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1 **Isolation and characterisation of microsatellite loci for the southern geoduck**
2 ***Panopea abbreviata* (Valenciennes, 1839) through 454 pyrosequencing**

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14 *pyrosequencing, population structure*

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19 Short title: *Panopea abbreviata* microsatellite markers

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24 **Abstract**

25 We have isolated the first, polymorphic microsatellite loci (21 in total) for the geoduck
26 clam *Panopea abbreviata* (Valenciennes, 1839) from San Matías gulf (Patagonia,
27 Argentina), using 454 GS-FLX Titanium pyrosequencing. We also developed
28 conditions for amplifying these markers in 5 multiplex and 7 individual reactions. Four
29 to 23 alleles were detected per locus across the 25 samples analysed. Observed and
30 expected heterozygosities ranged from 0.235 to 0.985 and from 0.528 to 0.937,
31 respectively. In the sampled population, only one locus deviated from Hardy–Weinberg
32 equilibrium. These markers are useful resources for future population structure studies
33 in this artisanal fishing species.

34

35 The genus *Panopea* Ménéard, 1807 (Bivalvia, Hiatellidae), has nine species, and its
36 populations naturally occur worldwide (Straus *et al.* 2008). These bivalves, called
37 geoducks, have one of the longest lifespans among exploited animals (Morsan *et al.*
38 2010). There is lucrative commerce based on geoduck fisheries, mostly from the North
39 Pacific Ocean. Probably due to this fact, the Pacific geoduck *Panopea generosa*,
40 previously known as *P. abrupta* (Vadopalas *et al.* 2010), was the first species of the
41 genus to have its genetic structure analysed (Vadopalas and Bentzen 2000; Vadopalas
42 *et al.* 2004).

43 Here, we focused on the southern Patagonian geoduck *Panopea abbreviata*
44 (Valenciennes, 1839), the largest bivalve found along the south-occidental Atlantic
45 coasts, with a distribution range from Rio de Janeiro, Brazil (23°S) to Nuevo gulf,
46 Argentina (43°S) (Scaravino 1977, Alfaya *et al.* in press). These geoducks live from
47 shallow waters to depths of 75 m, and are buried (up to 70 cm) in sand and muddy
48 sediments (Ciocco 2000). It has been an incipient resource for the artisanal fisheries in
49 the Patagonian gulfs since 1999 (Ciocco 2000; Ciocco *et al.* 2001). However, there is
50 not much basic ecological or biological information about *P. abbreviata*, and there is no
51 regulation for its management. Recently, its reproductive cycle was described as having
52 a continuous gametogenetic cycle (Van der Molen *et al.* 2007; Zaidman *et al.* 2012).
53 This lack of resting in reproduction coupled with a pelagic larval stage could indicate a
54 lack of differentiation, but some differences in growth and age classes were reported
55 among populations (Morsan *et al.* 2010).

56 To disentangle the basis of such differences, including factors that may influence
57 dispersal and gene flow (Acevedo *et al.* 2009), and to provide a new tool that will aid in
58 the sustainable management of this species, we have developed the first series of

59 microsatellite markers for *P. abbreviata*. Thus, we isolated and screened the
60 microsatellites using high-throughput sequencing techniques. Multiplex-enriched
61 libraries and 454 GS-FLX Titanium pyrosequencing are powerful tools for the isolation
62 of new markers in unknown genomes (Martin *et al.* 2010). This procedure has been
63 readily and successfully applied to a large variety of taxonomic groups (Malausau *et al.*
64 2011).

65

66 Genomic libraries were constructed at the Cornell Evolutionary Genetics Core
67 Facility (EGCF, USA). Total DNA was extracted from the siphon tissue of one *P.*
68 *abbreviata* specimen from San Matías gulf (Patagonia, Argentina), using the BioSprint
69 15 DNA Blood Kit (Qiagen, Hilden, Germany), according to manufacturer's tissue
70 protocol.

71 Five micrograms of extracted genomic DNA were completely digested with a
72 restriction enzyme (five-base cutter) that generated blunt-end fragments. Linkers were
73 ligated to the digested DNA, and the resulting fragments were enriched for
74 microsatellites by hybridization to and magnetic capture of biotinylated repeat probes
75 (representing two unique dimers –GT and TC; five unique trimers –TTC, GTA, GTG,
76 TCC and GTT; and five unique tetramers –TTTC, GATA, TTAC, GATG and TTTG).
77 Enriched genomic fragments captured by streptavidin-coated magnetic beads were then
78 amplified by PCR, ligated to Roche/454 Titanium Multiplex Identifier (MID) adapters
79 and size fractionated in an agarose gel.

80 Libraries with unique adapters were pooled, and sequences were generated with
81 Roche/454 GS FLX Titanium reagents, protocols and hardware. MID-sorted 454 reads
82 were trimmed of adapter sequences and assembled with SeqMan Pro (DNASTAR).

