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TECHNICAL NOTE

Microsatellite markers in the endangered Australian northern corroboree frog, *Pseudophryne pengilleyi* (Anura: Myobatrachidae) and amplification in other *Pseudophryne* species

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Abstract Seven microsatellite primer pairs were isolated and characterized in the endangered Australian northern corroboree frog (*Pseudophryne pengilleyi*). All seven were polymorphic (2–14 alleles) and displayed high heterozygosity (0.036–0.964) in 28 sampled individuals. We also tested the microsatellites on two closely related species. Four were polymorphic in the southern corroboree frog (*P. corroboree*) and Bibron's toadlet (*P. bibronii*). These primers will be useful in studies of conservation genetics and mating systems in *Pseudophryne* species.

Keywords Myobatrachidae · Frog · Anuran · Microsatellite primers · Australia

The northern corroboree frog (*Pseudophryne pengilleyi*) is one of the most endangered vertebrates in Australia (Osborne et al. 1999). It is currently confined to two disjunct areas in the Fiery Ranges and Brindabella Ranges in southeastern Australia. It is estimated that the wild population has declined from tens of thousands to under 1000 individuals over the last 30 years (Osborne et al. 1999). A recovery program including husbandry and captive breeding is underway, but levels of genetic diversity in both wild and captive populations are unknown. Here we report on the isolation and characterization of seven polymorphic microsatellite loci that will be used to determine the extent and distribution of genetic diversity in wild populations and also the potential range and magnitude of dispersal between wild breeding sites.

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Microsatellite loci were isolated from the genomic DNA of a male northern corroboree frog DNA. Genomic DNA (gDNA) was extracted using a modified protocol (Sambrook et al. 1989). A partial genomic library was made following a procedure outlined previously (Scott et al. 2001). An estimated 20,000 transformants were recovered and transferred to Hybond-NX nylon membrane (Amersham Biosciences). Using γ-dATP end labelled oligonucleotide mixture, clones were screened for microsatellites of the following repeats; $(ACTC)_7$ $(AACG)_7$ $(ACAG)_7$ $(AGAT)_7$ $(GA)_{15}$ $(GT)_{15}$ $(GTG)_{10}$ $(GTC)_{10}$ $(GTT)_{10}$ $(GTA)_{10}$ $(GCG)_{10}$ $(GAA)_{10}$ $(GAT)_{10}$ $(GAG)_{10}$ $(AGC)_{10}$ $(ATA)_{10}$. A total of 203 recombinant clones were picked, suspended in 50 µl of TE and boiled for 5 min. Each sample was used as template DNA for PCR amplification using universal M13 primers (F: 5′-TGTAAAACGACGGCCAGT-3′, R: 5′-CAGGAAACAGCTATGAC-3′). A total of 182 products of appropriate size were dotted on Hybond-NX membrane and secondarily screened with the same γ-dATP end labelled oligonucleotide mixture as above. Seventy-five positive clones were sequenced using ABI Big Dye terminator 3.1 (Applied Biosystems). Products were visualised on an ABI3100 Capillary Sequencer.

PCR primers for 35 loci were designed using PRIMER 3 (Rozen and Skaletsky 2000). The universal M13 Forward primer sequence (5′-TGTAAAACGACGGGCCAGTA) was added to each forward microsatellite primer. Fourteen microsatellite loci with specific and repeated PCR products were found and used for further polymorphism analysis. Loci were characterized in 24 northern corroboree frog individuals that were sampled (toe clip) from breeding sites within the Northern Brindabella Ranges, NSW, Australia. PCR amplifications were performed with each M13 forward primer labelled with a different fluorescent dye (FAM, VIC, NED or PET, GibcoBRI Life Technologies) in

Table 1 Locus name, repeat motif and forward and reverse primers for fourteen *Pseudophryne pengilleyi* microsatellite loci

Locus	Repeat motif	Primer sequence (5′–3′)	Genbank accession
Pc72	$(GTCT)_{13}(ATCT)_9$	GACTCGCTGCACTAACCACA CATGACATTCCTCTCAGCA	EU223382
Pc109	$(CACT)_7$	CCCTGCTGCCATTACAATTT ATTCAATTGTCCGTTTGG	EU223381
Pc177a	$(TAGA)_{19}$	GGCAAATCTAATGCAAACTGG CAGGTCGACTCTAGAGGAT	EU223380
Pc166a2	$(GATA)_{16}$	ACGACGAGTCGTTAGCACAG CGCTGAAGCCGGTAAGATAG	EU223379
Pc129	$(TCTA)_{17}$	GGTGCCTTCTTGTTGAAGGA CCTCAGGACCACCTACCTGA	EU223378
Pc116	$(AAT)_{16}$	CGTCTCATATGCGTCACGTT CAACTATGCCGGCACATAAA	EU223377
Pc110	$(TG)_{18}$	TGAATGAGCCGAAACTCACA GCTTCTAATCCTGGGTATG	EU223376

