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Highly polymorphic microsatellite markers for the Mediterranean endemic fan mussel *Pinna nobilis*

M. GONZÁLEZ-WANGÜEMERT[1], J. COSTA[1] , L. BASSO[2], C.M. DUARTE[2,3], E.A. SERRÃO[1] and I. HENDRIKS[2]

[1] CCMAR, CIMAR-Laboratorio Associado, Faculdade de Ciências e Tecnologias, Universidade do Algarve, Gambelas, 8005-139 Faro, Portugal

[2] IMEDEA (CSIC-UIB), Instituto Mediterráneo de Estudios Avanzados, C/ Miquel Marqués 21, 07190, Esporles (Mallorca), Spain
[3] UWA Oceans Institute and School of Plant Biology, The University of Western Australia, 35 Stirling Highway, Crawley 6009, Australia

Corresponding author: mwanguemert@ualg.pt

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Abstract

Pinna nobilis is an endemic bivalve of the Mediterranean Sea whose populations have decreased in the last decades due to human pressure; as a consequence, it was declared a protected species in 1992. Despite its conservation status, few genetic studies using mitochondrial markers have been published. We report on the isolation and development of 10 microsatellite loci for the fan mussel, *Pinna nobilis*. All loci (2 di-nucleotide, 5 tri-nucleotide, 2 tetra-nucleotide and 1 penta-nucleotide) are characterized by high levels of polymorphism in 76 individuals tested from two populations in the Balearic Islands (Spain, Western Mediterranean Sea). The number of alleles ranged from 4 to 24 and expected heterozygosity ranged from 0.4269 to 0.9400. These microsatellites could be very useful for the assessment of the genetic diversity and connectivity patterns of *P. nobilis* and the establishment of new conservation strategies.

Keywords: *Pinna nobilis,* conservation genetics, connectivity, Mediterranean

Introduction

The fan mussel *Pinna nobilis* (Linnaeus 1758) (Mollusca: Bivalvia) is an endemic Mediterranean species with great conservation interest due to regression of its populations as a consequence of overexploitation and habitat deterioration (Rabaoui *et al.,* 2011; Sanna *et al.,* 2013). It has been recognized as an endangered species in the Mediterranean Sea according to European Council Directive 92/43/EEC and is under strict protection (Annex IV of the directive); its capture is forbidden (92/43/EEC).

Pinna nobilis is the largest bivalve species in the Mediterranean Sea with maximum lengths of 1.20 m, occurring at depths between 0.5 and 60 m on soft bottoms mainly characterized by seagrass meadows, although it has also been observed on stands of *Caulerpa prolifera* (Hendriks *et al.,* 2012; per. observ. M. González-Wangüemert). *P. nobilis* presents veliger-stage larvae, which drift in the water column for 5-10 days before settling in the sediment, showing highly variable recruitment rates (Hendriks *et al.,* 2012). Despite its endangered conservation status, few studies have addressed population genetics and connectivity patterns in *P. nobilis* (Katsares *et al.,* 2008; Rabaoui *et al.,* 2011; Sanna *et al.,* 2013; 2014). These studies have used 2 mitochondrial (COI and 16S genes) and 2 nuclear (18S and 28S) markers, which were limited in their resolution to assess the current genetic structure of *P. nobilis* populations. In order to study the connectivity and dispersal patterns of the species, highly polymorphic genetic markers are needed. Therefore, we hereby report on the isolation and development of ten highly polymorphic microsatellites for *Pinna nobilis*.

Materials and Methods

Next generation '454' sequencing was performed by Biocant (Portugal) from genomic DNA of 8 adults of *Pinna nobilis* (Mallorca, Balearic Islands, Spain), isolated according to the Sambrook *et al.* (1989) protocol. This generated a total of 53,236 reads of which 2,460 contained di- to hexa-nucleotide microsatellites (4.6 %) with a minimum of ten repeats identified using MSATCOMMANDER (Faircloth, 2008). Primers were designed using PRIMER3 software (Rozen & Skalesty, 2000; http://

bioinfo.ebc.ee/mprimer3/) for 31 microsatellites. From a total of 17 primer pairs tested, 10 (Genbank accession numbers: KJ541808-KJ541817) revealed high polymorphism on a subset of 76 individuals from two different locations (Biniancolla in Menorca island and Cabrera island) in the Balearic Islands (Table 1).