83 Consensus files and singleton reads were exported as FASTA files, and simple repeats
84 were detected with MsatCommander software (Rozen and Skaletsky 2000; Faircloth
85 2008), with a limit of at least 8 perfect motif repeats.

86 We primarily focused on finding tetranucleotide repeats as they typically provide
87 clear allele assignment. From the 1004 perfect tetranucleotide repeats obtained, 208
88 showed the possibility of designing primers under the requested conditions (e.g., length
89 20 ± 2 bases, melting temperature 60 ± 2 °C, GC content between 30% and 70%). Then,
90 we discarded the ligated microsatellites (that had more than one group of repeats per
91 contig), leaving 140 viable microsatellites, with 21 different motifs. To have a good
92 representation of all motifs, when possible, we selected at least 2 of each. Finally, we
93 tested 41 potential microsatellites markers in 7 samples from 5 localities from the
94 Patagonian gulfs using a nested PCR protocol modified from Schuelke (2000). Twenty-
95 two microsatellites markers produced clear electropherogram patterns in these 7
96 samples and were selected for multiplex PCR and genotyping optimization.

97 Multiplex PCRs were performed in a total volume of 10 µl, which included
98 approximately 1 ng of DNA, 1X Qiagen Multiplex PCR Master Mix (Qiagen, Hilden,
99 Germany), and MgCl_2 at a final concentration of 3 mM. Primer concentrations ranged
100 from 0.2 to 0.8 µM (0.6 for Pa19 locus primers, 0.8 for Pa45 locus primers and 0.2 for
101 the all other primers). The forward primer from each primer pair was fluorescently 5'
102 end labelled with 6-FAM, NED, VIC or PET, while the reverse primers were pig-tailed
103 with 5'-GTTTCTT-3' (Brownstein, 1996) to facilitate genotyping. The cycling profile
104 began with an enzyme activation step at 95 °C for 15 min (Qiagen Multiplex PCR Kit
105 specifications), followed by 35 cycles at 94 °C for 30 s, 56 °C for 90 s (except for three
106 loci: Pa17, Pa31 and Pa33, for which the annealing temperature was 52 °C), 72 °C for

107 60 s and a final extension at 60 °C for 30 min. A Mastercycler gradient thermocycler
108 (Eppendorf AG, Hamburg, Germany) was used for all reactions.

109 Fluorescently labelled PCR products were run on an ABI PRISM 3730 DNA
110 Sequencer (Applied Biosystems) with the GeneScan-500 (LIZ) internal size standard
111 and analysed with the GeneMapper software (Applied Biosystems).

112 From the 22 loci surveyed, 21 loci were successfully amplified and genotyped in 25
113 specimens from San Matías gulf (40°50'S, 65°04'W; Patagonia, Argentina). Overall, 1
114 tetraplex, 2 triplex, 2 duplex and 7 monoplex were optimized to reduce the number of
115 PCRs performed to 12 (Table 1). Two of the amplified and genotyped microsatellites
116 were pentanucleotide repeats, while 19 were tetranucleotide repeats. These
117 microsatellite markers were then checked for accordance to Hardy-Weinberg
118 equilibrium (HWE) and the presence of linkage disequilibrium, using GENEPOP 4.1
119 (Rousset 2008) with a Markov chain method (5000 dememorization steps, 100 batches
120 and 5000 iterations per batch). Only one among the 210 tests was significant for linkage
121 disequilibrium, but no significance was found after sequential Bonferroni correction for
122 multiple testing (Holm 1979; Rice 1989). One locus (Pa 25) showed a significant
123 departure from HWE ($p = 0.0016 \pm 0.0003$), due to heterozygosity deficit, which could be
124 because of the presence of null alleles, as was found using MICRO-CHECKER (Van
125 Oosterhout *et al.* 2004). This program found that all loci lacked apparent errors of
126 scoring, large allele dropout and the existence of null alleles, except for the
127 aforementioned Pa25. Null alleles were also identified at locus Pa17 by MICRO-
128 CHECKER, but not at significant frequencies. Basic parameters of genetic variability
129 were calculated using GenAlex (Peakal and Smouse 2006). Allele richness ranged from
130 4 to 23, while observed and expected heterozygosities ranged from 0.235 to 0.985 and

0.528 to 0.937, respectively (Table 1). The lowest observed heterozygosity value was for the locus that showed the heterozygosity deficit (P25). Excluding this locus, the heterozygosity indices obtained here for *P. abbreviata* were higher than those observed in the 30 specimens of *P. generosa* (called before *P. abrupta*) analysed by Vadopalas and Bentzen (2000), even though the 5 microsatellite markers they isolated had a higher number of alleles per locus (7 to 33).

These markers will be valuable tools for population genetic studies that examine the dynamics, connectivity and variability of populations of *Panopea abbreviata*, thus helping managers to conserve and handle this artisanal fishery resource.

