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PRIMER NOTE

Isolation and characterization of polymorphic microsatellite loci for parentage analysis in a rhacophorid tree frog (*Chirixalus eiffingeri*) with unusual parental care

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Abstract

***Chirixalus eiffingeri* is an arboreal breeding rhacophorid frog with unique parental care behaviours. We developed 11 polymorphic microsatellites as genetic makers for parentage analysis to resolve the ecology of parental care. The numbers of alleles per locus ranged from two to 17. The observed and expected heterozygosities averaged 0.433 and 0.656, respectively. Total exclusionary probability of these loci is 0.984 when no parental genotypes are known, and is 0.999 when one of the parental genotypes is known. The results indicate that the 11 markers should provide sufficient resolution for inferring genetic parentage in *C. eiffingeri*.**

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Genetic studies of parentage have given revolutionary insights into evolutionary ecology of reproductive strategy and parental care behaviour in animals (Avise *et al*. 2002). *Chirixalus eiffingeri* is a rhacophorid tree frog that breeds in arboreal pools or phytotelmata with unique parental care behaviours. The males attend to the clutches during the embryonic period and females provide the obligatorily oophagous tadpoles in the nest by laying unfertilized eggs (Kam *et al*. 2000). The kinship between the care providers and the tadpoles in the nest, because of the difficulties in direct observation, is still a mystery, which becomes an obstacle to definitively reveal the strategy of parental care. We developed microsatellite loci as genetic makers to analyse parenthood of clutches in *C. eiffingeri*. Combining with behavioural observations, we anticipate resolving the detail in the parental care behaviours of this tree frog.

Genomic DNA for microsatellite isolation was prepared from a single adult according to the standard phenol–chloroform extraction procedures in Sambrook *et al*. (1989). We attempted two protocols to isolate microsatellite loci: enrichment and traditional methods (Sambrook *et al*. 1989). Originally, we attempted an enrichment method following Hamilton's protocol (available at http://bioserver.georgetown.edu/faculty/hamilton) and obtained 81 loci with sufficient flanking regions to design primers for amplifications. The online program PRIMER version 3.0 (Rozen & Skaletsky 2000) was used to design primers. However, we found that 62 microsatellite sequences isolated from this method shared a partial sequence with another locus, and all but four primer pairs developed from these sequences failed to amplify.

Consequently, we switched to constructing and screening the partial genomic libraries. Genomic DNA was digested with *Sau*3AI and fractioned on a 1% agarose gel. DNA of 300–1200 bp was eluted, purified with GFX Band Purification kit (Amersham) and ligated into plasmids PUC118/*Bam*HI/BAP (TaKaRa) according to the manufacturer's protocols. Ligated plasmids were transformed into the competent ECOS 101 cells (Yeastern Biotech). Recombinant clones containing inserts were transferred to Hybond-N$^+$ nylon membranes (Amersham), which were hybridized to a set of oligonucleotide probes, including $(AC)_{15}$, $(AT)_{15}$, $(AG)_{15}$, $(AAT)_{10}$, $(AAG)_{10}$, $(GATA)_6$, and $TC(TCC)_5$. Probes were labelled with digoxigenin (DIG) Oligonucleotide 3′-End Labeling Kit (Roche). Hybridization was performed at 50–53 °C for 16 h in a standard hybridization buffer, consisting of 5 × SSC, 0.1%

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Table 1 Characteristics of 11 polymorphic microsatellite loci in *Chirixalux eiffingeri*

Locus (GenBank Accession no.)	Repeat motif	Primer sequences (5′–3′)*	*T*a (°C)	*N*	Allele size range (bp)	No. of alleles	H_O	H_E	HWE *P* value	PE_1	PE_2
CEr00112 (AY853675)	i$(TG)_{12}$	GCAAAGAGGAGGCAGCAAAT GCTTGGCAAAACAGGTTTACTT^{HEX}	58.0	20	81–91	9	0.550	0.688	0.127	0.282	0.466
CEr00006 (AY853676)	i$(GA)_{88}$	ACATCCATGCTCATGCTCTG CCAATGACAAAGTTGGGGTT^{FAM}	60.0	16	267–297	9	0.250	0.700	0.000	0.295	0.484
CEr00009 (AY853677)	i$(GA)_{56}$	TCTGCATCCAAGTACAGGCTT^{TAMRA} GCCATGATGACCAACACCTA	56.5	19	224–290	12	0.526	0.836	0.000	0.483	0.655
CEr00118 (AY853678)	i$(GA)_{27}$	CCTGGTGTGAGGGGTTTTTA AACACATACCGTGTCTTTCGC^{HEX}	56.5	14	135–189	4	0.286	0.537	0.033	0.139	0.277
CEd02641 (AY853679)	i$(AC)_9$,$(CT)_{24}$	GTGTATCGCTTCCCCCTTTC^{TAMRA} TCCAGATTCTAGCGGCTCTG	56.0	16	258–280	6	0.500	0.734	0.126	0.314	0.497
CEd02747 (AY853680)	i$(TCC)_{11}$	AGTGATGCCCGTAACCTGAT^{FAM} TCAGGCCAGTCATTCACAAG	56.0	21	203–212	2	0.143	0.136	1.000	0.009	0.062
CEd06009 (AY853681)	$(AC)_8$	AAGTTAACCCTTGCAATGTCG^{HEX} TGCCTTGTTCTCCCCTAGAC	58.0	17	88–96	6	0.294	0.569	0.001	0.177	0.355
CEd08767 (AY853682)	i$(GA)_5$,$(GT)_6$,$(GT)_8$	ATATCAGTGCCCCAGTGACG^{HEX} GCGGGAGATTGAAGATGCT	56.0	20	243–251	2	0.800	0.492	0.005	0.115	0.182
CEd09258 (AY853683)	i$(GA)_{92}$	GGCTCTCACATCACCAAAA^{TAMRA} CTTACCTGTATGGGCCAGTT	52.0	5	364–370	4	0.000	0.800	0.003	0.298	0.472
CEd12365 (AY853684)	$(CT)_{24}$	GGCCTCTCCACACAAGTT^{HEX} CAGTGCCAGCCCTCAT	53.0	24	88–120	9	0.875	0.816	0.167	0.439	0.616
CEd15688 (AY853685)	$(GT)_{16}$	GAAAACCTGCAGCCAAACC^{TAMRA} TTGTGTGCAATGTGAAGTCAAC	60.0	24	82–128	17	0.542	0.902	0.000	0.625	0.769
Overall							0.433†	0.656†		0.984	0.999

