Conservation Genet Resour (2010) 2:51–54
DOI 10.1007/s12686-009-9141-5

TECHNICAL NOTE

Isolation and characterization of microsatellite loci in the rock scallop (*Spondylus calcifer*) (Bivalvia: Spondylidae) from the Northern Gulf California, Mexico

A. Munguia-Vega · G. Soria · T. Pfister · R. Cudney-Bueno

Received: 4 November 2009 / Accepted: 6 November 2009 / Published online: 26 November 2009

Abstract Twelve microsatellite loci were isolated from an enriched genomic library of the rock scallop (*Spondylus calcifer*). One locus was monomorphic. Overall polymorphic loci, the mean numbers of alleles per locus at one locality was 9.6 (range 3–16), and the average observed and expected heterozygosities were 0.650 and 0.707, respectively. Three loci deviated from Hardy–Weinberg equilibrium, and from these, one locus had and excess of heterozygotes and the other two loci showed deficits of heterozygotes likely due to the presence of null alleles. No evidence of linkage disequilibrium was found among loci. These loci are the first microsatellites ever reported for the monotypic family Spondylidae, and will be useful to validate the predictions of oceanographic larval transport models and connectivity between patchy reefs within fishing areas and marine reserves in the northern Gulf of California, Mexico.

Keywords Gulf of California · Fisheries · *Spondylus calcifer* · Rock scallop · Connectivity · Microsatellites

A. Munguia-Vega (✉)
Conservation Genetics Laboratory, School of Natural Resources and the Environment, BioSciences East 317, The University of Arizona, Tucson, AZ 85721, USA
e-mail: airdrian@email.arizona.edu

G. Soria · T. Pfister · R. Cudney-Bueno
School of Natural Resources and the Environment, BioSciences East 325D, The University of Arizona, 1311 E 4th st., Tucson, AZ 85721, USA

R. Cudney-Bueno
Institute of Marine Sciences, Long Marine Laboratories, University of California Santa Cruz, 100 Schaffer Road, Santa Cruz, CA 95060, USA

The rock scallop, *Spondylus calcifer*, is geographically distributed from de coast of Peru to the northern Gulf of California (NGC), Mexico (Poutiers 1995). In the Gulf of California, the species constitutes a fishing resource for small-scale fisheries throughout the coast of Sonora, Baja California, the midriff island region, and Baja California Sur (Villalejo-Fuerte and Muñetón-Gómez 2002; Cudney-Bueno and Turk-Boyer 1998; Cudney-Bueno and Rowell 2008). *S. calcifer* inhabits mainly rocky reef areas from the inter-tidal to 55 m in depth (Poutiers 1995). It is a gonochoric species and reaches sexual maturity between 2.5 and 4 years of age. The species can live to at least 10–12 years (Cudney-Bueno and Rowell 2008) and attain 25 cm in shell height. The fertilization is external and planktonic stages are followed by settlement of sessile individuals. In the Gulf of California, *S. calcifer* has a short spawning period from July to October (Villalejo-Fuerte et al. 2002; Cudney-Bueno and Rowell 2008).

A counterclockwise connectivity via dispersion of larvae among stocks has been hypothesized based on oceanographic circulation for the NGC (Marinone et al. 2008; Cudney-Bueno et al. 2009). In order to validate these hypotheses and provide management and conservation guidelines for this species, information related to population genetic structure is needed. Examining the level of biological connectivity among populations of harvested species is critical to design and establish networks of marine protected areas in the region.

An enriched genomic DNA library was constructed following a modified version of Glenn and Schable (2005). For that purpose, DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen) from five individuals collected from Angel de la Guarda island in the NGC. Genomic DNA was digested with *RsaI* (NEB), and fragments ligated to double-stranded SuperSNX-24 linkers.

