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TECHNICAL NOTE

The development and analysis of twenty-one microsatellite loci for three species of Amazonian poison frogs

Jason L. Brown · Mathieu Chouteau · Travis Glenn · Kyle Summers

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Abstract We describe polymerase chain reaction (PCR) primers and conditions to amplify one poly A, two trinucleotide and 18 tetranucleotide microsatellite loci isolated from two Peruvian poison frogs (*Ranitomeya imitator* and *R. variabilis*). All the primers developed for *R. variabilis* work on a closely related species, *R. ventrimaculata*. These primers yielded a high number of alleles (mean 14.4) and showed high levels of heterozygosity (mean 0.76 per locus).

Keywords *Dendrobates* · Microsatellites · Primers · *Ranitomeya imitator* · *R. variabilis* · *R. ventrimaculata* · Trinucleotide · Tetranucleotide

South American poison frogs of the genus *Ranitomeya* exhibit a wide array of parental types from single parental care (i.e. male parental care) to biparental care involving substantial effort from both parents and the provisioning of trophic eggs (Summers and McKeon 2004). The ecological and social factors contributing to the evolution of these parental care types are largely unknown. By characterizing the mating system of the different parental care strategies using microsatellites, we will be better able to understand how complicated parental care behaviors (i.e. biparental care) have evolved and are maintained. Here we present primers that amplify 21 microsatellite loci designed for two species of Peruvian poison frogs: *Ranitomeya imitator* and *R. variabilis*.

DNA was extracted from toe clips using a Qiagen DNeasy tissue kit. Extracted DNA was enriched for $(AG)_{12}$, $(TG)_{12}$, $(AAC)_6$, $(AAG)_8$, $(AAT)_{12}$, $(ACT)_{12}$, $(ATC)_8$, $(AAAT)_8$, $(AACT)_8$, $(AAGT)_8$, $(ACAT)_8$, $(AGAT)_8$ following a modified protocol of Glenn and Schable (2005, 2009). In brief, the DNA was digested with RsaI and BstU I, ligated to SuperSNX linkers, hybridized to biotinylated microsatellite oligonucleotides, captured on Dynabeads and Magnetic Particle Concentrator, unwanted DNA was washed away, captured DNA was recovered by polymerase chain reactions (PCR) using the SuperSNX24 Forward primer (5′GTTTAAGGCCTAGCTAGCAGAATC). To ligate the enriched DNA into a cloning vector we used a TOPO TA Cloning© Kit containing pCR© 2.1-TOPO© with TOP 10 cells and screened for inserts using the β-galactosidase gene. Positive colonies were amplified using M13 forward and reverse primers. PCR products of 500–1,000 base pairs (bp) were sequenced using BigDye Terminator v 3.1 ABI (Applied Biological Systems) chemistry and an ABI 377-96 sequencer.

Primers were designed from the flanking sequences of the unique clones to amplify the repetitive elements. PCR conditions were optimized for each primer pair and each locus was evaluated for polymorphism and heterozygosity. Each primer pair was tested on the species they were designed for. *Ranitomeya imitator* and *R. variabilis* were evaluated from three separate populations from North-Central Peru (numbers correspond to the number of each species evaluated at each population, respectively):

J. L. Brown (✉) · K. Summers
Department of Biology, East Carolina University,
East Fifth St, Greenville, NC 27858, USA
e-mail: jasonleebrown@gmail.com

M. Chouteau
Département de Sciences Biologiques, Université de Montréal,
C.P. 6128, Succ. Centre-Ville, Montréal H3C 3J7, Canada

T. Glenn
College of Public Health, University of Georgia,
Athens, GA 30602, Greece

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Table 1 Microsatellite loci for *R. imitator* (Rimi) and *R. variabilis* (Rvar) including: primer sequence, repeat motif, annealing temperature (T_a), observed size range of alleles in basepairs (bp), number of alleles, average observed population heterozygosity (H_O), average expected population heterozygosity (H_E), and GenBank Accession No.

