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TECHNICAL NOTE

Isolation and PCR-multiplex genotyping of 18 novel microsatellite markers for the threatened southern pygmy perch (*Nannoperca australis*)

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Abstract A set of 18 microsatellites was developed for the freshwater fish southern pygmy perch (SPP) *Nannoperca australis* (Percichthyidae). SPP is an endangered species endemic to South-eastern Australia. Applying a next generation DNA sequencing approach, we obtained 12,725 sequences containing putative microsatellite motifs using 1/8 of a shot-gun pyrosequencing reaction. Twenty-three microsatellite motifs with enough flanking sequence were selected for primer design. All 18 microsatellite loci were successfully genotyped using three multiplex reactions in 38 specimens of SPP rescued from the wild and kept in captivity. The number of alleles per locus varied from 3 to 15 alleles per locus (mean = 7.6) and the observed heterozygosity ranged from 0.368 to 0.921 (mean = 0.648). Evidence for null alleles was observed only for one locus (Nau33). No deviation from Hardy–Weinberg equilibrium or linkage disequilibrium between pairs of loci were detected. These polymorphic markers will prove useful for an ongoing conservation-breeding program of SPP aimed at minimizing kinship in captivity and for studies on landscape and restoration genetics.

Keywords Next Generation DNA sequencing · Endangered species · Percichthyidae · Microsatellites · Captive breeding program · Conservation

The Southern pygmy perch (SPP) *Nannoperca australis* (Percichthyidae) is a small sized freshwater fish found in South-eastern Australia. Regional populations of SPP have suffered declines due to altered flow regimes, loss of aquatic vegetation, introduction of exotic species and severe droughts, compounded by the loss of connectivity within associated rivers and creeks (Cook et al. 2007; Lintermans 2007). SPP is considered endangered in South Australia (Hammer et al. 2009), and, therefore, included in a "Drought Action Plan" (Hall et al. 2009) that intends to rescue fish populations from the lower Murray-Darling Basing (MDB) that are at very high risk of extinction. Moreover, cryptic species and distinct Management Units, unveiled by genetic studies, have been identified within SPP (Hammer 2001; Unmack et al. in press). Only five microsatellite markers are currently available for SPP (Cook et al. 2006) and additional highly resolving molecular markers are urgently needed to inform an ongoing SPP captive breeding program aimed at minimizing kinship and reintroducing genetically healthy individuals to the wild. In addition, more microsatellite markers are needed to conduct a large-scale landscape genetic analysis of SPP that would inform long-term management practices for the species.

To rapidly obtain a large number of microsatellite loci for SPP, a next generation DNA sequencing (NGS) was used. A total of 10 µg of genomic DNA was extracted from muscle tissues keep in −80°C from one SPP specimen and sent to the Australian Genome Research Facility (www.agrf.com.au) in order to perform a high throughput DNA

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Table 1 Characteristics of the 18 microsatellite loci isolated from *N. australis* and primers sequences

