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TECHNICAL NOTE

Development of 21 microsatellite markers for the threatened Yarra pygmy perch (*Nannoperca obscura*) through 454 shot-gun pyrosequencing

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Abstract Using a next generation sequencing approach, a set of 21 new microsatellites loci was developed from the threatened freshwater fish Yarra pygmy perch (YPP) *Nannoperca obscura* (Percichthyidae). All markers were successfully genotyped using 28 YPP kept in captivity. These animals represent an entire population rescued from the lower Murray-Darling Basin in Australia before its habitat dried out. As expected due to the critical conservation status of the population, we observed low genetic variation across most loci (mean number of alleles per locus = 2.76; mean heterozygosity = 0.28). No deviations from Hardy–Weinberg equilibrium or linkage disequilibrium were detected and only one locus (Nob30) showed evidence for null alleles. These molecular markers represent important resources for the ongoing YPP captive breeding program, and for upcoming restoration and landscape genetic studies of this species.

Keywords Microsatellite development · Next Generation DNA sequencing · Nannoperca obscura · Freshwater fish

The Yarra pygmy perch (YPP) *Nannoperca obscura* (Percichthyidae) is a small freshwater fish (<100 mm total length) endemic to a small coastal section of mainland southern Australia. YPP is listed as vulnerable (IUCN 2010) and as critically endangered in South Australia (Hammer et al. 2009). The species is include in a "Drought Action Plan" (Hall et al. 2009), which intends to rescue fish populations from the lower Murray-Darling Basing (MDB) that are at very high risk of extinction and implement captive breeding and reintroduction programs and long-term population management in the wild. Molecular markers capable of establishing kin relationships for guiding YPP captive breeding programs are not available for this species.

Next generation sequencing was used to characterize microsatellite markers for YPP. Approximately 10 μg of genomic DNA was extracted from muscle tissue of one specimen of *N. obscura* kept in −80°C and sent to the Australian Genome Research Facility (www.agrf.com.au) to obtain DNA sequences. The DNA was nebulised and ligated with 454 sequencing primers. Each fragment was tagged with a common sequence, which was used to separate the pooled sequences from the whole plate's fragment using post run bioinformatics tools. The sample was then subjected to high throughput DNA sequencing on 1/8 of a 70 9 75 PicoTiterPlate using the Roche GS FLX (454) system as described elsewhere (Margulies et al. 2005). The software MSATCOMMANDER 0.8.2 (Faircloth 2008), MICROFAMILY (Meglécz 2007) and PRIMER 3 (Rozen and Skaletsky 2000) were used to select contigs and design primer sets as previously described (Carvalho and Beheregaray 2010). From a total of 145,071 reads obtained through the NGS, 9,476 had putative microsatellite motifs. MICROFAMILY recovered 858 unique and UnBlastTable loci, from which the best 30 loci (24 di, 3 tri, 2 tetra and 1 penta-nucleotide) were chosen for polymerase chain reaction (PCR) trials. Amplifications and PCR conditions followed (Beheregaray and Sunnucks 2000) and used their 61 to 52°C touch down. After an initial PCR optimization with 30 markers, 21

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Table 1 Primers sequences and characteristics of 21 microsatellite loci isolated from *Nannoperca obscura*

Locus	Primer sequences (5′–3′)	Repeat structure	N_A	Size range (bp)	H_O/H_E	H–W	GenBank accesion no.
PlexA							
Nob2	F:GTTTAGGCGAGAGGCAAGC$^{6\text{-FAM}}$	(TG)17	3	164–170	0.357/0.448	0.1073	JF326246
	R:CCGAGCCTGAACCAAGAGG						
Nob12	F:CGAGGCGAATAACACTGATGG^{NED}	(CA)16	2	166–168	0.107/0.101	1.0000	JF326247
	R:AGTGGCCGCATGTATATTGAAC						
Nob17	F:TGTTTCGTCCTCAGGGAGC^{PET}	(GT)16	3	219–225	0.250/0.226	1.0000	JF326248
	R:TCCTTACGTCCATGTTGCAG						
Nob25	F:GGTCCATCCGCCAGAAGTG^{VIC}	(TG)17	4	190–214	0.296/0.377	0.1206	JF326249
	R:GCCTCAACACTGGAGCAAC						
Nob30	F:AGGTGGACTGCCTGGCTAAC^{VIC}	(CA)14	3	104–108	0.357/0.539	0.0463	JF326250
	R:CTGTCTGCTGGTGGGTCAC						
Nob37	F:TCATGTTGTTGACCCTCCTG^{PET}	(TG)11	1	310	–	–	JF326251
	R:CCCTCTCCTCTCCTGTTTCC						
Nob39	F:ATGTGAAGGACAGGGTGGAC^{VIC}	(TG)10	1	303	–	–	JF326252
	R:AAGATTACAATAAAATTGGTGCTCAG						
PlexB							
Nob11	F:GGGCTCAGATCAGGAAGGG$^{6\text{-FAM}}$	(GT)15	4	174–180	0.429/0.482	0.2823	JF326253
	R:TCCAGACAGGATGCCAAATC						
Nob13	F:ACCTTTAATTAGCTTCAATCTGCC^{PET}	(CA)13	1	170	–	–	JF326254
	R:GGCCTCAGATGTCCTTTATGC						
Nob18	F:GCATTCCCATATGACGCCC^{NED}	(GCA)9	1	169	–	–	JF326255
	R:CACCCATCCCTCGAAGAAC						
Nob21	F:TTGAGAAGAAGGAAAGAAACACC^{VIC}	(CA)18	5	168–176	0.679/0.610	0.3848	JF326256
	R:GGCCTACAACCTGCTCAAAG						
Nob29	F:CCTTTCTGAGGGCTAAACTGC^{VIC}	(AC)11	1	99	–	–	JF326257
	R:GCATGCTCTGGATTTTTGTTG						
Nob40	F:GGCCATTTAGTCTTGAATTTGG^{NED}	(TG)11	3	98–102	0.107/0.103	1.0000	JF326258
	R:CACACAGCAGCACAGGTAGAG						
PlexC							
Nob9	F:CCTCCTCTGACAACTCCCG^{VIC}	(CA)15	6	207–221	0.643/0.672	0.9020	JF326259
	R:AGGTAGGAAGCAGCTGTGG						
Nob16	F:CTGCATCGAGCCAGAACTC$^{6\text{-FAM}}$	(GT)14	2	164–176	0.179/0.163	1.0000	JF326260
	R:CAGCCAGCAGCTCAAATGG						
Nob20	F:TCCGTTAGCCATGATCCCG^{NED}	(CTG)9	1	171	–	–	JF326261
	R:AAGATGCAGTTCCAGTCCG						