Polymerase chain reaction (PCR) was performed in 20 µl total volume, which includes 10 ng of DNA, 1.5-2 mM MgCl2, 0.25 µM of each primer, 100 µM dNTP's and 0.5 U Taq polymerase (Table 1). Cycling conditions consisted of an initial denaturation step of 3 min at 95 °C, followed by 30 cycles of 50 s at 95 °C, 50 s at annealing temperature (55-63 °C; Table 1), 1 min at 72 °C and a final elongation step at 72 °C for 5 min. All PCR reactions were performed in a GeneAmp 9700 thermocycler (PE Applied Biosystems). Individuals were genotyped by assessing allele size using forward primers labelled with FAM (SIGMA), HEX (SIGMA) and NED (Applied Biosystems). Allele sizes were scored according to the protocols of the Molecular Biology Service (CCMAR, Faro, Portugal) using an ABI Prism 3130 automated genetic analyzer (Applied Biosystems).

Raw allele sizes were scored using the STRand software (v. 2.4.59 http://www.vgl.ucdavis.edu/STRand). The number of alleles per locus, observed (H_O) and expected heterozygosity (H_E) and linkage disequilibrium (Black & Krafsur's method; 10 000 permutations) were calculated in GENETIX v. 4.05 (Belkhir *et al.,* 2004). Deviations from Hardy-Weinberg equilibrium (HWE) across all samples were characterized by F_{IS} and tested using exact test in GENEPOP v. 4.0.10 (Rousset, 2008) software. In instances where the observed genotype frequencies deviated significantly from HWE, the Micro-Checker v.2.2.3 program (Van Oosterhout *et al.,* 2004) was used to test for null alleles. The genotypic (G-based) and genic (Fisher's method) differentiation for the pair of locations were estimated using the GENEPOP v. 4.0.10 software. The differentiation among locations was also quantified by F_{ST} (using the estimator θ of Weir & Cockerham, 1984) and tested for allele-frequency heterogeneity using an exact test (ARLEQUIN v.3.11; Excoffier *et al.,* 2005).

Results and Discussion

The number of alleles per locus ranged from 4 (P.n 2.2) to 24 (P.n 4.3) (Table 1) and expected heterozygosity (H_E) ranged from 0.4269 (P.n 3.5) to 0.940 (P.n 4.3) (Table 2). Therefore, microsatellite loci showed high levels

Table 1. Characterization of ten microsatellite loci for *Pinna nobilis*.

Locus	Repeat motif	Primer sequences (5´-3´) and the fluorescent dye to mark the 5´ end of the forward primer	T_a (ºC)	MgCl2 (mmol/L)	Nº Alleles	Size range (bp)
P.n 2.1	$(GT)_{14}$	F: FAM-TGCACCTTTTCTTGGACGG R: GGAACTGCACTCGATGACG	57	1.5	12	201-231
P.n 2.2	$(AC)_{10}$	F: FAM-GGCCATAAGTGCCGAACAC R: ACAGGAAAATTAGAACTTAGGAACG	55	2	4	230-236
P.n 3.2	$(AAC)_{12}$	F: FAM-CCGAGGTCCCGTATCACAG R: TGCCCTTTGTGTCATTATTTCG	63	1.5	15	194-228
P.n 3.3	$(ATT)_{13}$	F: HEX-CGAGACGGAGTTCCAAAGC R: TGGCCCTGAACAGTAGGTG	56	2	8	226-242
P.n 3.4	$(ATC)_{10}$	F: HEX-CCTCGTTCATTGCACCCTG R: ACAGTGCTTTCATATGTCGGG	55	2	8	123-155
P.n 3.5	$(AAT)_{13}$	F: FAM-CCTAGCCTACATTCCATATGTGC R: TCATGTCTATGTCAAATGAACTCG	55	2	11	149-183
P.n 3.6	$(AAT)_{13}$	F: NED-AGGGACTTAAATTGACCGCTTC R: CGGACTTTGTCAGTATGATCGG	55	2	13	208-236
P.n 4.2	$(AGAT)_{10}$	F: FAM-TCCTTTAATTCAGTGGGTCGC R: ATTCCCGCAAATCCATCGC	62	1.5	15	163-211
P.n 4.3	$(ATTT)_{17}$	F: HEX-TGGATCTAGACTCTTTGTTTGTCTTC R: ACAGTGCCATGCTATGTTGC	56	2	24	238-350
P.n 5.2	$(ATAGT)_{11}$	F: HEX-TTGCATGTGCCACCATAATC R: TTCATACCGATGAGCCAAATG	61	2	13	181-223

Table 2. Parameters of genetic diversity from the two *Pinna nobilis* populations (N: number of individuals; H_O: observed heterozygosity; H_E: expected heterozygosity; BIN: Biniancolla; CAB: Cabrera).

