S rDNA gene

A universal primer set (Palumbi 1996) was used for amplification of the 16S rDNA in both *P. nobilis* and *A. fragilis*. The reaction mixture contained template DNA (approximately 100 ng), 1× PCR buffer, 2.2 mM $MgCl_2$, 20 pmol of each primer, 0.25 mM of each dNTP and 0.5 U of Promega polymerase. Amplification was started by 94 ℃ for 3 min, followed by 31 cycles with 94 ℃ for 1 min, 50 ℃ for 50 s and 72 ℃ for 50 s. PCR products (approximately 600 bp) were electrophorised and visualized under UV in EtBr stained 2% (w/v) agarose gels. The PCR products were sequenced and the sequences (489 bp for *P. nobilis* and 532 bp for *A. fragilis*) aligned as described above. The new sequences were submitted to GenBank under the accession numbers DQ663473, EF536855 (*P. nobilis*) and DQ663474 (*A. fragilis*).

Statistic analyses

Molecular analyses were conducted using MEGA version 3.1 (Kumar et al. 2004) and the 2-parameter Kimura distance model (Kimura 1980). Population differentiation analyses were conducted using Arlequin v.3.11 (Excoffier et al. 2007).

Results and discussion

In total, 14 different haplotypes were found as far as the COI sequence is concerned, in the 25 individuals of *P. nobilis* and 5 different haplotypes in the 5 individuals of A. *fragilis* (Table 1). Only the haplotype from Corinthiakos Gulf was the same with one of the haplotypes from Aggelochori, while all the other haplotypes were unique. The polymorphic sites among the studied individuals in the COI sequences are shown in Fig. 2.

High values of haplotypic diversity (0.6667–0.900) were found in all studied population samples of *P. nobilis* (except for the population sample from Corinthiakos Gulf). This could probably be the result of the

Table 1. Sample number, haplotypes and haplotypic diversity based on COI sequences.

	Epanomi	Aggelochori	Chios island	Corinthiakos gulf	*A. fragilis*
N	8	9	5	3	5
H	6	3	4	1	5
h	0.8929	0.6667	0.9000	0.0000	1.0000
SD	0.1113	0.1048	0.1610	0.0000	0.1265

Explanations: N – sample size; H – number of haplotypes; h – haplotypic diversity; SD – standard deviation.

Table 2. Genetic distances among the studied population samples, based on COI sequences and the 2–p Kimura model. SD values are shown in brackets.

	Epanomi	Aggelochori	Chios island	Corinthiakos gulf	*A. fragilis*
Epanomi		(0.002)	(0.002)	(0.002)	(0.014)
Aggelochori	0.008		(0.001)	(0.001)	(0.014)
Chios island	0.008	0.003		(0.002)	(0.014)
Corinthiakos gulf	0.008	0.001	0.003		(0.014)
A. fragilis	0.193	0.187	0.188	0.188	

Table 3. F_{st} values among the studied population samples, based on COI sequences. Statistical P values are shown in brackets. (*) indicates the statistically significant values ($P < 0.05$).

	Epanomi	Aggelochori	Chios island	Corinthiakos gulf	*A. fragilis*
Epanomi		(0.00195*)	(0.09570)	(0.32129)	(0.00293*)
Aggelochori	0.19617		(0.01074*)	(0.46387)	(0.00098*)
Chios island	0.10790	0.39840		(0.13184)	(0.00781*)
Corinthiakos gulf	0.05931	0.10292	0.50239		(0.01660*)
A. fragilis	0.86057	0.90038	0.85587	0.82002	

pelagic larvae of the species, which lead in high gene flow among the different samples. After spawning and external fertilization, developing larvae spend a variable period of time as part of the plankton, which can be passively drifted by water currents, often over considerable distances (Seed 1969; Cho et al. 2007). Nevertheless, in the shellfish complex, the existence of both the planktonic larval stage and the sessile adult stage makes it difficult to predict genetic population structure and level of gene flow.

The genetic distances among all population samples are shown in Table 2. F_{st} and statistical P values among the studied population samples are shown in Table 3.