Table 2 Characterization of seven microsatellite loci in *Pseudophryne pengilleyi*, *P. corroboree*, and *P. bibroni*. *N*, number of individuals screened; Range, range of fragment length in base pairs; *A*, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; HWE, conformance to Hardy–Weinberg equilibrium: NS, nonsignificant ($P > 0.05$), *, significant probability test ($P < 0.05$); X, amplification failed

	P. pengilleyi					*P. corroboree*					*P. bibroni*				
Locus	*N*	Range	*A*	H_E/H_O	HWE	*N*	Range	*A*	H_E/H_O	HWE	*N*	Range	*A*	H_E/H_O	HWE
Pc72	28	365–415	10	0.864/0.821	NS	39	393–417	7	0.779/0.718	NS	60	361–425	15	0.887/0.633	*
Pc109	28	341–356	6	0.657/0.607	NS	39	347	1	n/a	n/a	59	347–383	10	0.817/0.814	*
Pc177a	28	164–208	11	0.864/0.964	NS	39	164–196	9	0.826/0.897	NS	55	166–218	13	0.845/0.473	*
Pc166a2	28	247–291	10	0.862/0.321	*	39	247–279	9	0.841/0.744	NS	60	227–299	15	0.885/0.783	*
Pc129	28	268–312	11	0.871/0.750	NS	39	268–296	8	0.719/0.769	NS	X	–	–		
Pc116	28	249–288	14	0.814/0.857	NS	X	–	–			X	–	–		
Pc110	28	254–268	2	0.035/0.036	NS	X	–	–			X	–	–		

a total volume of 20 µl. Reactions contained ~20 ng gDNA, 10 µmol each primer, 3 µmol $MgCl_2$, 1 µmol dNTPs, 1× PCR amplification buffer and one unit of PLATINUM® *Taq*-DNA polymerase (GibcoBRI Life Technologies). Reactions were amplified using a step-down PCR protocol on a Corbett PCR machine; initial denaturation was 94°C 5 min, then 94°C 30 s, 70°C 15 s, and 72°C 1.5 min, which was repeated twice. This cycle was subsequently repeated six times, dropping the annealing temperature by 5°C each cycle. The final stage consisted of 94°C 30 s, 35°C 15 s and 72°C 1.5 min, which was repeated 30 times, followed by a final extension step of 72°C 45 min. A maximum of four products of different colour and size were combined into a single well and run with GeneScan 500 LIZ internal size standard on an ABI3100 Capillary Sequencer. Fragment sizes were scored using GENEMAPPER version 3.7 software (Applied Biosystems). Population data were analysed using GenAlEx 6 (Peakall and Smouse 2005). Deviations from Hardy–Weinberg Equilibrium, observed and expected heterozygosities, and linkage disequilibrium were estimated using GENEPOP 3.3 (Raymond and Rousset 1995).

Seven of the fourteen loci screened showed moderate to high polymorphism with two to fourteen alleles (Table 1). Observed and expected heterozygosity ranged from 0.036–0.964 to 0.035–0.871 respectively. One locus (Pc166a2) deviated from Hardy–Weinberg equilibrium ($P < 0.05$). A deficiency of heterozygotes was observed at this locus. The presence of null alleles at this locus was detected using MICROCHECKER version 2.2.3 (Van Oosterhout et al. 2004). Null alleles or population fragmentation within the panel of frogs screened here may explain the observed departure from HWE at this locus. All pairs of loci exhibited linkage equilibrium ($P > 0.05$).

All seven loci were screened in 39 southern corroboree frogs (*P. corroboree*) and 60 Bribron's toadlets (*P. bibroni*) under the same PCR conditions (Table 2). Four loci were successfully amplified and polymorphic in each of these species. These microsatellites will provide a valuable tool for a range of conservation, ecological and evolutionary studies in the northern corroboree frog and are likely to be useful in other *Pseudophryne* species.

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