Locus	Primer sequences (5′–3′)	Repeat structure	N_A	Size range (bp)	H_O/H_E	H–W	GenBank Accession no.
PlexA							
Nau7	F: TGTCATTTGTTTGGGAGTGC$^{6\text{-}FAM}$ R: CCCTCGATACGTGTGCATAG	GT(13)	5	95–109	0.553/0.661	0.4988	JN007858
Nau14	F: TGTGATTTCTGTCTGCACCG$^{6\text{-}FAM}$ R: GCTGGAACAGCTGAGTGG	GT(13)	11	252–276	0.789/0.762	0.2396	JN007861
Nau24	F: TGACAGTAAGCAATTCTGCCAC^{PET} R: ACGGTCACGGAATCAACCC	CA(12)	7	168–210	0.763/0.695	0.5056	JN007864
Nau34	F: TTCTGTCTTTGTGCACTCTCC^{NED} R: CAATCAGGTCAAGGTCATGC	AC(12)	6	82–94	0.553/0.631	0.3503	JN007868
Nau42	F: ACTTGTGTTTGATTAGTTTGAACGC^{NED} R: CTGGAGGAGGAACTCTGTGG	GATT(17)	11	189–233	0.737/0.838	0.0746	JN007872
PlexB							
Nau3	F: TGACCTTTTAGTTTCACCATCC^{NED} R: CAACAAGCCGTCCTCGTC	TTG(9)	11	96–132	0.921/0.894	0.6848	JN007856
Nau15	F: GCGAAATAAGGAAAATGTCCAC^{VIC} R: TTCCGGTAACGATGGAAGTC	CAA(11)	5	183–195	0.684/0.687	0.1543	JN007862
Nau21	F: TTCCTCTGCACCAGTTCCC^{PET} R: TCTGTAGCGAAGCCAGCTC	CCAT(9)	7	227–259	0.579/0.596	0.6698	JN007863
Nau33	F: CCGTTTCACTGTTCCAGGC^{NED} R: GGTCGGAGTCAAACAATCAGG	GT(14)	15	251–283	0.730/0.895	0.0583	JN007867
Nau37	F: GTGGTTCATCTGCTCAATGC$^{6\text{-}FAM}$ R: GCTAAGTCAATTGCATCTTTTCTC	CA(14)	5	72–80	0.526/0.523	0.3630	JN007869
Nau40	F: GAGCAGGGTTGTGAATGCC^{PET} R: ACTATACCTCTTCCCATCGCC	GAGGA(17)	10	119–169	0.737/0.832	0.0530	JN007870
Nau41	F: GATCCGTAGGAAATCCCAGTC$^{6\text{-}FAM}$ R: TCATCCAGCAAGTATGAAGCTG	CAGA(8)	6	248–280	0.658/0.668	0.8569	JN007871
PlexC							
Nau1	F: TGGTTGCGCCATTATTCCAC$^{6\text{-}FAM}$ R: ACGTGGTCCCTCCTCTAAAC	GT(15)	8	187–207	0.711/0.765	0.7998	JN007855
Nau5	F: ATACTGCTGCGAGGTGAGC^{NED} R: AGGCTCTCCGTCTATTACCG	TG(19)	5	170–180	0.368/0.377	0.6697	JN007857
Nau8	F: TCATGTGGACTCGTCTCTGC$^{6\text{-}FAM}$ R: GTTGGACGGTCATCACTGC	CA(9)	3	299–303	0.474/0.467	0.9251	JN007859
Nau13	F: GCACGTACTGAGGTGAAGTTG^{VIC} R: TGTTGTCCCATTCCCAGATG	AAT(10)	5	220–238	0.553/0.599	0.2102	JN007860
Nau25	F: GCACAGCTTCCAAGAGTTCC^{NED} R: CTGCTGAGAAACCACTGACC	GAT(16)	10	298–334	0.842/0.787	0.8534	JN007865
Nau30	F: TGACATTCAGCCAAGGCTTTC^{PET} R: GGCTGGTGGCAAACAAGG	CA(12)	6	163–179	0.500/0.493	0.6232	JN007866

Observed (H_O) and expected (H_E) heterozygosity, Number of alleles (N_A), range of allelic size, and Hardy–Weinberg *P* values (H–W) are based on 38 specimens. Types of fluorescence used to label forward primers are indicated (6-FAM, NED, PET, VIC)

sequencing. That was conducted on 1/8 of a 70 9 75 PicoTiterPlate using the Roche GS FLX (454) system as described elsewhere (Margulies et al. 2005). The command "Design Primers" of MSATCOMMANDER 0.8.2 (Faircloth 2008) was used to screen contigs for microsatellites using the default settings. A total of 171,350 reads were obtained, from which 12,725 sequences contained putative microsatellite motifs. The software MICROFAMILY (Meglécz 2007) and PRIMER 3 (Rozen and Skaletsky 2000) were used to select contigs and design primer sets as described in Carvalho and Beheregaray (2011). Aiming at multiplexing genotyping, primers were manually designed to a specific

size range using PRIMER 3 (Rozen and Skaletsky 2000), depending on enough flanking region of each locus. MULTIPLEX MANAGER (Holleley and Geerts 2009) was used to design the multiplex PCR.

MICROFAMILY recovered 1,021 unique loci, from which the best 23 loci were chosen for polymerase chain reaction (PCR) trials. Amplifications and PCR conditions followed Beheregaray and Sunnucks (2000) using 61 and 52°C treatments. From an initial PCR optimization with the panel of 23 markers, 18 amplified consistently and were selected for further analyzes. Fin clips obtained from 38 SPP individuals were used for microsatellite characterization. These animals originated from a threatened population in Mundoo Drain on Hindmarsh Island, Lake Alexandrina, South Australia (35.5482°S, 138.9156°E) and are all currently kept in captivity.

The PCRs were performed in three separated runs, namely PlexA, PlexB and PlexC (Table 1) and detected on an ABI 3130 Sequencer (Applied Biosystems). The resulting profiles were examined using GENEMAPPER 4.0 (Applied Biosystems) and peaks were scored manually. GENEPOP v4 (Rousset 2008) was used to estimate expected (H_E) and observed (H_O) heterozygosity, number of alleles (N_A), linkage disequilibrium and Hardy–Weinberg proportions. Bonferroni corrections were applied when conducting multiple statistical tests (Rice 1989). The program MICROCHECKER (Van Oosterhout et al. 2004) was used to check for null alleles and scoring errors.

All 18 loci were polymorphic, with an average of 7.6 alleles per locus (ranging from 3 to 15 alleles per locus) and H_O ranging from 0.368 to 0.921 (mean = 0.648). No significant deviation from Hardy–Weinberg equilibrium or linkage disequilibrium was observed. MICROCHECKER detected evidence for null alleles only for Nau33, but with no evidence of scoring errors or large allele dropout. These microsatellite markers will be a valuable resource for the ongoing SPP captive breeding program, and for future restoration of natural populations.

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