

PRIMER NOTE

Isolation of simple and compound polymorphic tetranucleotide microsatellites for the neotropical leaf litter frog *Eleutherodactylus ockendeni* (Leptodactylidae)

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Abstract

Few studies of population structure and genetic diversity exist for frogs in the Amazon of South America, an area renowned for exceptionally high species richness. We isolated seven highly variable tetranucleotide microsatellite loci for the neotropical leaf litter frog, *Eleutherodactylus ockendeni* using an enrichment method. Three of the repeats are simple, three are compound and one is imperfect. We screened all loci with 175 individuals from one geographical area in the upper Napo of Ecuador and found high polymorphism in all loci (> 14 alleles/locus). These markers are suitable for population genetics studies of *E. ockendeni* and perhaps other leaf litter frogs of the same genus.

Keywords: *Eleutherodactylus ockendeni*, 1, microsatellites, primers, upper Amazon

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The frog genus *Eleutherodactylus* is extremely speciose in South America. Although the Amazon Basin is vast, the greatest proportion of anuran endemics is in the far west, on the Andean eastern slopes of Peru and Ecuador, an anuran biodiversity hotspot (Duellman 1999). The genus *Eleutherodactylus* is the greatest contributor to the higher anuran species richness in upper rather than central and lower Amazonia (Zimmerman & Simberloff 1996).

Neotropical frog species generally have higher genetic diversity and among population differentiation than temperate species: low vagility, high environmental sensitivity of amphibians and heterogeneous habitat and microclimate likely notably restrict gene flow (e.g. Nevo & Beiles 1991; Garcia-Paris *et al.* 2000). There have been few studies of intraspecific diversity in tropical frogs, and those that do exist rely on mitochondrial sequences or allozymes (e.g. Gascon *et al.* 1998; Lougheed *et al.* 1999). For fine-scale genetic studies, DNA microsatellites have proven appropriate markers because they are codominant, often highly polymorphic, comparable among studies, and can reliably estimate gene flow (see Sunnucks 2000).

To assess the scale of gene flow for one species in a global amphibian hotspot, we designed tetranucleotide microsatellite

primers for a common upper Amazonian leaf litter frog, *Eleutherodactylus ockendeni* (*unistrigatus* group). These are the first microsatellites we are aware of that have been successfully developed for an Amazonian terrestrial frog.

We designed an enriched microsatellite library using a modified Hamilton *et al.* (1999) protocol. Briefly, genomic DNA from liver/muscle tissue for two individuals of *E. ockendeni* was extracted using standard phenol–chloroform methods (Sambrook *et al.* 1989). Fifty micrograms of genomic DNA was digested with 150 U each of *NheI* (Fermentas Life Sciences), *RsaI* (Gibco-BRL) and *HaeIII* (New England BioLabs) restriction enzymes. Single-stranded overhangs were cut to blunt ends using 50 U T4 DNA polymerase (Fermentas), reaction buffer and 0.1 mM dNTPs. DNA of 250–900 bp was excised from a 1% 1× TAE agarose gel visualized with ethidium bromide, then cleaned with QIAquick Gel Extraction Kit (QIAGEN). Genomic DNA ends were dephosphorylated with calf intestinal alkaline phosphatase (0.01 U/pmol DNA) and then ligated to SNX linkers. Ligated DNA was enriched by hybridization of biotinylated (GATA)₇ and (AAAG)₇ probes at 63 °C for 3 h. Streptavidin-coated magnetic beads (Dynabeads M-280 Streptavidin; Invitrogen) were used as a substrate to bind the microsatellite-enriched genomic DNA, following manufacturer's washing instructions. Microsatellite-enriched DNA was ligated into the *XbaI* site of pBluescript II

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SK+ (Stratagene). ElectroTen-Blue Electroporation-Competent (Stratagene) *Escherichia coli* cells were electroporated to accept the plasmid. Cells were grown on agar medium treated with ampicillin and X-Gal. Probe hybridization was facilitated with a DIG Easy Hyb kit (Roche Diagnostics) following manufacturer's protocols. Target DNA was detected with CDP-Star (Roche Diagnostics) and chemoilluminated on film. Fifty-one positive clones were grown in LB ampicillin-broth. Total DNA was extracted and cleaned with QIAprep Spin Miniprep Kit (QIAGEN). Microsatellite inserts were amplified by polymerase chain reaction (PCR) in a GeneAmp 9700 (Applied Biosystems) thermal cycler with M13 primers and run out on a 1× TBE 2% agarose gel to size-select clones of 200–600 bp in length. Twenty-eight of the 41 putative positive clones were sequenced with M13F primer and BigDye Terminator version 3.1 cycle sequencing reactions on an ABI 3100 capillary sequencer. Six sequences were effectively redundant.

