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MICROSATELLITE LETTERS

A set of microsatellite markers for the threatened Murray hardyhead, *Craterocephalus fluviatilis* (Pisces: Atherinidae) from the southern Murray–Darling Basin

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Abstract A new set of 16 microsatellite markers was isolated and characterized for the threatened Australian freshwater fish Murray hardyhead *Craterocephalus fluviatilis* (Atherinidae) using a next generation sequencing approach. Seventy-eight fish from wild and captive populations were genotyped at all markers. All markers were polymorphic, with average allelic diversity of 5.7 and heterozygosity of 0.46. These markers will benefit substantially the ongoing conservation program of a critically endangered lineage of *C. fluviatilis* that includes captive breeding, relatedness and paternity analyses, reintroduction, and landscape genetics.

Keywords Australian fish · Conservation genetics · Landscape genetics · Restoration genetics

The Murray hardyhead (MH) *Craterocephalus fluviatilis* (McCulloch 1912) (Atherinidae) is a small freshwater fish (~75 mm total length) endemic to southeastern Australia. It was considered widely distributed in the Murray–Darling Basin (MDB) covering New South Wales (NSW), Victoria (VIC.) and South Australia (SA). After recent population declines the species is now extinct in NSW, considered Endangered (IUCN 2011) and listed as Vulnerable under the Australian Government *Environment protection and biodiversity conservation act* 1999.

Threats to the survival of MH include altered flow regimes and increasing fragmentation in the River Murray that have reduced the suitable areas for feeding, shelter and breeding (Hammer et al. 2013). We have implemented a conservation program for MH that involves captive breeding, relatedness and paternity analyses, reintroduction, and landscape genetics. In order to accomplish the aims of the conservation program, highly resolving molecular markers capable of establishing kin relationships and guiding captive breeding are needed.

Here we characterize the first set of microsatellite markers for MH using a next generation sequencing approach. Genomic DNA (≅10 μg) was extracted from one specimen of *C. fluviatilis* from the lower MDB (Rocky Gully Wetland, SA; 35.111°S, 139.264°E). This sample was subjected to high throughput DNA sequencing on 1/8 of a PicoTiterPlate of a Roche GS FLX (454). Methods used to select contigs, design primers and build multiplex polymerase chain reactions (PCRs) followed Carvalho and Beheregaray (2011). From the 303,160 reads obtained, 3,168 had putative microsatellite motifs. The best 21 loci were chosen for PCR. Procedures for PCR followed Beheregaray et al. (2004) and used their 63–55 and 63–59 °C touch downs. Sixteen microsatellite primer sets amplified consistently and were used in two distinct multiplex PCRs, PlexA and B (Table 1). Sixty-one wild fish breeders from the lower Murray (SA) held in captivity and 17 fish from Elizabeth Lake (VIC) were used for genotyping. Microsatellite profiles were examined using GENEMAPPER 4.0 (Applied Biosystems) and peaks scored manually. GENEPOP

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Table 1 Characterization of 16 microsatellite loci for *Craterocephalus fluviatilis*

