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PRIMER NOTE

Characterization of microsatellite markers in a threatened species, the White Sands pupfish (*Cyprinodon tularosa*)

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Abstract

We report the characterization of nine new microsatellite markers for a threatened species, the White Sands pupfish (*Cyprinodon tularosa*), using an enriched library method. These markers show moderate levels of variation (two to five alleles per locus) in the two native populations of this species and reveal substantial divergence between these two populations as indicated by a high percentage of private alleles. These markers will prove very useful in the conservation management of this rare species.

Keywords: Cyprinodontidae, *Cyprinodon tularosa*, microsatellite, pupfish, simple sequence repeat

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The White Sands pupfish (*Cyprinodon tularosa*) is found in only four locations in southern New Mexico: Salt Creek, Malpais Spring and Mound Spring (located on White Sands Missile Range) and Lost River (located on Holloman Air Force Base). The Salt Creek and Malpais Spring populations are native (Pittenger & Springer 1999) and have been recognized as separate evolutionarily significant units (Stockwell *et al*. 1998). Genetic research on this species has been hampered by a lack of polymorphic markers. Here, we report the isolation and characterization of a set of nine microsatellite markers and their characterization in both evolutionarily significant units of *C. tularosa*.

Genomic DNA pooled from six pupfish (four Malpais Spring and two Salt Creek individuals) was restricted with *Mbo*I and 300–1000-bp fragments excised after gel electrophoresis. The DNA was purified through Qiaquick columns (Qiagen) and 2.5 μg used for ligation to 7.5 μg of an *Mbo*I adaptor molecule (Kandpal *et al*. 1994; Iyengar *et al*. 2000). The ligated DNA was column purified and a polymerase chain reaction (PCR) performed using either one of the two adaptor oligonucleotides as a primer. The PCR was carried out in a 50-μL volume using the following conditions: 94 °C for 4 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 120 s and then 72 °C for 10 min. The PCR product (2 μg) was denatured and hybridized to 1 μg of biotinylated $(CA)_{15}$ oligonucleotide overnight at 50 °C in 100 μL 0.5 M sodium phosphate, pH 7.4, and 0.5% sodium dodecyl sulphate. Enrichment was carried out using streptavidin-coated magnetic beads and a magnetic particle concentrator (Promega). The beads were first washed three times in 1× Tris buffer (100 mM Tris, pH 7.5, 150 mM NaCl) and the hybridization mixture added to the beads and incubated for 30 min at room temperature. Washes used for the enrichment procedure were as follows: 1× Tris buffer at room temperature, 1× Tris buffer at 50 °C, 0.1× Tris buffer at 50 °C, 0.1× Tris buffer at 65 °C and water at 65 °C. We have observed that although the most enriched fraction is frequently in the final wash it can, on occasion, be in the penultimate wash.

Consequently, DNA from both the fourth and fifth washes was column purified and amplified in PCRs as described above. The PCR products were column purified and cloned into pGEM-T Easy (Promega) following an additional A-tailing step by addition of 10 mM dATP and 0.5 U *Taq* DNA polymerase (ABgene) upon completion of PCR and incubation at 72 °C for 15 min. Recombinant clones were spotted onto a gridded agar plate and also swirled in 50 μL water (heated to 95 °C for 15 min and stored at –20 °C). A volume (1 μL) of this solution was then used in screening for repeats using the PCR-based isolation of microsatellite arrays method described by Lunt *et al*. (1999). Clones found to possess a strong extra band, or sometimes

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Table 1 Microsatellite markers isolated in White Sands pupfish