140

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Table 1 Characterisation of 21 microsatellite loci in *Panopea abbreviata*. Primer sequences, repeat motifs, clone size (bp), alleles size range (bp). PCR reaction indicates multiplex (loci with the same number in this column were amplified and genotyped together) or individual reactions. Na= number of alleles, H_O= observed heterozygosity, H_E= expected heterozygosity. GenBank accession numbers for each microsatellite. *indicates significant deviation from Hardy-Weinberg equilibrium.

Locus name	Primer sequences (5'-3')	Repeat motif	Clone size	PCR reaction	Alleles range	Na	H _O	H _E	Accession number
Pa6	F VIC-CATGTTTACAGAAGTTAGGC R ACAGCAAGATGTTGAAACT	(TACTC) ₁₁ (TACCC) ₅ (TACTC) ₂	329	1	318-368	10	0.840	0.866	JX416866
Pa8	F 6-FAM-ATAACATGTAAATGTATCATTAGAG R TATTGACGTTAGGACGTTT	(CCATT) ₈	163	2	154-179	6	0.680	0.747	JX416867
Pa13	F 6-FAM-CGTTTACTCAAACATGGTAT R TGAACATCTTTCTATAATTTTATCT	(CAGA) ₁₂	143	3	123-179	11	0.760	0.859	JX416868
Pa14	F 6-FAM-AAAGGCAAGGTGGCTTGT R TTTCACGGATAGTGAATTTTCG	(CATA) ₁₁	166	4	161-315	22	0.880	0.924	JX416869
Pa16	F 6-FAM-CAATAGCTCGCCTTATTAC R CTGACCGTCTGATAGCTC	(GTTT) ₈	131	5	130-154	5	0.440	0.607	JX416870
Pa17	F PET-TTTGTAATATGACGTTCTTG R AATAAAACGTTACACAGAGAC	(TTAC) ₉ (TTAA) ₄ TTAC TTAA (TTAC) ₃	246	6	203-679	14	0.720	0.899	JX416871
Pa18	F VIC-CGTTTGTCTAGTGTTGAG R GTACACCTGTAAATCAGACC	(TCCA) ₁₂	367	7	356-420	15	0.840	0.910	JX416872
Pa19	F 6-FAM-ATTTATAACCTCCATAATGC R ACAAACACAATTTAATAACG	(CATT) ₁₃	179	7	155-199	11	0.667	0.744	JX416873
Pa20	F PET-TTGGACTGAGTTATTAAAGG R CCATGAGACATGACATTG	(CCGT) ₈ CCAT (CCGT) ₃	252	1	241-261	4	0.560	0.573	JX416874
Pa23	F VIC-GACGTAATAATAGCGTGTTTC R ATAAGACATTGAACGGAAG	(CGCA) ₁₂	363	4	345-417	16	0.920	0.903	JX416875
Pa25	F PET-TTCTGTGTAAATGCATAGG	(GCGT) ₃ GTGT GGTT (GCGT) ₈	212	7	197-217	4	0.235	0.528*	JX416876

	R AGTAACGCGCTTATAGGT									
Pa27	F6-FAM-TTTCTGAGCTTGTATCTGTT R GTTATACGGAATAATCGTGA	(CGTG) ₁₂	177	8	162-194	9	0.840	0.842	JX416877	
Pa28	F PET-CTCGATGACATAATACGG R ACGTACTTTGTTTATAGGTCA	(CAAT) ₁₀	205	9	189-217	7	0.680	0.693	JX416878	
Pa29	F 6-FAM-GCCAGTATTGACTATTTTGT R GACGTGAACAATTAAGTAGAG	(TGCG) ₉	194	1	194-226	7	0.520	0.576	JX416879	
Pa31	F PET-TCCTTTATCCCTGTATTTG R TATTATTGTACTGTCATGCAC	(GAGT) ₉	237	10	247-391	23	0.880	0.937	JX416880	
Pa32	F NED-GACGTGACATAAAACAC R AATGTCACTTTTATTACTTC	(CATT) ₉	115	9	119-179	11	0.880	0.874	JX416881	
Pa33	F NED-TTAAATGCTGCATATTTTG R AATTTAAAATAGGCAATTACTC	(TCTG) ₁₃	145	11	133-185	14	0.985	0.898	JX416882	
Pa35	F 6-FAM-TTAGGAGATTGTAACAGAGC R ATTATACTACGCAGGAGGA	(GGAT) ₉	161	9	157-189	8	0.640	0.774	JX416883	
Pa36	F 6-FAM-TTCACCATCATCTTTAAAAC R GAAGAAGGACATTACATTGA	(CGCA) ₁₂	161	12	136-164	8	0.800	0.812	JX416884	
Pa39	F NED-AGTGACGTTACATTTACAGG R GTTCGATCATTTTAACATCT	(GACT) ₃ (GGCT) ₈	137	8	146-210	14	0.880	0.890	JX416885	
Pa45	F VIC-GATTATTTAATAGTCTTAAATGG R CTAGTTAAAAGCAATGCTAA	(AAAC) ₁₁	268	9	240-292	8	0.640	0.589	JX416886	