Table 1 Characteristics of twelve microsatellite loci isolated from *Spondylus calcifer*, including: locus name, GenBank accession number, cloned repeat motif, primer sequences, size of cloned allele, size range of allelic variation, number of different alleles (N_A), and observed (H_O) and expected (H_E) heterozygosities

Locus GenBank	Repeat motif	Primer sequences (5′–3′)	Clone size (bp)	Size range (bp)	N_A	H_O	H_E
Spca4	$(CA)_{19}$	F: CCAGAACTTGGGGCAAGATA	236	129–239	14	0.783	0.868
GU080200		R: GCTGCTAGAGCATGGTGGAT					
Spca9	$(CA)_{13}$	F: TGACGAAAACGCAGTAAACG	229	204–236	8	0.696	0.702
GU080201		R: AATCCCCTCCATTGTTGTGA					
Spca16	$(CA)_{25}$	F: TTGTGTTTTAGAGAATTTTGTTCCA	197	160–198	14	0.957	0.890
GU080202		R: TGATGTAACAAAGTCATATTGTAGAGC					
Spca34	$(GT)_{12}$	F: ATCTATGACGAAAAGAATCACCAG	181	118–200	13	0.826	0.887
GU080203		R: CCAGCATTTGTACCCCGTA					
Spca36	$(GT)_3GAGG$	F: AGAAAGAGAGAGGGAGTG	196	159–198	5	0.913*	0.586
GU080204	$(GT)_5$	R: TACAATATATCACTACACATACACAGT					
Spca50	$(GT)_{11}$	F: TGCCTCAGCAGTCTCAAAGA	207	192–240	15	0.435*	0.858
GU080205		R: ACCTCAACCACGAGATGTCC					
Spca61	$(GA)_2(GT)_9$	F: AGTGTATTTATTCATTTCATTTTGAGG	272	248–282	6	0.609	0.633
GU080206		R: CACCATCAGTGGCCATTTTA					
Spca1A	$(CA)_{16}$	F: CAGGTTCAAACCAATTAATAATGA	186	212–290	16	0.696	0.914
GU080207		R: TGATGCGGTATTTTCACAAAG					
Spca9B	$(CA)_6$	F: TTCACAATACTAAGAACAATGATGTGA	214	213–217	3	0.435	0.355
GU080208		R: AGATGAGCAAAGGGCGATAA					
Spca12	$(GT)_5GAGAGG(GT)_7$	F: TAAAAAGACCGCATCGCCTA	268	209–280	8	0.200*	0.525
GU080209		R: CCGTTGGCACCAAACTTACT					
Spca24	$(GT)_9$	F: TGTGAGGGGATGTGTTGTGA	268	272–278	4	0.600	0.561
GU080210		R: TCCCTGCAGAGTGAAACAGA					
Spca39	$(CA)_2CC(CA)_5$	F: CCCACCCTGTCAAAGGTCT	244	240	1	0.0	–
GU080211		R: TGTTCCGTGTGCACTGTTTT					

Twenty-five individuals sampled at San Luis Gonzaga, Baja California, were successfully amplified and scored for all loci

* Loci that deviated significantly from HWE after Bonferroni correction (adjusted critical $P \leq 0.0045$)

Linker-ligated fragments that ranged from 300 to 1,200 bp were recovered by means of the polymerase chain reaction (PCR), SuperSNX-24 forward primer, and Platinum high-fidelity Taq DNA polymerase (Invitrogen). Later, "linker-ligated" fragments were hybridized to 5′-biotinylated microsatellite oligonucleotide probes $(GT)_{15}$ and $(CT)_{15}$. Hybridized fragments were captured on streptavidin-coated paramagnetic beads (Dynal), recovered by PCR, ligated into the vector PCR4-TOPO (Invitrogen), and transformed into TOP10 chemically competent *E. coli* cells (Invitrogen) following the manufacturer's protocol. Clones were amplified by PCR using M13 primers, visualized on 2% agarose gels and selected for sequencing based on checking for successful amplification and differing amplicon sizes. One-hundred and ninety-six clones were directly sequenced in both directions on an Applied Biosystems 3730XL DNA Analyzer using the BigDye Terminator Cycle Sequencing Kit. One-hundred and twenty (62.5%) of the clones had recognizable microsatellite sequences but from those only 42 had adequate flanking regions to design primers using PRIMER3 (Rozen and Skaletsky 2000). The universal M13 primer was added at the 5′ end of the forward primers to allow fluorescent labeling of the amplicons using M13 labeled oligonucleotides (Schuelke 2000). Reverse primers were designed with a "pig-tail" at the 5′ end to reduce variability in adenylation of amplification products (Brownstein et al. 1996).