* primer labelled with fluorescent dye: FAM, HEX or TAMRA; and †, average across loci.

i, interrupted repeat motif; H_O, observed heterozygosity; H_E, expected heterozygosity; HWE, Hardy–Weinberg equilibrium; PE_1 and PE_2, exclusion probabilities without information of parents and with only one known parent, respectively; T_a, annealing temperature; and *N*, sample size.

sodium-N-lauroylsarcosine, 0.02% SDS, and 1% blocking reagent (Roche). The membranes were washed twice, each for 5 min at 50 °C with a solution of 2 × SSC, 0.1% SDS, and then twice, each for 15 min at 60 °C with a solution of 0.1 × SSC, 0.1% SDS. Chemiluminescent detection was performed with DIG Luminescent Detection Kit (Roche). A total of 185 positive clones was sequenced using a MegaBACE 500 automated sequencer. Twenty-nine clones contained repeat motifs with repeats numbered more than five and contained sufficient flanking region to design primers. The approximate proportion of positive clones number to total number of screened clones obtained by this method was 1.1%, which was lower than the average of 2–3% in many taxa (Zane *et al*. 2002).

Polymerase chain reaction (PCR) conditions were optimized for each primer pair. Each reaction mixture (10 μL) contained 50–100 ng template DNA, 0.5 unit of *Taq* DNA polymerase (Promega), 0.9–1.5 mM Mg^{++}, 0.2 mM of each dNTP, 10 × Buffer (Promega: 10 mM Tris-HCl (pH 9.0), 50 mM KCl, and 0.1% Triton X-100), and 0.2 μM of each primer, with the forward or reverse primer being end-labelled with fluorescent dye (Table 1). Amplification was carried out by the thermal profile: 95 °C for 7 min, followed by 40 cycles at 95 °C for 30 s, optimal annealing temperature (Table 1) for 30 s, 72 °C for 30 s, and a final extension step at 72 °C for 30 min. PCR products were run on linear polyacrylamide (LPA) gels with a MegaBACE 500 automated sequencer. ET-400-R Size Standard (Amersham) was used as size marker to determine the allele sizes. Individual genotypes were determined and the individuals with ambiguous genotypes were amplified and scored at least twice to determine the allele sizes.

Eleven microsatellite loci, four from the enrichment method (*Cer00006*, *00009*, *00112*, and *00118*) and seven from the traditional method, were polymorphic among *C. eiffingeri* individuals from a bamboo thicket of *c*. 10 ha (Table 1). The number of alleles averaged 7.27 (2–17). The observed and expected heterozygosities averaged 0.433 and 0.656, respectively (Table 1). Hardy–Weinberg expectation for each locus was tested with the program GENEPOP version 3.3 (Raymond & Rousset 1995). The observed genotypes deviated from Hardy–Weinberg expectation at six of the 11 loci ($P < 0.01$) (Table 1), five resulting from heterozygote deficiency and one (*Ced08767*) from heterozygote excess. The deviation from Hardy–Weinberg expectation was somewhat expected because our frogs were from small patchy habitats and therefore a certain degree of inbreeding cannot be avoided. However, the small sample size and existence of null alleles cannot be ruled out either.

Linkage analysis revealed no significant evidence of linkage disequilibrium ($P > 0.01$), indicating that each locus could be viewed as an independent genetic marker (Raymond & Rousset 1995). The power of these loci to resolve parentage was estimated by the total exclusion probability, PE_1 and PE_2 (Table 1). PE_1 was the combined power of the set of loci to exclude a random unrelated candidate parent from parentage, when only the genotype of the offspring is given, and PE_2 was the power when the genotypes of the offspring and of one of the parents are given (Marshall *et al*. 1998). Both of PE_1 and PE_2 were high, 0.984 and 0.999, respectively, indicating that these 11 loci can provide powerful tools to infer the parenthood of clutches in *C. eiffingeri*.

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