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PRIMER NOTE

Polymerase chain reaction primers for polymorphic microsatellite loci from the túngara frog *Physalaemus pustulosus*

H. PRÖHL,*† R. M. M. ADAMS,* U. MUELLER,* S. RAND† and M. J. RYAN*
*Section of Integrative Biology, University of Texas at Austin, Austin, TX 78712, USA; †Smithsonian Tropical Research Institute, Balboa, Panama

Abstract

We developed eight PCR–primer pairs of polymorphic microsatellite loci for the túngara frog *Physalaemus pustulosus*. Genomic libraries were enriched for one of four microsatellite repeat sequences (CA_n, GA_n, ATG_n and $TAGA_n$). Following characterization of microsatellite loci by sequencing, primers were designed and PCR conditions optimized. Microsatellite PCR-amplification was tested in 37 frogs from 8 populations in Costa Rica and Panama. Primer sequences, PCR conditions, allelelic diversities and observed as well as expected heterozygosities in the screened populations are described.

Keywords: anuran, genotyping, microsatellite marker, PCR, *Physalaemus pustulosus*, primer

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The túngara frog, *Physalaemus pustulosus* (family Leptodactylidae) is an abundant species distributed through much of Central America and northern South America (Colombia, Venezuela, Trinidad). Because of its local abundance, interesting communication system and behaviour (complex call, lek mating, foam nest construction), and broad distribution, the túngara frog has been become a model system to test hypotheses of sexual selection (Ryan 1985), mating signal variation and evolution (Ryan & Rand 1995; Ryan *et al*. 1996), population dynamics (Marsh *et al*. 1999), and phylogenetic history of the *P. pustulosus* group (Cannatella *et al*. 1998). To test biogeographical and population genetic hypotheses of behavioural evolution in *P. pustulosus*, we developed primer pairs for PCR-amplification of eight polymorphic microsatellite loci. To date, microsatellite primers have been developed for only a few anuran amphibians (e.g. Arens *et al*. 2000; Rowe & Beebee 2001), and applications in population genetic, behavioural and conservation biological studies are still lacking for frogs (but see Scribner *et al*. 1994).

A túngara frog genomic library was constructed by Genetic Identification Services (GIS, Chatsworth, CA) from pooled DNA extracted from five Panamanian frogs collected near Panama City, Republic of Panama. Pooled DNA was digested with the restriction enzyme *Hin*dIII, then DNA fragments from 350 to 650 bp were selected. This size-selected library was split fourfold, and each of the four libraries was enriched for one specific microsatellite repeat, $(CA)_n$, $(GA)_n$, $(ATG)_n$ or $(TAGA)_n$. Enriched DNA was ligated into the plasmid pUC19 and plasmids were propagated in the *Escherichia coli* strain DH5α, then suspended in 20% glycerol stock for cryostorage.

To isolate colonies for sequencing, cells from the glycerol stock were spread on X-gal/IPTG/Ampicillin plates. After incubation, cells were selected from the resulting colonies and heated to 100 °C for 10 min in PCR tubes containing 10 μL PCR master mix to lyse cells and release the plasmids. Per reaction, the master mix contained 1 μL 10× PCR Buffer (Promega), 30 nmol $MgCl_2$, 3 nmol of each dNTP, 15 pmol M-13 cloning site primers and ddsH_2O to final volume of 10 μL. Five microlitres *Taq* polymerase solution [0.075 μL 5 U *Taq* DNA polymerase in storage buffer B (Promega), 0.5 μL 10× PCR buffer, 4.425 μL H_2O] was then added to amplify the region containing the *P. pustulosus* insert on a PTC-200 Cycler. Thermal cycling began with denaturing at 94 °C for 1 min, followed by 35 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 57 °C, elongation for 30 s at 72 °C, followed by a final extension at 72 °C for 2 min. PCR products were electrophoresed on 2%

Correspondence: H. Pröhl. Fax: +1 512 471 3878; E-mail: heike@biosci.utexas.edu, hproehl@hotmail.com

agarose gels to confirm single-product amplification and expected fragment size (350–650 bp).

From 348 PCR products examined [147, 95, 51 and 55 for the $(CA)_n$, $(GA)_n$, $(ATG)_n$ and $(TAGA)_n$ libraries, respectively], 107 strongly amplifying loci were selected for sequencing [31, 34, 26 and 16 for the $(CA)_n$, $(GA)_n$, $(ATG)_n$, and $(TAGA)_n$ libraries, respectively]. PCR products were purified using the QIA Quick Spin Purification Procedure (Quiagen) and then amplified by Cycle Sequencing using the DNA Sequencing Kit [Big Dye™ Terminator Cycle Sequencing Ready Reaction with AmpliTaq® DNA polymerase, 2 μL per 10 μL reaction (Applied Biosystems, Perkin-Elmer)] and forward primer (3.2 pmol per l0 μL reaction). Between 10–100 ng of DNA were used per cycle sequencing reaction. Amplifications were performed under the following conditions: 25 cycles for 10 s at 96 °C, for 5 s at 50 °C, for 4 min at 60 °C. Reactions were cleaned with Centri-Sep Spin Columns (Princeton Separations) and Sephadex Solution (Sigma). Sequence information was generated on an ABI 3100 Genetic Analyser (Applied Biosystems, Perkin-Elmer) and analysed with SEQUENCING ANALYSIS SOFTWARE Version 3.6.1. Forty-three of the 107 inserts [20, 3, 14 and 6 for the $(CA)_n$, $(GA)_n$, $(ATG)_n$ and $(TAGA)_n$ libraries, respectively] contained microsatellite sequences with at least 10 uninterrupted repeats of the targeted core motif. Primer sets were designed for 21 loci [8, 3, 8 and 2 for the $(CA)_n$, $(GA)_n$, $(ATG)_n$ and $(TAGA)_n$ libraries, respectively] using PRIMER3 software (Rozen & Skaletsky 1996).

