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TECHNICAL NOTE

Characterization of novel microsatellite markers in the Antarctic silverfish *Pleuragramma antarcticum* and cross species amplification in other Notothenioidei

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Abstract We characterized nine polymorphic microsatellites by an enriched library from the Antarctic silverfish *Pleuragramma antarcticum*, a key species in the high Antarctic zone of the Southern Ocean. The number of alleles scored ranged from 7 to 39, whereas the observed and expected heterozygosity ranged from 0.4000 to 0.9750 and from 0.3943 to 0.9782, respectively. Cross-amplification was tested in the 3 species of the genus *Chionodraco* (Channichthyidae). These new microsatellites could potentially be useful in further investigations on *P. antarcticum* for which many questions on population structure and exposition to global environment change are still open to debate.

Keywords *Pleuragramma antarcticum* · Antarctic silverfish · Notothenioidei · Southern Ocean · Microsatellite · FIASCO protocol

The Antarctic silverfish *Pleuragramma antarcticum* Boulenger, 1902 is a key species in the high Antarctic zone of the Southern Ocean. This species has a circumpolar distribution and occurs at depths between 0 and 1,000 m (Eastman 1993). Among notothenioids, *P. antarcticum* is the only species in which all life stages occur throughout the water column (Regoli et al. 2005; Vacchi et al. 2004). Moreover, *P. antarcticum* represents the most abundant fish in Antarctic waters and a major contribution to the diet of most Antarctic vertebrates such as whales, seals, penguins, flying birds and benthic fish (La Mesa et al. 2004). Unfortunately, this species is experiencing a dramatic decline and has disappeared from a significant part of its range probably as a result of an high sensitivity to the global climate change, especially on the western side of the Antarctic Peninsula, with its warmer water and reduction in sea-ice coverage (Massom and Stammerjohn 2010 and references therein). This work reports on the isolation of new, polymorphic, molecular markers for population genetics analysis of *P. antarcticum*. In particular, these markers will be useful to detect possible loss of genetic variability for this species and further investigate the weak genetic structure, with significant differences between samples both in space and time, that have been found within *P. antarcticum*'s distribution range using the mitochondrial control region (Zane et al. 2006).

Microsatellites were isolated by an enriched library using the FIASCO (Fast Isolation by AFLP of Sequences COntaining repeats) protocol (Zane et al. 2002) as also described in Susana et al. (2007). Genomic DNA was extracted from 10-100 mg of a muscle tissue sample collected in Terranova Bay in 1997 (Ross Sea, 13th Italian expedition, PNRA) and 90% ethanol preserved.

Among the 81 sequenced clones (sequencing facility at http://www.bmr-genomics.com), 22 sequences contained a microsatellite with appropriate flanking regions and sequence quality for primer design. Primer pairs for amplification of microsatellite loci were designed with the software OLIGOEXPLORER (http://www.genelink.com). Primer pairs were used to amplify genomic DNA from 40 *P. antarcticum* individuals all collected at Terranova Bay

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Table 1 Amplification conditions and descriptive statistics for 9 novel microsatellite loci of *Pleuragramma antarcticum*

Locus	*S. R.*	Primers (5′–3′)	Repeat motifs	Initial T_A (°C)	Core T_A (°C)	N/N_A	H_O	H_E	PHWE	GB acc. no.
26Pl	144–172	F: FAM-CGGATTAACGCCACAGCAAA R: TCGTTTGGATTACTGGTCTGG	$(AC)_5GTATAG(AC)_{13}$	57	51	40/11	0.5250	0.6582	0.0213	HQ393884
41Pl	254–344	F: FAM-AGAATCACGGACCAATAGCC R: TGTGTAGTGGAATGAAGGGG	$(CA)_{15}TATG(CA)_3$	57	51	40/32	0.9500	0.9620	0.1196	HQ393886
42Pl	81–108	F: FAM-TTCGGATATTCAGGTACAGCC R: GGTCACACTTCTACGGTATGT	$(GT)_6GCTT(GT)_3$	57	51	40/9	0.7500	0.7025	0.3336	HQ393885
78Pl	263–400	F: TAMRA-GAGACTGCGTTGGTTAGACT R: CCTTCCATTGCTTTCCGAGT	$(CA)_{30}$	56	52	40/38	0.6750	0.9782	0.0004	HQ393887
101Pl	166–262	F: TAMRA-AAAGCCAGAGGACAGCAGGAGGA R: CCGTCGTGGGAAGGTGTTTGGGA	$(GT)_4TAT(GT)_{10}$	60	55	40/20	0.6000	0.9237	0.0001	HQ393888
132Pl	168–271	F: HEX-ATATTCAGATGGCTGCTTATG R: CGTGTCAGAACAGATTACATATAA	$(GA)_{37}$	58	52	40/32	0.9250	0.9703	0.2513	HQ393889
206Pl	97–205	F: HEX-TGTTCCAATCCATGAGTCCAAGC R: TACCAAACAAACTACCCAACCCA	$(GT)_{15}G(GT)_5$	60	55	40/38	0.9250	0.9759	0.0620	HQ393890
211Pl	213–331	F: HEX-TCATAGTCAGTCAGACATCAGTAT R: CGTCTCACCTCTAATCTACC	$(GA)_{44}$	58	52	40/39	0.9750	0.9778	0.4743	HQ393891
221Pl	172–194	F: TAMRA-AGAGGTAGGACAAAAAGGACAGAT R: GAAAAGGGAAAGCATGATGAGTGG	$(AC)_5AT(AC)_3AG(AC)_4$	60	55	40/7	0.4000	0.3943	0.6512	HQ393892