Nineteen primer pairs for 15 loci were designed in PRIMER 3 (Rozen & Skaletsky 2000) and by eye. Primers were screened by PCR in 10 µL reactions of 0.5 mM of each dNTP, 0.5 µM of each forward and reverse primer, 1× MgCl₂-free PCR standard buffer [75 mM Tris-HCl (pH 9.0), 50 mM KCl, 20 mM (NH₄)₂SO₄; Biotools], 2.50–7.50 mM MgCl₂, 0.25 U *Taq* polymerase (Biotools) and 5–10 ng DNA. Amplification profiles were 94 °C for 2 min; 35 cycles of 94 °C for 30 s, 52–66 °C for 20 s and 72 °C for 20 s; 72 °C for 5 min followed by a 4 °C hold (MgCl₂ concentrations and annealing temperatures in Table 1) in either an Applied Biosystems 2700 or GeneAmp 9700 (Applied Biosystems) thermal cycler. A subset of 10 DNA samples (extraction method below) was screened for variability by visualization on a 3% 1× TBE agarose gel stained with ethidium bromide. Primer pairs that reliably amplified variable loci were tagged with VIC, 6-FAM, PET or NED fluorescent labels (Applied Biosystems) in the forward direction.

Eleutherodactylus ockendeni DNA for 175 individuals from a single locality (Napo, Ecuador) was extracted from liver and/or muscle tissue stored in ethanol using a DNeasy Tissue Extraction Kit (QIAGEN) following manufacturer's protocols, amplified with the previous PCR conditions for seven loci and genotyped on an ABI 3100 Automated Capillary DNA Sequencer. Allele sizes were determined according to GeneScan 500-LIZ Size Standard (Applied Biosystems) and GENESCAN ANALYSIS software version 3.7.

Calculation of observed and expected heterozygosities and tests for conformance to Hardy–Weinberg (HW) and linkage equilibrium expectations were performed in GENEPOP on the Web (Raymond & Rousset 1995).

All loci are highly polymorphic, with 14–32 alleles per locus, and show strong homozygote excess (obs/exp homozygotes = 1.09–11.09). Five of seven loci show significant discordance from HW expectations. We attribute this

Table 1 Locus, microsatellite motif, primer sequence (label in superscript), PCR annealing temperature, PCR MgCl₂ concentrations, size of cloned allele, number of individuals scored, range of allele sizes, number of alleles, observed/expected heterozygosity ratio, deviation from HW expectations (full sample/subsample) and clone GenBank Accession no.

Locus	Repeat Motif	Primer sequence (5'–3')	Annealing temperature (°C)	MgCl ₂ concentrations (mM)	Size of cloned allele	No. of individuals	Range of observed allele sizes	No. of alleles	H _o /H _e	Deviation from HW	Clone GenBank Accession no.
Eloc – Bert & Ernie	(CTAT) ₁₉ (CCAT) ₃	ElocBert (F): GAAAAGAAATGGAGCTGTNED ElocErnie (R): 5' AGCTAGAGATAGATATACAGATG	52	2.50	154	169	102–202	29	0.860	**/NS	DQ359136
Eloc – Beauty & Beast	(CTAT) ₁₉	ElocBeauty (F): GGTTACCTCCACTCTATATCVC ElocBeast (R): GTTGACCAACTATATAGGCAACA	61	6.25	169	175	141–245	23	0.900	**/NS	DQ359131
Eloc – JeanPaul & Simone	(CTAT) ₁₁ (CTAC) ₁	ElocJeanPaul2 (F): CCTCCACTCTATATCATGACAG6FAM ElocSimone (R): CTCCACTGACAGTTGACCA	62	5.00	175	175	151–251	22	0.943	NS/NS	DQ359132
Eloc – Romeo & Juliet	(GATA) ₁₇	ElocRomeo2 (F): CACAAGATGACTACATGAANED ElocJuliet2 (R): CCTCCACTCTATATCATGAC	56	7.50	183	173	167–259	22	0.824	**/**	DQ359133
Eloc – Thelma & Louise	(CTCT) ₅ (TCTG) ₃	ElocThelma (F): AATCTGTGCTCTGTGTCAGAGVIC ElocLouise (R): CAGTACTTTTCAGCCACACAG	57	2.50	223	170	191–243	14	0.977	NS/NS	DQ359135
Eloc – Laurel & Hardy	(TCTA) ₁₁ (TCTG) ₅	ElocLaurel (F): CACGAGGAGCAITGAGACTGPEI ElocHardy (R): CCCATGAGCCCTGTAGCTTACG	66	5.00	186	142	162–274	32	0.367	**/**	DQ359130
Eloc – Batman & Robin	(GATA) ₂ (GAT) ₃ (GATG) ₁₁ (AATA) ₁ (GATA) ₂ (GAT) ₂ (GATA)	ElocBatman (F): CCTTTGGGTTGGCTTACCTC6FAM ElocRobin (R): GACCAACAATGTTTTCCTTGG	56	5.00	224	169	204–286	27	0.814	**/**	DQ359134

Deviations from HW expectations: full sample/subsample; ns, not significant ($P > 0.01$); **, significant ($P < 0.01$).

to the geographical sample area ($\sim 6 \times 4$ km) being larger than a biological population of small terrestrial frogs like *E. ockendeni*. Consequently, we analysed a subsample of 36 geographically more proximate individuals and found four of the seven loci to be in accordance with HW expectations (Eloc – Bert & Ernie, Eloc – Beauty & Beast, Eloc – JeanPaul & Simone and Eloc – Thelma & Louise). After Bonferroni correction, two locus pair comparisons exhibited significant linkage disequilibrium (Eloc – Beauty & Beast vs. Eloc – Romeo & Juliet and Eloc – JeanPaul & Simone vs. Eloc – Romeo & Juliet; $P < 0.0026$).

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