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Brief Report

Isolation and characterization of polymorphic microsatellite loci in plainfin midshipman fish

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Plainfin midshipman, *Porichthys notatus* (Batrachoidiformes; Batrachoididae), migrate from deeper waters to the intertidal zone along the Pacific coast from Alaska to the Gulf of California to spawn (Feder et al. 1974; Walker and Rosenblatt 1988; Bass 1990). They are nocturnal and hide themselves in sand or mud of the intertidal zone during the day but swim just above the seabed to feed at night (Arora 1948; Thompson et al. 1988). Mating in this species depends on auditory communication; males during the breeding season broadcast a humming sound, generated by rapid contractions of the sonic muscles attached to the lateral sides of their swim bladders (Bass et al. 1999). The female usually lays more than 100 eggs, which typically are attached to the underside of a rock, while the male fertilizes them (Arora 1948; Thompson et al. 1988). After depositing eggs, the female leaves the nest and the male remains and provides parental care. Care behaviour includes fanning the eggs until they hatch and then guarding the larvae until they become free-swimming in about 45 days (Arora 1948).

Male plainfin midshipman are sexually dimorphic with two reproductive morphs known as type I and type II (Brantley and Bass 1994; Bass 1996). Type I males are territorial, acoustically court females and then provide parental care for the eggs and larvae. In contrast, type II males are smaller than type I males and frequently steal fertilizations using sneaking or female mimicry behaviour (Brantley et al. 1993; Brantley and Bass 1994; Bass 1996). Although type II males have sonic muscle with contractile apparatus, the ratio of sonic muscle mass to body mass is six times greater in type I males than in type II males; type II males do not hum but do occasionally produce low-amplitude grunts (Bass 1996; Lewis et al. 2003). Type II males also provide no care for their offspring, but instead leave that to the type I males that they parasitize. The plainfin midshipman fish thus provides an opportunity to assess the determinants and evolutionary consequences of alternative mating tactics. To date, however, the molecular markers essential for such studies have not been available. We now describe the development of eight novel and polymorphic microsatellites for this species.

MATERIAL AND METHODS

Sample collection

During June 2007, midshipman were sampled from three sites in the intertidal zone along Vancouver Island. Two sites, Ladysmith Inlet and Mill Bay, were on the east side of the island and separated by about 50 km. The third site, Bamfield Inlet, was on the west side of the island and separated from the other two by about 230 km (nautical distance).

Microsatellite isolation

We first constructed a microsatellite DNA-enriched library following the method of Hamilton et al. (1999). Total genomic DNA was extracted from caudal peduncle tissue and digested with *Hae* III*, Rsa* I *and Nhe* I (New England Biolabs). Fragments 400–900 bp in length were ligated to a specific double-stranded linker (SNX; 5′-3′ forward: CTAAGGCCTTGCTAGCAGAAGC; reverse: GCTTCTGCTAGCAAGGCCTTAGAAAA) using T4 DNA ligase (New England Biolabs). The fragments were recovered by polymerase chain reaction (PCR) using the single-stranded forward and reverse linkers as primers. The PCR product was then hybridized to biotinylated $(CT)_{12}$, $(GT)_{12}$, $(GATA)_7$, $(GATC)_7$ and $(GACA)_7$ probes. Bound fragments were recovered using streptavidin-coated magnetic beads (Dynabeads M-280 Streptavidin, New Eng-

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Table 1. *Characterization of eight polymorphic microsatellite loci for midshipman, Porichthys notatus. Data comprise locus name, GenBank accession number, repeat motif, forward (F) and reverse (R) primer sequences, number of alleles per locus, allele size range, and for each of three collection sites, the number of individuals examined (n), and observed (H_O) and expected (H_E) heterozygosity.*

						Ladysmith Inlet			Bamfield Inlet			Mill Bay		
Locus	GenBank	Repeat motif	Primer sequences (5′-3′)	Alleles	Size range	n	H_O	H_E	n	H_O	H_E	n	H_O	H_E
Pon10	1236971	$(CA)_{15}$	F: AGCGACAAATG TGGCAAATAC R: ATATACCCAAC TGACAAGCTC	4	184–238	20	0.40	0.41	23	0.61	0.48	10	0.80	0.67
Pon22	1237010	$(CA)_{11}$	F: CACGCTCTGTG CATTTGCCAG R: GCATGATGAC ATCACAGGCAG	7	242–279	22	0.41	0.58	20	0.95	0.82	10	0.80	0.75
Pon23	1238048	$(TG)_{11}$	F: CTCTCTTTCAG CAGCTCACCC R: CCTCCGACAC CTGTCAAAGTC	7	193–212	24	0.54	0.73	22	0.46*	0.61	10	0.60	0.78
Pon25	1238047	$(CA)_{10}$	F: CTCCGCAAC ATCAGGGATTTG R: GAGGAGCAAC GAAAGCAAGAG	3	217–227	23	0.61	0.52	21	0.57	0.51	10	0.30	0.27
Pon29	1238054	$(CA)(GA)_3$~$(CA)(GA)_3$ ~$(CA)_7$~$(GACA)_3$	F: AGGTGGAGGG TTTGGACTG R: TTTTCAGACTG TTTCTGGTGG	7	223–277	27	0.67	0.75	20	0.75	0.75	10	0.50	0.67
Pon30	1238057	$(GC)_8(AC)_{12}$	F: AACATGCGTG AAAGGCAGTCAG R: GTTTGCTTCTC CTCACTACCAG	10	242–279	23	0.57	0.64	22	0.68	0.81	10	0.90	0.74
Pon32	1238058	$(GA)_3$~$(GC)_7(GT)_8$	F: CAGGCGAGCC TTCATAAACTG R: GTGCGTAATC GTGCGTAATGG	4	166–180	26	0.65	0.61	24	0.38	0.59	10	0.70	0.58
Pon47	1238059	$(GT)_{13}$	F: AGCTGACACA GCATGAAGCGG R: GTTCACTGCT GTCCTGCTGTG	6	186–196	23	0.61	0.65	20	0.60	0.68	8	1.00	0.73