Locus	Repeat sequence	Primer sequences (5′–3′)	Annealing temp. (°C)	No. of alleles (sizes)	Malpais Spring heterozygosity (sample size $n = 10$)		Salt Creek heterozygosity (sample size $n = 10$)		GenBank Accession no.
					H_O	H_E	H_O	H_E	
WSP20*	$(GATA)_7$	F: GAGGACTCCAGCAGCAAAAC		2	0.00	0.00	0.00	0.00	AY340795
		R: GTTTGGGTTGTTATAGATGCTGC	60	(100–108)					
WSP23	$(TG)_{23}G(GT)_4$	F: ACCCATTCACACAACCTTCAC		4	0.00	0.00	0.50	0.51	AY340801
		R: AGAGAGGGCACAAAGCTGAC	52	(303–317)					
WSP24	$(AC)_{15}$	F: TCACTGATACTCCTGTTGAG		3	0.50	0.39	0.40	0.51	AY340803
		R: AGTCTGCATGAATGCCTGC	55	(117–133)					
WSP25	$(ACA)_2(CA)_{12}(N)_{29}$ $(AC)_2AG(AC)_{10}$ $TGAG(AC)_5$	F: CCCGTCCATCAAACTGAGAC		3	0.20	0.19	0.00	0.19	AY340800
		R: CAGGGTTCTGAAGATGGTGAA	52	(291–299)					
WSP26	$(CA)_3(AC)_{20}$	F: ACACAGTTCCTCGCATCCTT		2	0.40	0.53	0.00	0.00	AY340799
		R: ACTTGGCGTTTCTGCTCTGT	50	(183–205)					
WSP30	$(AC)_{16}$	F: TTCCCCTTCATCTACACATGC		2	0.20	0.34	0.00	0.00	AY340807
		R: TCTGGAGCTGCAGCGTACAG	59	(101–105)					
WSP32	$(CA)_{15}$	F: CATCGCTCAGCCTTCCAG		2	0.20	0.19	0.00	0.00	AY340802
		R: GCTGCAGACAACAAGTGTTCA	59	(217–221)					
WSP33	$(GT)_{12}$	F: CGTCTCTCCATCCATGCTATT		2	0.00	0.00	0.60	0.51	AY340808
		R: ATGTCTCTAGGCAAGCTGC	55	(132–136)					
WSP34	$(TG)_{16}$	F: GGGGAGTGTGTGAGGTAGGA		5	0.50	0.66	0.50	0.39	AY340809
		R: AAGCAGGCAGCTGCAGGGTG	61	(195–205)					

*WSP20 was identified fortuitously and was not a result of the enrichment procedure.
Additional sequences not shown here have been deposited in GenBank [Accession nos AY340796, AY340797 and AY340804 (not fully optimized) and AY340798, AY340805 and AY340806 (monomorphic in our samples)].
H_O, observed heterozygosity; H_E, expected heterozygosity.

a 'smear' of extra bands, as a result of CA repeat specific primer binding were identified as positive. Forty-six of 320 positive clones were identified in the fourth wash as opposed to 26 of 244 in the final wash. The 46 clones from the fourth wash were sequenced on an automated sequencer (3700; Applied Biosystems) and 16 of them were found to contain usable CA microsatellite sequences. Sequences containing < 12 perfect CA repeats (19 of 46) were not used for primer design, one clone was found to be too close to the linker to allow primer design and two clones were found to contain the same microsatellite locus. Primers were designed for 15 microsatellite loci either manually or using the MICROSATELLITE TARGET IDENTIFICATION PROGRAM (http://www.abcc.ncifcrf.gov/staff/bstephens.shtml).

The microsatellite primers were tested on 20 individuals, 10 each from Malpais Spring and Salt Creek, using genomic DNA extracted with the DNeasy tissue kit (Qiagen). The results are summarized in Table 1. Nine polymorphic markers are reported here, three were found to be monomorphic (after screening of 13 individuals from each population) and three were found to be difficult to score accurately and abandoned. All 15 microsatellite sequences have been submitted to GenBank.

Microsatellite PCRs were performed in 10 μL containing ~50 ng template DNA, 1 × buffer, 2.5 mM $MgCl_2$, 10 pmol of each primer, 200 μM dNTPs and 0.4 U of Amplitaq Gold® *Taq* polymerase (Applied Biosystems). Thermal cycling consisted of 35 cycles at 94 °C for 30 s, primer-specific annealing temperature (Table 1) for 30 s and 72 °C for 30 s, preceded by 3 min at 94 °C and followed by 3 min at 72 °C. The PCR products were electrophoresed on 6% polyacrylamide gels followed by silver staining. Once a locus appeared polymorphic, the forward primer was labelled with Dye D2, D3 or D4 (Beckman®) and run on an automated sequencer (CEQ® 2000XL; Beckman). Allele sizes were determined using the CEQ® DNA ANALYSIS SYSTEM software package. We used the program GDA (Lewis & Zaykin 2001) to calculate allele frequencies, expected and observed heterozygosities and to test for departures from Hardy–Weinberg equilibrium and linkage disequilibrium within each population.

The microsatellite markers had between two and five alleles per locus. Others have observed similar levels of variation at microsatellite markers in *Cyprinodon* sp. (e.g. Burg *et al*. 2002). Only one test revealed a departure from Hardy–Weinberg equilibrium, the Salt Creek population at locus WSP-25 ($P = 0.05$). Nine and one individuals were

homozygous for the 299 and 297 alleles, respectively, suggesting the possibility of a relatively high frequency null allele for this locus. Linkage disequilibrium among markers was not observed. WSP-20 appears to show a fixed difference and five loci were variable in one population but fixed in the other population. Further, we observed an unusually high percentage of private alleles (72%, $n = 25$). These observations suggest that both populations have experienced high levels of drift. The markers reported here will prove useful in monitoring genetic variation of actively managed populations of this rare fish.

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