Isolation and characterization of polymorphic microsatellite loci in the razor clam *Ensis siliqua*

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Abstract

Five polymorphic microsatellite loci in the razor clam *Ensis siliqua* are described. A collection consisting of 34 individuals from Finisterre, Spain, was analysed. Loci were isolated from the sequences of intersimple sequence repeat (ISSR) markers. Detailed analysis of 42 ISSR markers led to the design of 16 primer pairs. Five of these yielded consistent and polymorphic products. The number of alleles ranged from five to 23 per locus with the observed heterozygosity ranging from 0.46 to 0.94. Linkage equilibrium was observed in all loci and three of them showed significant deviations from Hardy–Weinberg equilibrium.

Keywords: *Ensis siliqua*; ISSR; microsatellites; razor clam

The razor clam *Ensis siliqua* is a species of the family Pharidae distributed along the European Atlantic coast, from Norway to Spain, and in some parts of the Mediterranean and northwest Africa. This species has important commercial value and it is exploited in many countries. However, little is known about the population structure and genetic variability of this species, and in this sense, it is important to obtain and develop highly polymorphic markers to properly manage natural stocks and design breeding programmes. To these ends, we present the characterization of five polymorphic microsatellite loci.

Microsatellite loci were isolated from the sequences of intersimple sequence repeat (ISSR) markers (Fisher et al. 1996). ISSR markers are generated from nucleotide sequences located between two microsatellite priming sites inversely orientated on opposite DNA strands and near enough to be amplified by polymerase chain reaction (PCR) (Zietkiewicz et al. 1994). We amplified ISSR markers from one individual using the following anchored primers: $YG(CA)_9$, $YG(GA)_9$, $GATC(CGT)_7$, $YG(CT)_9$, $G(CT)_9$, $HVG(TG)_7$ and $KKVRVRV(CT)_6$. DNA extraction was carried out as in Winnepenninckx et al. (1993). PCR amplification of ISSR markers was performed using a touchdown protocol with the following conditions: initial denaturation at 94 °C (2 min), followed by 94 °C (20 s), 66 °C (30 s) and 72 °C (2 min). The annealing temperature was dropped 1 °C for each of the subsequent 10 cycles, followed by 30 cycles at 94 °C (20 s), 55 °C (30 s) and 72 °C (2 min), with a final extension at 72 °C (5 min). Amplification of the ISSRs was

conducted in 25-µL volumes consisting of 1× NH4 reaction buffer [16 mM $(NH_4)_2SO_4$; 67 mM Tris-HCl, pH 8.8; 0.1% Tween 20], 1-µm primer, 0.2 mM of each dNTP, 5.2 mM $MgCl_2$, 0.75 U *Taq* DNA polymerase (Bioline) and 20 ng of genomic DNA.

Pools of ISSR markers amplified using each of the anchored primers were ligated directly into plasmid pCR2.1 TOPO TA vector, and transformed into *Escherichia coli* TOP10F′ competent cells (Invitrogen). Clones were selected on LB X-Gal/IPTG/ampicillin plates and 271 white colonies were chosen for further screening. We determined the insert length of clones by colony PCR using M13 forward and reverse primers and picked clones with different insert length to avoid redundant sequencing. Plasmid DNA was prepared from 42 unique clones with the QIAprep spin miniprep kit (QIAGEN) and sequenced using a capillary array electrophoresis sequencer CEQ 8000 Genetic Analysis System (Beckman Coulter). To identify microsatellites in the genomic inserts, sequences were screened with the program TANDEM REPEATS FINDER 2.02 (TRF) (Benson 1999) using default parameters and 20 as the minimum alignment score. The search criteria was set to find microsatellites containing three or more units of repeat motifs consisting of two to six nucleotides. The analysis of these sequences revealed that each of the 42 clones (EMBL Accession nos AM182570–AM182611) contained a microsatellite at the 3′ and 5′ ends of the insert, and 25 had internal ones as well. OLIGO 6.3 (Molecular Biology Insights) was used for designing primers to amplify the 16 internal microsatellites with sufficient flanking sequence.