Table 1 continued

Locus	Primer sequences (5′–3′)	Repeat structure	N_A	Size range (bp)	H_O/H_E	H–W	GenBank accesion no.
Nob26	F:GAGGGTCCTGAAGTGGAGC^{VIC}	(AGAT)12	6	335–387	0.643/0.786	0.7781	JF326262
	R:GCCGTTGCTTCAAATTACCG						
Nob32	F:GTTCAATGCCCTCTTCCAGC^{PET}	(GT)8	1	315–331	–	–	JF326263
	R:GGTTCTCGCAGCGGTTATC						
Nob34	F:TGTGGTTTGGGACAATAAGCC^{NED}	(CA)11	2	301–303	0.679/0.484	0.0586	JF326264
	R:GGAGCGATGTTGTGAGATCC						
Nob35	F:ACTAGGTATCAATAACAGAGGAATGAC^{6FAM}	(CA)13	5	92–102	0.786/0.668	0.2283	JF326265
	R:CGTAATAATCACAGCCCTGTTACC						
Nob36	F:TCCACGATGTTGCACCTAAC^{VIC}	(GTT)7	2	101–104	0.321/0.357	0.6057	JF326266
	R:GCCTTGGTTCTCCTCGTTTAG						

Number of alleles (N_A), range of allele size, observed (H_O) and expected (H_E) heterozygosity and Hardy–Weinberg P values (H–W) are based on 28 specimens from a critically endangered population (details in text). For multiplex purpose, the forward primers were label using four different fluorescence as indicated (i.e. 6-FAM, NED, PET, VIC)

microsatellite primer sets amplified consistently and were selected for further work. MULTIPLEX MANAGER (Holleley and Geerts 2009) was used to design the multiplex PCR. Three distinct multiplex PCRs were optimized: PlexA (7 loci), PlexB (6 loci) and PlexC (8 loci) (Table 1). A total of 28 YPP from a native but critically endangered population in Steamer Drain on Hindmarsh Island, Lake Alexandrina South Australia (−35.53°S, 138.91°E), was used for microsatellite genotyping. These animals are currently in captivity and are the target of an ongoing conservation breeding and restoration program. They were rescued before their habitat dried out in 2007 and represent the entire local population (Hammer 2008), being among a handful of captive representatives of an Evolutionarily Significant Unit feared extirpated (Hammer et al. 2010).

Amplification products were detected on an ABI 3130 Sequencer (Applied Biosystems) at the SouthPath and Flinders Sequencing Facility. The resulting microsatellite 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 MICRO-CHECKER (Van Oosterhout et al. 2004) was used to check for null alleles and scoring errors.

Out of 21 loci, six were monomorphic and 15 polymorphic for the critically endangered population sample, with an average of 2.76 alleles per locus (2–6 alleles per locus) and generally low heterozygosity (0–0.786; mean = 0.28). The lack of polymorphism observed at six loci (Nob37, 39, 13, 18, 29, 20, 32) is likely due to the critically small size of the rescued population. As expected, after genotyping a few additional individuals ($n = 5$) from other YPP populations that are not under as severe extinction risk, loci Nob13, Nob20 and Nob29 were polymorphic (data not shown). This suggests that these markers and perhaps the other three monomorphic loci reported here would be useful for YPP population genetic studies conducted over large spatial scales.

No significant deviation from Hardy–Weinberg equilibrium or linkage disequilibrium locus-pair/population was observed between loci. Moreover, MICROCHECKER detect significant evidence of null alleles only at locus Nob 30, but with no evidence of scoring errors or large allele dropout. These molecular markers represent important resources for the ongoing YPP captive breeding program, and for landscape genetic studies aimed at assessing the influence of environmental factors on long-term population persistence in the wild.

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