	Population	
Locus	**BIN**	**CAB**
N	34	42
P.n 2.1		
Allele number	11	11
H_O	0.5588	0.4615
H_E	0.7845	0.7449
F_{IS}	0.2907***	0.3835***
P.n 2.2		
Allele number	3	3
H_O	0.7187	0.9750
H_E	0.4876	0.5420
F_{IS}	-0.4854	-0.8172
P.n 3.2		
Allele number	14	13
H_O	0.7647	0.8250
H_E	0.8665	0.8864
F_{IS}	0.1191	0.0701
P.n 3.3		
Allele number	6	7
H_O	0.5454	0.4500
H_E	0.6606	0.6870
F_{IS}	0.1766*	0.3479***
P.n 3.4		
Allele number	6	8
H_O	0.3529	0.4634
H_E	0.6866	0.6703
F_{IS}	0.4897***	0.3113***
P.n 3.5		
Allele number	11	6
H_O	0.4412	0.4500
H_E	0.4569	0.4269
F_{IS}	0.0351	-0.0548
P.n 3.6		
Allele number	10	11
H_O	0.2593	0.2059
H_E	0.8763	0.8310
F_{IS}	0.7081***	0.7550***
P.n 4.2		
Allele number	8	14
H_O	0.6061	0.5238
H_E	0.8149	0.8858
F_{IS}	0.2593***	0.4116***
P.n 4.3		
Allele number	20	21
H_O	0.9412	0.8809
H_E	0.9337	0.9400
F_{IS}	-0.0081	0.0636
P.n 5.2		
Allele number	8	12
H_O	0.6969	0.6098
H_E	0.6103	0.5869
F_{IS}	-0.1446	-0.0395

of polymorphism in *P. nobilis*, which is consistent with the number of alleles observed in other bivalve species (Martínez *et al.,* 2009; Pereira *et al.,* 2010).

Significant heterozygote deficiency was detected in five loci (Table 2). Such deviations from HWE may be due to the Wahlund effect, non-panmixia (inbreeding, groupings of relatives, selection against heterozygotes) or to genotyping errors (null alleles and other scoring errors) (González-Wangüemert & Vergara-Chen, 2014). Inbreeding seems an unlikely explanation in bivalves with large populations and pelagic larvae. Non-random mating is also unlikely in our case, as deficits were heterogeneous among loci (significant and non-significant F_{IS} values). Selection against heterozygotes cannot be demonstrated from our results; although microsatellite loci are typically recognized as neutral genetic markers, it is possible that one or more loci are linked to genes or gene groups under selection (Astanei *et al*., 2005). Moreover, none of the microsatellite loci showed significant probability ($P > 0.05$) of "large allele dropout" or "stuttering". However, three of the loci with significant heterozygote deficiency (P.n 3.4, P.n 3.6, P.n 4.2) showed significant values ($P < 0.05$) for "null alleles". These are thought to be common in bivalve species and possibly associated with high mutation rates (Miller *et al.,* 2013). The Walhund effect could also explain the deficit of heterozygotes due to the recruitment of genetically variable cohorts of larvae (González-Wangüemert *et al.,* 2007), such as could occur in *P. nobilis* (Hendriks *et al.,* 2012). Only 1 pair of loci out of 45 tests showed significant linkage disequilibrium after Bonferroni correction.

The pattern of differentiation, based on F_{ST} statistic, pointed to non-significant differences between the two sampled localities (F_{ST}=0.0067; P=0.1113). However, significant genic differentiation was found among localities for every loci and for each population pair across all loci (χ^2 = 71.1878; P=0.0000). Similar results were detected on genotypic differentiation (χ^2 = 51.8727; P=0.0001). F_{ST} is frequently used as a summary of genetic differentiation among groups. It depends on the allele frequencies at a locus, showing peculiar properties linked to genetic diversity: higher values for biallelic single-nucleotide polymorphisms (SNPs) than for multiallelic microsatellites, low values among high-diversity populations viewed as substantially distinct, and low values for populations that differ primarily in rare alleles (Jakobsson *et al.*, 2013). Due to these reasons, several authors argued that measures of F_{ST} may be poor measures of genetic differentiation when the level of diversity is high (Jakobsson *et al.*, 2013 and references therein). This could explain the non-differentiation detected among our locations using F_{ST}, considering the high diversity showed by our microsatellites and the high number of rare alleles. However, genic and genotypic differentiation was detected between localities, stressing the importance of genetic studies at small spatial scales (González-Wangüemert & Vergara-Chen, 2014).

The 10 microsatellite loci developed from the *P. nobilis* genome show high genetic diversity, making them useful for connectivity studies aimed to establish conservation strategies for this endangered species. Caution is required for the loci that exhibited significant heterozygote deficiency and evidence of "null alleles".

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