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 2 to 17. The observed and expected heterozygosity 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 to infer the genetic parentage in *C. eiffingeri*.

Genetic studies of parentage have given revolutionary insights into evolutionary ecology of reproductive strategy and parental care behavior(s) in animals (Avise *et al.* 2002, Griffith *et al.* 2002, Ross 2001). *Chirixalus eiffingeri* is a rhacophorid tree frog that breeds in arboreal pools or phytotelmata with unique parental care behaviours (Kam *et al.* 1996). The males attend the clutches during the embryonic period and females provision 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, due to the difficulties in direct observation, is still a mystery, which becomes an obstacle to definitively reveal the strategy of parental care (Kam *et al.* 2000). We developed microsatellite loci as genetic makers to analyze parenthood of clutches in *C. eiffingeri*. Combining with behavioral observations, we hope to resolve the detail in the parental care behaviors in this tree frog.

Genomic DNA for microsatellite isolation was prepared from a single adult according to standard phenol-chloroform extraction procedures in Sambrook *et al.*(1989). We attempted two protocols to isolate microsatellite loci, enrichment (Hamilton *et al.* 1999) 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 on-line program Primer 3.0 (Rozen and 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 4 primer pairs developed from these sequences failed to amplify, as in Koblizkova et al.(1998).

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 size range 300-1200 bp was eluted, purified with GFX^{TM} Band

Purification kit (Amersham) and ligated into plasmids PUC118/BamHI/BAP (TaKaRa) according to 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 labeled with Digoxigenin (DIG) Oligonucleotide 3'-End Labeling Kit (Roche). Hybridization was performed at 50-53℃ for 16 hrs in a standard hybridization buffer, consisting of 5X SSC, 0.1% Sodium N-lauroylsarcosine, 0.02% SDS, and 1% Blocking Reagent (Roche). The membranes were washed twice, each for 5 min at 50℃ with a solution of 2X SSC, 0.1% SDS, and then twice, each for 15 min at 60℃ with a solution of 0.1X 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 number more than 5 and contained sufficient flanking region to design primers. The approximately 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 PCR reaction mixture (10 μL) contains 50-100 ng template DNA, 0.5 units of *Taq* DNA polymerase (Promega), 0.9-1.5 mM Mg^{++}, 0.2 mM of each dNTP, 10X Buffer (Promega: 10 mM Tris-HCl (pH9.0), 50 mM KCl, and 0.1% Triton X-100), and 0.2 μM of each primer, with the forward or reverse primer being end-labeled with fluorescent dye (FAM, HEX or TAMRA). Amplification was carried out by the thermal profile: 95℃ for 7 min, followed by 40 cycles of 95℃ for 30s, optimal annealing temperature (Table 1) for 30s, 72℃ for 30s, and a final extension step at 72

℃ for 30 min. PCR products were run on linear polyacrylamide (LPA) gels with a MegaBACE 500 automated sequencer. ET-400 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.

Finally, eleven microsatellite loci, 4 (Cer00006, 00009,00112, 00118) from the enrichment method and 7 from the traditional method, were polymorphic among *C. eiffingeri* individuals from a bamboo thicket of ca. 10 ha. (Table 1). The number of alleles averaged 7.27 (2 to 17). The observed and expected heterozygosity 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 and Rousset 1995). The observed genotypes deviated from Hardy-Weinberg expectation (HWE) at six out of the eleven loci ($P < 0.01$) (Table 1), five resulting from heterozygote deficiency and one (Ced08767) from heterozygote excess. The deviation from HWE was somewhat expected because our frogs were from a small patchy habitats and, therefore, certain degree of inbreeding can not be avoided. However, the small sample size and existence of null alleles can not 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 and Rousset 1995). High degree of polymorphic information content (PIC) was found in each locus, and all loci averaged 0.597 (Table 1). The power of these loci to resolve parentage was estimated by the total exclusionary probability, PE_1 and PE_2 (Table 1). PE_1 was the combined power of the set of loci to exclude a randomly-selected unrelated candidate parent from parentage, given only the genotype of the offspring, and PE2 was the power when given the genotype of the offspring and of one of the parents (Marshall *et al.* 1998). Both of PE_1 and PE_2 were

high, 0.984 and 0.999, indicating that these eleven loci can provide powerful tools to infer the parenthood of clutches in *C. eiffingeri*.