We genotyped túngara frogs of three Panamanian (Cañaza, Galique, Bugaba) and five Costa Rican (Golfito, Cortéz, La Junta, Filadelfia, Santa Rosa) populations for analyses of heterozygosities and allele diversities per locus. DNA was extracted from toe tissue of frogs collected in 2000 and stored in 20% DMSO/0.25 M EDTA buffer (pH = 8.0) until extraction with the DNeasy Tissue Kit (Quiagen). Extraction yielded between 0.3 μg and 2 μg genomic DNA per toe. Ten primers amplified loci in the size range (150–300 bp) expected from the original (cloned) microsatellite sequence. Annealing temperature was optimized for these primers using a temperature gradient programme with the following reaction conditions: 1 cycle of 94 °C for 3 min, 39 cycles of 93 °C for 30 s, 50 °C to 65 °C gradient for 1 min, 72 °C for 45 s, and finally 72 °C extension for 5 min. Allelic variation and other characteristics at the nine loci were examined in 27–38 frogs from all populations. Each PCR reaction (10 μL) contained 1 μL 10× PCR buffer, 2 nmol of each dNTP, 15 nmol $MgCl_2$, 0.075 μL 5 U *Taq* DNA polymerase (Promega), 5.0 pmol of each primer, and ≈ 10–30 ng DNA. Each forward primer was labelled with a fluorescent dye (6-FAM). A portion (0.2–1.0 μL) of the PCR product was mixed with 11 μL 20:1 Hi-Di-Formamide: GenScan™/500 Rox™ size standard and denatured at 100 °C. Fragments were analysed on the ABI 3100 Genetic Analyser, then scored using GENE SCAN ANALYSIS Version 3.5 and GENOTYPER Version 3.6 NT software. We did not test whether the newly developed primer pairs amplified microsatellite loci in other species.

Eight of the 10 loci showed allelic polymorphism between individuals and populations (Table 1). In comparison with microsatellite loci from other anuran species,

Table 1 PCR primers for eight polymorphic and one monomorphic microsatellite loci in the túngara frog *Physalaemus pustulosus*

Locus	Repeat structure*	Primer sequence (5′ → 3′) forward reverse	°C†	Length of PCR product	No. ind. scored	No. alleles	H_O	H_E	GenBank Accession no.
CA 120	$(CA)_{14}$	CCAAAATTGTGGACACCAGA GACCGGCAGATAATGTGTGG	61	211–247	29	11	0.28	0.75	AF481827
CA 298	$(CA)_8CG(CA)_{20}$	CATAATGCTGAGCCCTCCTC AACAGAGTGGCTAACACATCCA	59	148–180	38	13	0.55	0.75	AF481825
GA 240	$(GA)_{13}$	CCGACTCTGGTCTGGGATTA CCCCTGACCAGCAGAATAAA	60	195–275	36	17	0.78	0.89	AF481824
ATG 159	$(TAGA)_{20}$	ATGTGCCCTGCTGAAGTTTT GTGCTGACCAATGACACCAG	62	202–294	33	15	0.82	0.89	AF481823
A# 3.11	$(CA)_{16}$	GACAGGTTCAGCCCCTTTCT CTGCAGAAAGCAGTCGACAA	59	175–244	30	17	0.70	0.86	AF481820
A# 19.11	$(CA)_{22}$	AGGTAAATTGTTGTAAAACATTAAAGG ACTGCAGTGGAAGGGTCTCT	60	177–277	27	20	0.44	0.93	AF481821
C# 30.11	$(TAGA)_{17}$	TCCATTCCCTTTGAAGACATC CAGGAAGAAATTGTGATTTTGGA	61	204–300	27	18	0.78	0.93	AF481826
ATG 263	$(TAGA)_{15}$	CTTCACAAGCTGCTGACCAA GTCCCCTTATGTGCTGTGCT	62	200–216	29	5	0.45	0.54	AF481822

*The length given is for the original sequence.
†Annealing temperature for PCR amplification.

allelic diversity is rather high (5–20 alleles per locus in ≈ 30 individuals scored). This high diversity might be due to the genetic difference between northern Costa Rican populations and southern Costa Rican/western Panamanian populations which are separated by an ≈ 200 km long gap along the Pacific coast of Central Costa Rica. This reproductive barrier in Central Costa Rica may also explain that observed heterozygosities are consistently lower than expected ones when calculating expectations across the entire range (Wahlund effect). A more comprehensive analysis including additional populations along a transect spanning northern Costa Rica to Central Panama is currently in progress (Pröhl *et al*. unpublished).

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