Locus	Primer sequences (5′–3′)	Repeat motif	T_a (°C)	Range of products (bp)	No. of alleles	H_O	H_E	GenBank Accession No.
RvarA09	CTGCATGAATCAGTTAACTGCC CGGGATTGTGACCGGTGATTTC	AGAT	58	98–186	13	0.77	0.86	GQ273464
RvarB01	ATGGAACTATTGAGTGCTGCC AAAAGGGGCCACACTGGATCC	AGAT	52	112–150	10	0.70	0.89	GQ273462
RvarC01	GAAAGAAGTGGGGCTGTCTG TCACCATCAATCCTAGGGAAA	GAT	58	231	1	–	–	GQ273465
RvarD01+	GAAAAAGCATTACAGCTCATCAA GCCGAAACATTGCCATAAA	TCTA	58	180–272	14	0.89	0.76	GQ273466
RvarD04+	TCATCATGAACTGCGAGTGA TGATGGCACATTCACACTTG	TCA, TCT	58	183–246	3	0.52	0.69	GQ273463
RvarE04	ACAAATGAGGCCATGTGTCA CAAGCCGTTTTCCCATTAGA	Poly A and T rich	58	414–421	2	0.43	0.40	GQ273467
RvarF01	TTTCCGTACCCACTGTATATTTATCTC GGTAATTGGCACTGAGAGCTG	TCTA	58	232–281	13	0.84	0.89	GQ273468
RvarF08	CATCATGAAAGCTGACAGATCG GATTGAATAGATAATGGATGGC	TCTA	52	174–374	30	0.92	0.96	GQ273469
RvarG12	CAGTTCACATTGTGATTCAAC ATTGTATGGAGATGCTGGAC	TCTA	52	99–225	22	0.92	0.95	GQ273470
RvarA08	ATTGTTGGGGGATTTTCTATC TGTAAAAGTATGTAAATTGTGT	TCTA	52	94–118	8	0.70	0.90	GQ273461
RimiA06	CTTAATTGAGTAATTGTCAAG GCTTTTGGATAATCAGTATCG	TCTA	52	98–158	16	0.89	0.90	GQ273471
RimiA07	TTCTTAATTGAGTAATTGTC TCCTTAATATACCAGTTAAGC	CATA	52	118–178	16	0.86	0.89	GQ273472
RimiB01	TAATTGTATTTGTCACTGAC ATTTTTGCGGGCATATTCGG	TCTA	52	240–288	13	0.73	0.83	GQ273473
RimiB02a*	TCGAGATTTTAGCAGTGTTTTATCC CATGAAAACCATATTTCGGACA	AGAT	58	116–207	26	0.92	0.94	GQ273474
RimiB02b*	TAGCAGTGTTTTATCCACTTAC AAAACCATATTTCGGACAATG	AGAT	52	104–204	26	0.92	0.94	GQ273474
RimiB07a	CACCGTGCACTGGTTATCTATC GTTTCGCTCAACCCTAGTGC	TCTA	58	268–316	17	0.83	0.85	GQ273475
RimiB07b	CACCGTGCACTGGTTATCTATC CGTTCCGAGTGTCATGCG	TCTA	58	188–252	17	0.83	0.85	GQ273475
RimiB11	GTAAGTCCGTATATGTCGATG CCTGAGAGTGTAATGGATAGAC	TCTA	52	180–236	15	0.86	0.90	GQ273476
RimiC05a	CGTTTCGCTCAACCCTAGTC ATGGAGGCAATCCACAAATC	TCTA	58	208–331	18	0.70	0.74	GQ273477
RimiC05b	TAGTCATTAGTGTGTGTTTC CTTGGCCAGATATGGAGGC	TCTA	52	204–280	18	0.70	0.74	GQ273477
RimiE02a	GCAGAGGGGATTAGGGACTC TGGGTAGCTGTGTTCCATGA	TCTA	58	312–395	23	0.89	0.64	GQ273479
RimiE02b	TGGGAAAACCTGGTCACAAG GAGTTCACATGGGTGGACC	TCTA	52	208–308	23	0.89	0.64	GQ273479
RimiE03*	GTTCAGGCTCAGCAGCTCTT GGTGATGGAGAGGTAATAGATTGG	TCTA	58	416–560	20	0.89	0.88	GQ273480

Table 1 continued

Locus	Primer sequences (5′–3′)	Repeat motif	T_a (°C)	Range of products (bp)	No. of alleles	H_O	H_E	GenBank Accession No.
RimiD04	CTCCAAAACACACCCCAAAC AGAGGTGCTGCCCTTTTGTA	AGAT	58	210–228	3	0.05	0.05	GQ273478
RimiF06	TTGATATTCTGAGGTATG GTAGCTTATGGCAGCTACG	TCTA	52	172–252	21	0.89	0.91	GQ273481

Note: + and * are loci that are linked. Loci ending in "a" and "b" (i.e. RimiB02a and RimiB02b) are primer pairs that amplify the same locus

Cainarachi Valley (45, 61), Chazuta (65, 2), and Pongo de Cainarachi (151, 26). The forward primer of each pair was labeled at its 5′ end with one of the four following fluorescent labels for G5 sequencers (VIC, 6-FAM, NED, PET). The conditions for PCR were 10 µl 10× Buffer (Invitrogen), 20 mM $MgCl^2$, 4 mM dNTP (Promega), 0.05 u of Taq polymerase (Invitrogen), and 10 µmol of both primers in a final reaction volume of 20 µl. Between 25–75 nanograms of DNA were used as a template. We also performed reactions using a final of volume 10 µl (using half the previously mentioned amounts). Two PCR amplification temperature profiles were used: (1) 94°C for 3 min, 32 cycles of 92°C for 30 s, T_a for 30 s, 72°C for 2 min and a final extension of 72°C for 5 min or, alternatively, (2) 92°C for 10 s, 44 cycles of 92°C for 10 s, T_a for 30 s, 68°C for 30 s and a final extension of 68°C for 5 min. PCR products were diluted (1:10) and then 1–2 µl of each dilution were multiplexed with other loci (up to 4). We added 10 µl formamide and 0.1 ul size standard (ABI Genescan 600 Liz) to the total volume of multiplexed mixture. Products were resolved on an ABI 3130 Genetic Analyzer capillary DNA sequencer and were scored and binned using ABI Genemapper 4.0 software.

Twenty polymorphic loci were found. The number of alleles ranged from 2 to 30 per locus (mean 15.5). All primers designed for *R. variabilis* function on *R. ventrimaculata* individuals from San Martin, Peru. Observed and expected heterozygosities and estimates of linkage disequilibrium between loci were calculated using GenePop 4.0 (Rousset 2008). Observed heterozygosity ranged from 0.05 to 0.92 (mean = 0.76). Two of the 21 loci, RimiE02 and RvarD01, were determined to be significantly ($P < 0.05$) out of Hardy–Weinberg equilibrium. For both loci this could be due to heterozygote deficiency ($P < 0.001$) and may indicate the presence of null alleles at these loci. We detected evidence of linkage between RvarD01 and RvarD04 ($P = 0.031$) and RimB02 and RimiE03 ($P = 0.031$). No other loci appear to be in linkage disequilibrium. For four *R. imitator* loci (B02, B07, E02, C05), we present two sets of primer pairs that amplify the same locus, but yield different sized products (Table 1). To check our results, we used Micro-Checker v2.2.3 (van Oosterhout et al. 2004) and found no evidence for scoring errors.

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