Acknowledgments

This study was supported by National Science Council grants to YCK (92-2311-B-029-003) and to HTY (92-2317-B-002-011). We thank the staff of the Experimental Forest of the National Taiwan University at Chitou for providing accommodation and permitting us to collect specimens. We especially appreciated Pei-Yi Lin's assistance in sample collection.

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Table 1 Characteristics of 11 polymorphic microsatellite loci in *Chirixalux eiffingeri*. i, imperfect repeat motif; H_o, observed heterozygotsity; H_e, expected heterozygotsity; HWE, Hardy-Weinberg equilibrium; PIC, polymorphic information content; PE_1 and PE_2, exclusion probabilities without information of parents and with one known parent, respectively; T_a: annealing temperature; N: sample size.

Locus*	Repeat motif	Primer sequences(5'-3')	Ta (°C)	N	Allele Size range (bp)	No. of alleles	H_o	H_e	HWE P value	PIC	PE_1	PE_2
CEr00112	$i(AC)_{13}$	GCAAAGAGGAGGCAGCAAAT GCTTGGCAAAACAGGTTTACTT	58.0	20	81-91	9	0.550	0.688	0.127	0.641	0.282	0.466
CEr00006	$(CT)_{78}$	ACATCCATGCTCATGCTCTG CCAATGACAAAGTTGGGGTT	60.0	16	267-297	9	0.250	0.700	0.000	0.654	0.295	0.484
CEr00009	$i(CT)_{51}$	TCTGCATCCAAGTACAGGCTT GCCATGATGACCAACACCTA	56.5	19	224-290	12	0.526	0.836	0.000	0.796	0.483	0.655
CEr00118	$i(CT)_{27}$	CCTGGTGTGAGGGGTTTTTA AACACATACCGTGTCTTTCGC	56.5	14	135-189	4	0.286	0.537	0.033	0.460	0.139	0.277
CEd02641	$i(AC)_9,(TC)_{24},(TG)_{14}$	GTGTATCGCTTCCCCCTTTC TCCAGATTCTAGCGGCTCTG	56.0	16	258-280	6	0.500	0.734	0.126	0.677	0.314	0.497
CEd02747	$i(TCC)_9$	AGTGATGCCCGTAACCTGAT TCAGGCCAGTCATTCACAAG	56.0	21	203,212	2	0.143	0.136	1.000	0.124	0.009	0.062
CEd06009	$(AC)_8$	AAGTTAACCCTTGCAATGTCG TGCCTTGTTCTCCCCTAGAC	58.0	17	88-96	6	0.294	0.569	0.001	0.527	0.177	0.355
CEd08767	$iCT)_5,(CA)_6,(CA)_8$	ATATCAGTGCCCCAGTGACG GCGGGAGATTGAAGATGCT	56.0	20	243,251	2	0.800	0.492	0.005	0.365	0.115	0.182
CEd09258	$i(GA)_{94}$	GGCTCTCACATCACCAAAA CTTACCTGTATGGGCCAGTT	52.0	5	364-370	4	0.000	0.800	0.003	0.672	0.298	0.472
CEd12365	$(CT)_{24}$	GGCCTCTCCACACAAGTT CAGTGCCAGCCCTCAT	53.0	24	88-120	9	0.875	0.816	0.167	0.773	0.439	0.616
CEd15688	$(GT)_{16}$	GAAAACCTGCAGCCAAACC TTGTGTGCAATGTGAAGTCAAC	60.0	24	82-128	17	0.542	0.902	0.000	0.873	0.625	0.769
Overall							0.433#	0.656#		0.597#	0.984	0.999

- GenBank Accession numbers are in the following order: --------and .
- # Average across loci