PCRs were performed in 15 µl volumes containing ~40 ng genomic DNA, 1× PCR buffer, 0.2 mM each dNTP, 0.02 µM of the unlabeled M13-tailed forward primer, 0.2 µM of the reverse and 0.2 µM of the fluorescently-labeled M13 primers, 1.5 mM $MgCl_2$, and 0.5 U taq DNA polymerase (Invitrogen), and 0.2% BSA. All loci were amplified with a touchdown protocol that included an initial denaturation at 94°C for 5 min, 15 cycles at 94°C for 30 s, annealing at 65–50°C for 30 s (1°C decrease in each cycle), 72°C for 30 s, followed by 40 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 30 sec and a final extension of 72°C for 5 min. Loci were genotyped on an ABI PRISM 3730 Genetic Analyzer (Applied Biosystems), alleles were scored using GENOTYPER 3.7 (Applied Biosystems) and their sizes classified into bins with FLEXIBIN (Amos et al. 2007). We used GENALEX 6.2 (Peakall and Smouse 2006) to calculate observed and expected heterozygosities and probabilities of identity, GENEPOP 3.4 (Raymond and Rousset 1995) to estimate deviations from Hardy–Weinberg equilibrium (HWE) and to test for linkage disequilibrium (LD) between pairs of loci, and MICRO-CHECKER 2.2.3 (Van Oosterhout et al. 2004) to estimate the frequency of null alleles and other genotyping errors. Adjusted *P* values were obtained using a sequential Bonferroni test for multiple comparisons (Rice 1989). The experiment-wise error rate was predefined at 0.05.

Twelve di-nucleotide loci were successfully amplified and scored on 25 individuals sampled from San Luis Gonzaga, Baja California (Table 1). One locus (*Spca*39) was monomorphic, but the size of the observed allele differed from the cloned allele, suggesting polymorphism is present elsewhere. Overall polymorphic loci, the mean number of alleles per locus was 9.6 (range 3–16). Average observed and expected heterozygosities were 0.650 and 0.707, respectively. Three loci (*Spca36*, *Spca50*, *Spca12)* deviated from HWE. Locus *Spca36* had an excess of heterozygotes, while loci *Spca50* and *Spca12* showed deficits of heterozygotes and evidence of null alleles according to the distribution of homozygote-size classes. The estimated frequencies of the null alleles were 0.245 and 0.270, respectively (Van Oosterhout et al. 2004). Although null alleles at microsatellite loci seem to be commonly reported for some invertebrates, including bivalves (Geist et al. 2003; Kaukinen et al. 2004), they could also be confounded with Wahlund effects, population bottlenecks and inbreeding. MICROCHECKER did not detect any other genotyping error among the loci (e.g. stuttering, large-allele dropout). No evidence of significant LD was found among the tests for each pair of loci (all adjusted *P* values > 0.0009). The probability of identity calculated was 1.4×10^{-12}. These markers are the first microsatellite loci reported for the monotypic family Spondylidae.

Acknowledgments We acknowledge the financial support provided from The David and Lucile Packard Foundation, The Wallace Research Foundation, The Nature Conservancy and the Conservancy's RJ KOSE Grant Program. AMV received a CONACYT graduate scholarship (#158622). We wish to thank commercial divers of the Northern Gulf of California for their support with the collection of samples. Research was conducted under permit #SGPA/DGVS/13159 of the Dirección General de Vida Silvestre; Secretaría de Medio Ambiente y Recursos Naturales (SEMARNAT) of Mexico. This is a scientific contribution of the PANGAS project: www.pangas.arizona.edu.

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