Microsatellite loci were characterized in 34 razor clams (*Ensis siliqua*) from Finisterre, located on the Atlantic coast of Spain. Microsatellite fragments were amplified under the following conditions: 94 °C (2 min), followed by 30 cycles at 92 °C (1 min), 60 °C (1 min) and 72 °C (30 s) with a final extension step at 72 °C (10 min). PCRs were carried out in a total volume of 25 µL containing 1× Roche *Taq* PCR buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl), 0.2 µm each of forward and reverse primers, 0.2 mM of each dNTP, 2-2.4 mM $MgCl_2$, 0.75 U *Taq* DNA polymerase (Roche) and 20 ng DNA template. An initial screening of the 16 primer pairs was carried out to evaluate each primer pair for robust amplification and polymorphism using an Agilent 2100 Electrophoresis Bioanalyser (Agilent Technologies). Five primer pairs fit these criteria and reverse primers were 5′-labelled with fluorescent dyes (WellRED oligos, Proligo). Amplification products labelled with different fluorescent dyes were pooled and electrophoresed on a CEQ 8000 Genetic Analysis System apparatus (Beckman Coulter). Five to 23 alleles per locus were detected. The analyses of these loci are shown in Table 1. GENEPOP version 3.3 (Raymond & Rousset 1995) was used to calculate observed and expected heterozygosities and linkage disequilibrium between loci. Observed heterozygosities ranged from 0.46 to 0.94, all loci were unlinked and two (Es177 and Es136) conformed to Hardy–Weinberg equilibrium (HWE) at a 5% significance level. Even after applying Bonferroni correction for two loci (Es263 and Es129), significant deviations from HWE were observed due to deficiency of heterozygous genotypes ($P < 0.01$), whereas Es128 showed a significant excess of heterozygosity ($P < 0.01$). The deficiency of heterozygosity suggests the presence of null alleles at Es263 and Es129, although this should be confirmed by further studies involving segregation analysis. In summary, a survey of 34 individuals showed numerous alleles and high H_O. The genetic variation observed in these five microsatellite markers and the lack of evidence for linkage disequilibrium indicate that the markers presented here will be useful for future studies on the population structure of *E. siliqua* in the wild and the aquaculture of this species.

Table 1. Characterization of five microsatellite markers in the razor clam *Ensis siliqua*

Locus	EMBL no.	Repeat motif (5'-3')	Primer sequence (5'-3')	[$MgCl_2$]	*N*	Alleles	Sizes (bp)	H_O	H_E
Es263	AM182601	$(TAG)_{29}(GTT)_7$	F: AATTACTTCTGGAACCTTATTTACGCA R: CTATTTACCCGAACATATACTGCCG	2.4 mM	32	23	203–317	0.5313*	0.9390
Es177	AM182587	$(GA)_{10}$	F: ATTACCTCCAATACTAGGAGAGCCG R: CCGTAACCGTGTTCTTCTCCG	2 mM	33	11	173–197	0.9394	0.8108
Es136	AM182580	$(CT)_3GTATGT(CT)_5$	F: TGACCAACACTACCACCCCATC R: AGAAGGGTGTGAATGAGAGATAGGG	2.4 mM	33	6	129–139	0.4545	0.5432
Es129	AM182574	$(ATT)_{10}$	F: TAATGCATACCCGTCTCTGATAAGC R: AATTAGCCTAAATTGTGCAGAAACG	2.4 mM	33	13	114–162	0.7576*	0.8815
Es128	AM182578	$(GA)_{40}$	F: GAAAGAGAGAAGGGAGATAATTGGG R: GTTTTTGTGTATGTGTGTGCGTCTT	2.4 mM	33	5	126–136	0.8788*	0.5533

N, number individuals tested; H_O, observed heterozygosity; H_E, expected heterozygosity.

* Significant departures from Hardy–Weinberg equilibrium after Bonferroni correction ($P < 0.01$).

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