Locus	Primer sequences (5′–3′)*[a]	Repeat structure	N_A	Size range (bp)	Captivity		Wild		GenBank accession no
					H_O/H_E	H–W	H_O/H_E	H–W	
PlexA									
Cf13	F-AGTTGCGAAGTTTGCTTTGG^{FAM}	(GGA)10	5	162–177	0.72/0.50	*	0.44/0.38	1.000	
	R-CGCGTTTTATCTGGGATCTG								
Cf18	F-CTGAAGGTGCAAGATGAACG^{FAM}	(TCTA)11	6	225–267	0.50/0.50	0.460	0.44/0.52	1.000	
	R-CCAACTACAGCATCCAGGTG								
Cf7	F-CTCATTTGCTTCCAAAACACC^{VIC}	(AC)15	15	162–197	0.77/0.76	0.766	0.25/0.23	1.000	
	R-GACCCATTCCACCTTTTGTC								
Cf19	F-AAAGGTGTCTTCGGTTGACG^{VIC}	(TAATA)14	24	387–481	0.65/0.91	*	0.87/0.91	0.711	
	R-GGATACTCGTGCGGAATGTC								
Cf16	F-TGTGTATGTTTTCTGGTTTTTGG^{NED}	(TG)12	6	121–171	0.26/0.26	1.000	0.33/0.30	1.000	
	R-ACACTGTAGAAGCGGCACAC								
Cf6	F-CCTTCAGGCTGACGTGATG^{NED}	(CA)11	8	221–246	0.67/0.67	0.876	0.33/0.43	0.403	
	R CGTGGTGGAGGAGTACCG								
Cf5	F-ATGCATGGGCTTACAGCAG^{PET}	(AC)15	11	165–191	0.78/0.77	0.207	0.66/0.62	1.000	
	R-CCATATGTGTTTTGCCTTTTTG								
Cf20	F-GGTCAAAAGTTACACAGCATCC^{PET}	(AAT)13	7	322–355	0.68/0.68	0.064	0.62/0.67	1.000	
	R-CACATACCTTCTCCCCCTTG								
Plex B									
Cf1	F-TCTGACACCTCACCATCTGC^{FAM}	(CA)16	5	181–194	0.62/0.65	0.309	0.25/0.23	1.000	
	R-TGCCATATTGTAATCCTGTTACC								
Cf2	F-GGATGGCAGACTTTTTGAGG^{FAM}	(CA)15	11	231–276	0.37/0.66	*	0.00/0.57	0.0051	
	R-GCTGGCAAGGGAAATAGATG								
Cf8	F-CGGATTATAGAGCCGCACAG^{VIC}	(AC)13	6	104–116	0.70/0.70	0.118	0.37/0.33	1.000	
	R-CCGGGCCGAATATAGGATAG								
Cf11	F-AAACTATGAAACTTCTGACCT GAAAC^{VIC}	(CTAT)13	45	168–427	0.91/0.94	0.010	0.87/0.96	0.365	
	R-CACAGATTATTGGGGCAAGAG								
Cf3	F-TTACCTTGGGTGGGTATTCG^{NED}	(TAA)13	9	112–190	0.83/0.59	*	0.37/0.32	1.000	
	R-CAACACTGAAGGCAGCACAG								
Cf12	F-TCATGAAGAGAAAAACAGAAA GGTC^{NED}	(ATCT)17	15	168–280	0.26/0.86	*	0.12/0.46	0.005	
	R-CAAGTTCACACCATGTGTTGC								
Cf15	F-TGCTGCATTTCTTTTGGTTG^{PET}	(CA)15	23	155–215	0.78/0.89	*	0.87/0.86	0.960	
	R-CAATATGAACTGAGGCGAAGG								
Cf9	F-GGGGACGAGTACGAGAAGTG^{PET}	(AC)15	8	220–295	0.34/0.69	*	0.11/0.41	0.0039	
	R-GCTACTGCCTGAGGAGGCTA								

N_A number of alleles; H_O observed heterozygosity; H_E expected heterozygosity; *H–W* Hardy–Weinberg *P* values

* $P < 0.05$

[a] Tagged with 5′M13 universal sequence (5′TGTAAAACGACGGCC)

v4 (Rousset 2008) was used to estimate genetic diversity, linkage disequilibrium and Hardy–Weinberg equilibrium (HWE). MICROCHECKER (Van Oosterhout et al. 2004) was used to check for null alleles and scoring errors.

All loci were polymorphic with an average of 5.7 alleles per locus (2–26) and mean observed heterozygosity of 0.46. Deviations from HWE and linkage disequilibrium (between loci CF11 and CF15) were detected only for the captive population. These markers are an important resource for the ongoing conservation program of the critically endangered lineage of *C. fluviatilis* from the lower MDB.

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