*locus departed from Hardy–Weinberg equilibrium in this sample.

land Biolabs). DNA was amplified at 94°C for 10 min, followed by 35 cycles of 30 s at 94°C, 45 s at 58°C, 1 min at 72°C, and a final extension time of 72°C for 10 min using SNA forward primer. PCR products were ligated into the pGEM-T Easy Vector (Promega), transformed into competent *Escherichia coli* DH5α cells (Invitrogen), and spread onto LB agar plates. Approximately 300 positive clones were sequenced with T7 and SP6 universal primers at the Genome Quebec Innovation Centre (McGill University, Montreal, Canada).

Microsatellite amplification and analyses

Sequences were used to design primers using Primo Pro 3.4 (<www.changbioscience.com/primo/primo.html>). The loci were then initially screened with 11 individuals from the Ladysmith Inlet site. PCR amplifications were carried out in 10 µl reactions, containing ~50 ng template DNA, 3 mM $MgCl_2$, 1×PCR buffer (Invitrogen Life Technologies), 0.25 mM of each deoxynucleotide (Sigma-Aldrich), 0.25 units Taq DNA polymerase (Invitrogen) and 0.25 µM of each forward and reverse primer. Forward primers were labeled with Beckman Dye D2, D3 and D4 (Sigma-Genosys, Woodland, TX, USA). Reactions were performed on T1 Thermocycler (Whatman-Biometra): 94°C for 10 min, 35 cycles of 30 s at 94°C, 30 s either at 58°C (*Pon29*) or 61°C (all other loci), 30 s at 72°C and final elongation at 72°C for 10 min. The fragment analyses were conducted on a CEQ 8000 (Beckman Coulter).

The utility of the polymorphic loci was then examined using a broader sample of 63 individuals encompassing all three sampling sites. For each site, the observed and expected heterozygosity, Hardy-Weinberg equilibrium, and the linkage disequilibrium were calculated using Genepop version 3.4 (Raymond and Rousset 1995). The test for the presence of null alleles was conducted using MICRO-CHECKER ver. 2.2.3 (Van Oosterhout et al. 2004). Population differentiation among the three sites was examined using Genepop and pairwise F_{ST} estimates. Hierarchical genetic structuring was analyzed by assessing the relative contributions of among-population and within-population components using an analysis of molecular variance (AMOVA, Excoffier et al. 1992). The AMOVA was performed using the program Arlequin 2.0 (Schneider et al. 1997).

RESULTS AND DISCUSSION

Of the about 300 clones sequenced, sufficient flanking sequence was available to design primers for a total of 62 of the clones. Of these 62 loci, 34 (55%) did not amplify consistently, 18 (29%) were monomorphic, 2 (3%) were linked to other loci in our sample, and 8 (13%) amplified reliably and were polymorphic.

Based on the larger sample of 63 individuals from three sites, the number of alleles for each locus ranged from three to 10; the observed and expected heterozygosity ranged from 0.30 to 1.00 and from 0.27 to 0.82, respectively (Table 1). Only one locus, *Pon23*, in Bamfield Inlet was found to be out of Hardy–Weinberg equilibrium following Bonferroni correction. As no deviation was found in the other two sites for *Pon23*, it is unlikely that the deviation at the Bamfield site was due to a null allele. No linkage was detected between any pair of the eight loci (^{2}-tests $p > 0.07$). Interestingly, the three sites did not show any significant genetic differentiation (pairwise $F_{ST} = 0.024$–0.029; $p > 0.09$ for all comparisons), which suggests that the three sites may be derived from a single breeding population. The hierarchical AMOVA revealed that most of the genetic variance was found within populations compared to a smaller proportion among populations (percentage of variance; among populations: 2.6%, within populations: 97%, $p < 0.05$).

The lack of detectable genetic divergence among the populations examined could relate to the frequent genetic exchange between the Georgia Straight and west coast of Vancouver Island. The population structure around Vancouver Island may be influenced by seasonal dispersal patterns. The bifurcation of the Subarctic Current off the west coast of Vancouver Island into the southerly flowing California Current and northerly flowing Alaska Current may allow differentiation of dispersal areas (Thomson et al. 1989), as has been reported in dungeness crab (Beacham et al. 2008). Additional surveys of other species are required to fully detail the genetic structure of populations in this area. Nevertheless, our data suggest little genetic structure in populations of plainfin midshipman fish around Vancouver Island.

The eight loci developed here will be useful for broader analysis of population structure and gene flow, as well as for evaluation of reproductive success of males that utilize alterative mating tactics. Indeed, the eight loci have a single-parent and parent-pair exclusion probability of 0.90 and 0.99, respectively, which will allow accurate calculation of parentage in this system (Neff et al. 2000). The loci may also be useful in closely related species within the Batrachoididae.

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