It is obvious that the *A. fragilis* sample is clearly differentiated, while only the population sample of Aggelochori is statistically differentiated from those of Epanomi and Chios Island (Table 3). This differentiation of Aggelochori population sample (the northernmost of the studied ones) could be probably the result of a kind of a geographical isolation due to the specific geomorphologic and hydrodynamic conditions of this habitat area where the water exchange is minimal for most wind directions (Barber et al. 1997), as well as due to the winds that influence the water movements, obstructing gene flow from south to north (Nittis & Lascaratos 1992).

The genetics of marine populations with pelagic larval development has often been characterized by low genetic differentiation among populations, a pattern driven by high dispersal capabilities and large scale oceanic mixing (Reichow & Smith 2001; Rivera et al. 2004). However, passive larval dispersal may not fully explain the conclusion of high genetic homogenization, because it has been suggested that larvae with pelagic dispersion are subject to a high death rate (Cameron 1986). The genetic differentiation among *P. nobilis* population samples revealed in this study was low and in accordance with the above statements, although the high values of haplotypic diversity based on the analysed COI mtDNA sequences.

Two different haplotypes for 16S rDNA were found for *P. nobilis*, while all analyzed *A. fragilis* individuals had the same 16S rDNA haplotype. The individuals of the studied *P. nobilis* population samples had the same 16S rDNA sequence, apart from the individuals from the population sample of Chios island, in which the 16S rDNA sequence differentiated only in a single base (in nucleotide position 480C→T, considering only *P. nobilis* haplotypes, or in nucleotide position including *A. fragilis* sequence in the alignment). The polymorphic sites among the studied individuals in the 16S rDNA sequences are shown in Fig. 3. This lack of polymorphism in 16S rDNA sequences could be attributed to the fact that 16S rDNA gene is a relatively conserved gene, used mainly as an interspecific marker at genus and family level (Saavedra & Pena 2003).

In pinnid species, very few data about their genetic background could be obtained. Yokogawa (1996) had examined and calculated the genetic divergence of non-