S. R. size range of amplified fragments in bp, *Initial* T_a (°C) annealing temperature of the first cycle, *Core* T_a (°C) annealing temperature of following thirty cycles, N/N_a number of individuals assayed/number of alleles detected, H_O and H_E observed and expected heterozygosities; *PHWE* the probability of Hardy–Weinberg equilibrium; *GB acc. no.* the GenBank accession number

Table 2 Cross-amplification of the *Pleuragramma antarcticum* microsatellite loci in three additional notothenioid species

Species name	Collection cruise and year	Locus 41Pl			Locus 101Pl			Locus 221Pl		
		S.R.	N_A	PHWE	*S.R.*	N_A	PHWE	*S.R.*	N_A	PHWE
Chionodraco hamatus	11th Italian expedition 1995–1996[a]	289–371	22	0.0070	181–337	23	0.5986	161–166	3	0.0200
Chionodraco myersi	5th Italian expedition 1989–1990[b]	305–388	24	0.0013	181–312	23	0.0259	161–180	4	0.1734
Chionodraco rastrospinosus	ANT-XIV 2 Cruise 1996[c]	318–380	20	0.0002	188–343	27	0.0134	161–177	7	0.7494

[a] 11th Italian expedition, Ross Sea, Terranova Bay 1995–1996, PNRA

[b] 5th Italian expedition, Ross Sea, Terranova Bay 1989–1990, PNRA

[c] ANTARKTIS expedition, PFS "Polarstern" ANT-XIV/2 Cruise 1996/1997 at the Antarctic Peninsula

S. R. size range of amplified fragments in bp, N_a number of alleles detected, *PHWE* the probability of Hardy–Weinberg equilibrium

in 1997 (Ross Sea, 13th Italian expedition, PNRA). PCRs were carried out in 20 μL total volume containing: Taq buffer 1X (Promega, 50 mM KCl, 10 mM Tris–HCl pH 9 at 25°C, 0.1% TritonX-100), 1 mM $MgCl_2$, 150 nM of each primer, 70 μM dNTPs, 0.04 U/μL of Taq polymerase (Promega) and 50 ng of genomic DNA. Loci were amplified with a touchdown PCR profile (for differences between starting and final annealing temperatures, see Table 1): (1) pre-denaturation: 94°C 2 min; (2) 8, 10 or 12 touchdown cycles: denaturation 94°C 30 s, annealing 60–56°C 30 s decreased of 0.5°C each cycle, extension 72°C 30 s; (3) 30 cycles: denaturation 95°C 30 s, annealing 55–51°C 30 s, extension 72°C 30 s; (4) additional extension for 5 min at 72°C.

Forward primers were labeled with different fluorescent dyes and a fraction of the PCR product was loaded on an Abi Prism 3100 or 3700 automated sequencer (Rox400 as size standard, genotyping facility at http://www.bmr-genomics.com) and allele sizes were assigned using Genotyper 3.7 (Applied Biosystems). Binning was automated with the software Flexibin ver. 2 (Amos et al. 2007) and all input files for further analysis were produced with Create (Coombs et al. 2008).

Among the 22 primer pairs tested, all produced amplified product, nine produced polymorphic microsatellite markers (Table 1), and the remaining were monomorphic or produced bad quality profiles.

The number of alleles scored for the nine polymorphic loci ranged from 7 to 39 and the observed heterozygosity ranged from 0.4000 to 0.9750. Hardy–Weinberg equilibrium (Fisher's exact test) and genotypic disequilibrium for pairs of loci (Fisher's exact test) within the sample were tested with the software Genepop, online version (Raymond and Rousset 1995) (nominal significant threshold $\alpha = 0.01$). No significant deviation from Hardy–Weinberg equilibrium was detected at seven out of nine loci. A general excess of homozygotes was observed for loci that were found to be in disagreement with the Hardy–Weinberg model. Among the general reasons, this pattern could be explained by Wahlund effect due to mixing of geographically or temporally differentiated genetic pools or null alleles. The presence of null alleles is a common phenomenon in microsatellites (Dakin and Avise 2004) and their frequency was shown to increase by the transfer of microsatellite loci to new species (Li et al. 2003). Cross species amplification results (see below and Table 2) were not consistent with the presence of null alleles for locus 101Pl. All loci resulted in linkage equilibrium at a 1% nominal significance level.

Cross-amplification of primers was evaluated in 3 species of the genus *Chionodraco* (Channichthyidae). DNA was extracted as in Patwary et al. (1994) from 95% ethanol stored muscle tissue of *Chionodraco rastrospinosus*, *C. hamatus*, and *C. myersi* collected during three different Antarctic cruises and campaigns (Table 2). Of the nine loci tested, three cross-amplified and were found to be polymorphic in 20 individuals of each species (Table 2). The loci represent key markers for further investigations on *P. antarcticum* for which many questions on population structure and exposition to global environment change are still open to debate.

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