PERMANENT GENETIC RESOURCES NOTE

Development of 13 microsatellite markers for the threatened galaxiid fish *Aplochiton zebra* (Jenyns, 1842)

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Abstract

Aplochiton zebra (peladilla, zebra trout) is a galaxiid fish endemic to Patagonia and the Falkland Islands, where populations are threatened by salmonid introductions. Here we describe the isolation and characterization of 13 polymorphic microsatellite DNA markers that can be used for assessing the genetic variation and conservation status of this species. The variability of these markers was assessed in six rivers in Chilean Patagonia. The number of alleles ranged from 5 to 80 per locus and observed heterozygosities varied between 0.168 and 0.910. A protocol for multiplexing these markers is described.

Aplochiton zebra (known as 'peladilla' or 'zebra trout') is a diadromous galaxiid fish that inhabits rivers and lakes of Patagonian South America (Chile, Argentina) and the Falkland Islands, where it can also form resident, land-locked populations (McDowall 2006). Ecological studies suggest that *A. zebra* is in decline as a consequence of predation and competition from non-native salmonids (Arismendi *et al.* 2009; Lattuca *et al.* 2008; McDowall *et al.* 2001; Young *et al.* 2009; Young *et al.* 2010), but there is little or no information on the genetic diversity or connectivity of threatened populations. Here we describe the isolation and characterization of 13 polymorphic microsatellite loci for *A. zebra* which can be used to analyze the genetic diversity and structure of this species, and help to clarify the conservation status of diadromous and resident populations.

Microsatellite loci were isolated from Inter Simple Sequence Repeats (ISSR) sequences by amplification of the region between two microsatellite priming sites inversely orientated (Zietkiewicz *et al.* 1994). ISSR regions were amplified from a single zebra trout using the following nine ISSR-PCR primers: $AAC(CCA)_5$, $(GAA)_7CT$, $(CA)_8RG$, $GATC(GTAG)_7$, $GGT(TC)_7$, $(AG)_9TT$, $(AG)_9CT$, $GCGC(GAC)_5$ and $(GA)_9CC$. Genomic DNA was extracted from the pelvic fin clip using the Wizard® SV 96 DNA Purification Kit (Promega, USA). A single ISSR-PCR primer was used in each PCR that consisted of 20 ng of DNA, 1x Standard Taq (Mg-free) Reaction Buffer, 1.5 mM $MgCl_2$, 0.8mM dNTP mix, 1µM primer and 0.04 units of Taq DNA Polymerase (5u/λ) in a total reaction volume of 25 µL. PCR amplifications were carried out under the following conditions: 5 min at 94℃, followed by 35 cycles of; 94℃ for 60s, 54℃ for 60s, 72 ℃ for 90s and a final extension at 72 ℃ for 20 min. A 5 µL aliquot of the ISSR-PCR product was resolved on a 1.5% agarose gel stained with ethidium bromide to confirm amplification of ISSR regions. The remaining product was purified with the Qiaquick PCR purification kit (Qiagen, UK),

pooled and ligated in a pCR®4-TOPO plasmid vector (Invitrogen, USA). Following the ligation, 2 µL of the constructs were transformed into 50 µL of One Shot® Top10 chemically competent *E.coli* cells using the kit protocol suppled by Invitrogen, USA. Cells were plated onto agar plates containing ampicillin (100 mg/mL; Sigma, UK) and grown at 37℃ overnight.

A total of 278 positive colonies were sequenced using M13 vector specific primers, and from these, 72 ISSR fragments were identified as containing tandem repeats (≥5 repeats in length). Microsatellite repeats were identified using TANDEM REPEATS FINDER (Benson 1999) with default parameters and 0.95 as minimum SSR match. Primer3 (Rozen& Skaletsky 1999) was then used to design primers flanking the SSR. A total of 30 primer pairs were tested for amplification and variability on 16 individuals sampled from 4 different rivers in Chilean Patagonia (Region X, Los Lagos). Although 19 microsatellite markers amplified reliably, only 13 were clearly polymorphic over all samples. The forward primer of each of the markers was labelled with a fluorescent dye (FAM, VIC, PET or NED) and amplified in three separate multiplex PCR reactions (multiplex1: Aze1, Aze2, Aze3, Aze4, Aze5, Aze6; multiplex 2: Aze8, Aze9 and Aze10; multiplex 3: Aze11, Aze12, Aze13 and Aze14) using the QIAGEN Multiplex PCR kit (QIAGEN, Sussex, UK). PCR mixes for each reaction contained 1X HotStart Taq Polymerase MasterMix (Qiagen, UK), 2 µM of each primer and 5-20 ng DNA. Touchdown PCR was performed on a 2720 Thermal Cycler (Applied Biosystems, USA) using an initial denaturing step of 15 min at 95℃ followed by 8 cycles of 95℃ for 45s, 64℃ - 56℃ annealing for 90s and extension at 72℃ for 1 min. Another 25 cycles were then performed using an annealing temperature of 56℃ and a final extension at 72 ℃ for 10 min. PCR products were resolved on an ABI3130xl sequencer with GeneScan-500

LIZ size standard (Applied Biosystems, USA) and analyzed using the software programme GeneMapper v 4.0 (Applied Biosystems, USA).