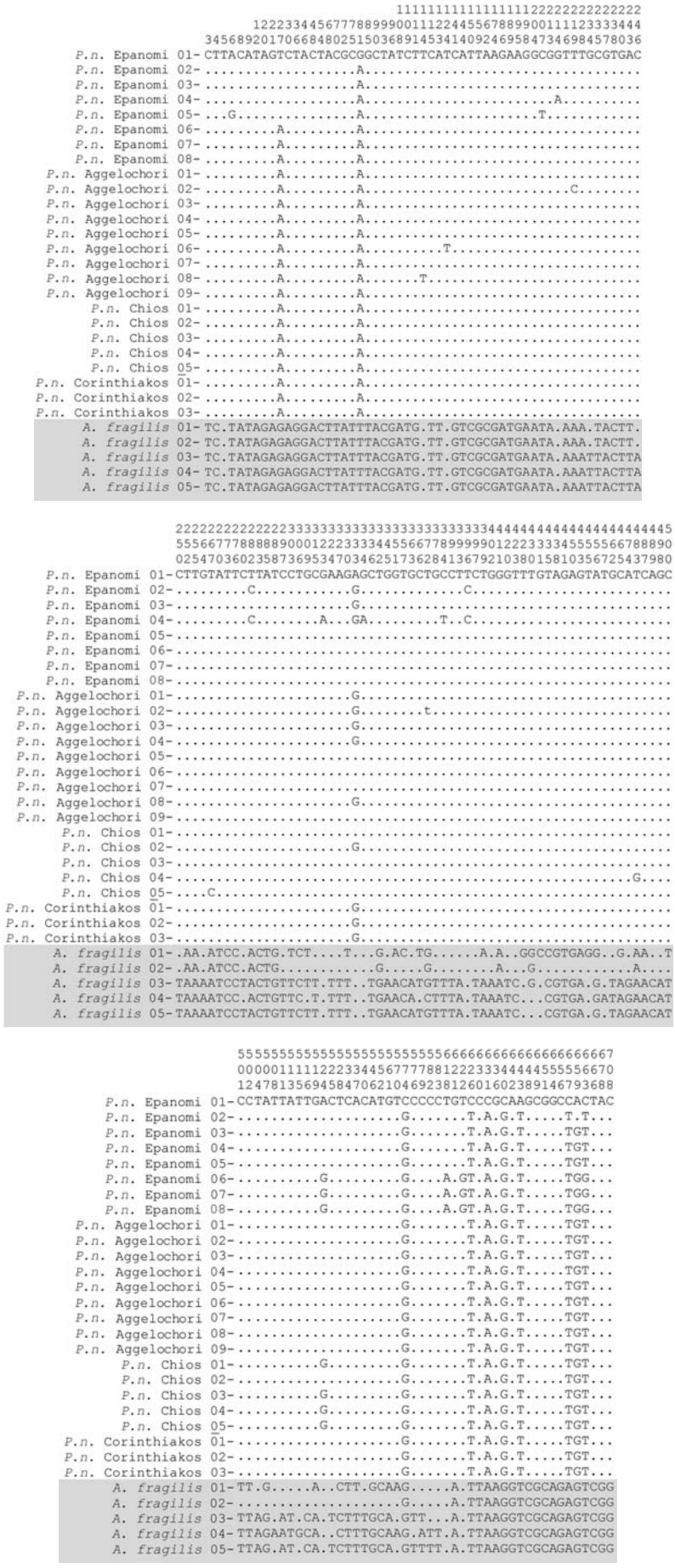

Fig. 2. Polymorphic sites in the partial COI gene of the analyzed population samples of *P. nobilis* and *A. fragilis*.

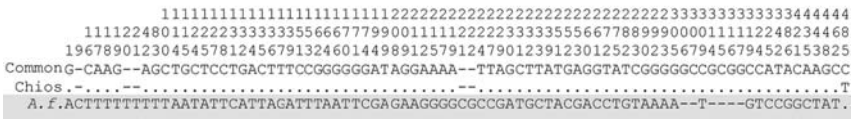

Fig. 3. Polymorphic sites in the partial 16S rDNA gene of the analyzed population samples of *P. nobilis* and *A. fragilis*.

scaly and scaly pen shell *Atrina pectinata* (L., 1767) distributed in partial Japanese marine region, by isozyme electrophoresis, suggesting that there could be two taxonomically distinguished forms. In a recent study (Yu et al. 2004), RAPD analysis was used to detect among four forms of *A. pectinata*. The results of these studies (Yokogawa 1996; Yu et al. 2004) indicate that previously regarded as one species *A. pectinata*, should be a conspecific complex composed of at least two species.

The present study is one of the few in the literature, dealing with the genetic structure of an endangered bivalve mollusc based on informative mitochondrial DNA sequences. Furthermore, COI and 16S rDNA mitochondrial sequences of *P. nobilis* are the first DNA sequences of the species submitted to any Genetic Database. The high values of intrapopopulation haplotypic diversity that was found in most of the population samples of *P. nobilis*, take place in case of not endangered populations as possibly the Greek populations are, with a density of 0.800–1.300 ind. m^{-2} (Galinou-Mitsoudi et al. 2006), comparing to the highest density (0.020–0.200 ind. m^{-2}) of a studied Mediterranean "recovered" population in the East Adriatic Sea (Šiletić & Peharda 2003).

In the last decade, there is an increasing interest and effort in culturing pinnid species for enrichment of declined natural populations. The objective is to breed new recruits that can be used to recover endangered populations. Further research in this field is under development (Garcia-March 2006). Knowledge of the genetic background could certainly facilitate the cultivation of *P. nobilis* individuals.

Studies providing mtDNA sequence information, could be useful for further evolutionary and conservation work, possible cultivation strategies, conservation plans contribution etc. Sequencing analysis of more *P. nobilis* individuals for both mtDNA genes, could undoubtedly give a more clear idea about the status of the populations.

Acknowledgements

Funding of the project by the State Scholarships Foundation of Greece, as a postdoctoral scholarship for Dr. A. Tsiora, is gratefully acknowledged. The authors are grateful to Dr. L. Kokokyris for helping with *A. fragilis* sampling.

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Received May 22, 2007
Accepted November 25, 2007