Microsatellite loci were examined for potential genotyping errors including presence of null alleles, allele drop outs and dubious stutter peak calls (confidence interval 95%) using Micro-Checker (Van Oosterhout *et al.* 2004) and for evidence of gametic disequilibrium using GENEPOP ON THE WEB (Raymond& Rousset 1995). The program TFPGA (Miller 1997) was used to estimate Hardy Weinberg proportions (HW), allele diversity (A) and observed and expected heterozygosities (Ho and He). Levels of significance were adjusted by sequential Bonferroni correction for multiple tests (Rice 1989).

All 13 microsatellite markers were screened on 168 fish from six rivers around the Island of Chiloé (River Huincha, n = 30), Lake Ranco (River Quiman, n = 27; River Punahue, n= 21; River Pitreno, n = 30; River Futangue, n = 30), and Lake Panguipulli (River Blanco, n = 30). All microsatellite loci were shown to be polymorphic in these samples with the number of alleles ranging from 5 to 80 (Table 1). Only 1 of the 13 microsatellites (i.e. Aze14) showed significant deviations from HWE (P = 0.0001) for two of the six populations analysed. Evidence of null alleles was detected in five microsatellites but only in one population (Aze6, Aze8, Aze9) or two populations (Aze11, Aze14) and the frequencies were always less than 0.2. There was no evidence of allele drop outs in any of the microsatellites in any of the populations.

In summary, the screening of six Chilean populations of *Aplochiton zebra* employing 13 novel microsatellite markers indicates the existence of moderate to high heterozygosity and allelic diversity with no evidence of gametic disequilibrium. These results suggest that these markers will prove useful for examining the genetic population structure and

connectivity of zebra trout populations. Moreover, they will facilitate an assessment of the conservation status of *A. zebra* in areas impacted by salmonid invasions.

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Table 1. Motif and primer sequences (including fluorescent dye label), size range of the alleles, annealing temperature (Tm); number of individuals successfully amplified; number of alleles (A); expected heterozygosity (He) and observed heterozygosity (Ho). Genbank accession numbers are in shown brackets.

Locus	Motif	Primer sequence (5'-3')	Size range (bp)	Tm (°C)	n	A	He	Ho
Aze1	$(TG)_{14}$	F: NED-TCATGTAGTGAAGGCAGAAAGC	121-141	59.5	164	9	0.408	0.372
(HM997136)		R: TGGACCTGAATTTTCCTGCT		59.5				
Aze2	$(GT)_{21}$ N $(GA)_3A(GA)_4N(TG)_4$	F: PET-CAAACACTGGGCCTCTGG	123-169	60	166	16	0.801	0.590
(HM997137)		R: TGCACACTCTCGAACAAACA		59				
Aze3	$(CT)_{13}$	F: VIC-GACCTAGGGCTGGCTTTACC	75-91	60	168	5	0.526	0.381
(HM997138)		R: CGTCAAAGAGAAGCCAAGGA		60.5				
Aze4	$(TA)_3GAC(AT)_{14}ACA(TCC)_3$	F: 6FAM-CCATGACAAAACAAGCTGGA	99-115	59.5	152	9	0.587	0.204
(HM997139)		R: GCCTCACATCCTCACTAGGC		60				
Aze5	(AGAAG)17A(CAAAGA)10	F: VIC-GTGGAGACAGTCAACCTTGC	123-316	58	167	80	0.975	0.910
(HM997140)		R: CCTGTCCTTTTCTGTTCTTGG		59				
Aze6	(TG)19	F: 6FAM-TCCTTCCCCTTCTCTCTAAGG	151-185	52	167	16	0.884	0.778
(HQ003931)		R: ATACCAAGGGGGCACTAAGC		58				
Aze8	(GT)25	F: NED-GTGCTTGGCCACCTAACCT	173-307	58	168	34	0.943	0.768
(HM997142)		R: AGGGCAACGTTTAGCTATGG		50				

Aze9	(ACAG)16	F: VIC-GAGAGAGACCGACAGACAGACC	79-259	59	168	41	0.962	0.827
(HM997143)		R: CGCCTAGACAACGCCTTTAG		55				
Aze10	(CA)14	F: 6FAM-CCGGGTGTAAGTGCTACTGAA	152-190	52	168	15	0.898	0.804
(HM997144)		R: TCTGCAGTTTGACTGCCTGT		50				
Aze11	(GT)15	F: 6FAM-ACTAAGGCCGGAGAGAGACT	108-156	55	153	20	0.727	0.588
(HM997145)		R: TATGAATAGGTGCCCGTCGT		50				
Aze12	(TG)43	F: PET-TCCCCTGTGGGTGTTTATGT	129-235	50	167	38	0.937	0.904
(HM997146)		R: GGCCCCTCTGTAGTCAGACA		60				
Aze13	(GT)9	F: NED-GAAAGCGTGTTTCCCTTGAC	124-174	50	139	16	0.764	0.712
(HM997147)		R: TGGGAGGCAGTTTTAGACAGA		47				
Aze14	(AC)11	F: VIC-TGCATCCTTTCCCTCTTCTC	92-112	50	161	5	0.455	0.168
(HM997148)		R: TTCGCTGTGAGGTCTGTCAC		55				

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