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PERMANENT GENETIC RESOURCES

Isolation of microsatellite loci from the coqui frog, *Eleutherodactylus coqui*

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Abstract

Thirteen microsatellite loci were isolated from the coqui frog (*Eleutherodactylus coqui*) and optimized for future research. The loci were screened across 37 individuals from two Puerto Rican populations. Loci were variable with the number of alleles per locus ranging from three to 38. Polymorphic information content ranged from 0.453 to 0.963 and observed heterozygosity for each population ranged from 0.320 to 0.920.

Keywords: amphibian, conservation genetics, invasive species, population structure, primer, Puerto Rico

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The most abundant and widely distributed frog species endemic to Puerto Rico, *Eleutherodactylus coqui*, has recently invaded Florida and several islands in the Caribbean, and was accidentally introduced to Hawaii in the late 1980s (Kraus *et al*. 1999), where is it considered a pest species. In both Puerto Rico and Hawaii, *E. coqui* reaches densities of > 20 000 individuals/ha (Woolbright *et al*. 2006). Direct development, lack of a breeding chorus, and year-round breeding are thought to contribute to its ability to invade new areas (Beard & O'Neill 2005). Despite an apparently continuous distribution, pronounced spatial genetic structure has been described in Puerto Rico: the eastern and central-western regions of the island are occupied by distinct mitochondrial cytochrome *b* clades (approximately 5–7% sequence divergence) (Velo-Antón *et al*. in press). Phylogeographical patterns in nuclear markers have not been investigated. Here, we describe microsatellite loci that can be used to investigate nuclear genetic structuring, assess demographic expansions and bottlenecks, characterize fine-scale landscape genetic patterns, and potentially identify source populations in this important species.

For initial microsatellite development, we used toe-clips from two individuals: one from El Yunque Caribbean National Forest (eastern part of Puerto Rico) and one from the Maricao Forest Reserve (western part of Puerto Rico). All tissues were collected into 95% ethanol and DNA was extracted using a salt–chloroform protocol with isopropanol precipitation (Mullenbach *et al*. 1989). To develop microsatellite loci, pooled DNA was serially enriched twice for microsatellites using three probe mixes (2, 3 and 4) following Glenn & Schable (2005; see www.uga.edu/srel/DNA_Lab/protocols.htm for updates and probe mixes). Briefly, DNA was digested with restriction enzyme *Rsa*I (New England Biolabs) and simultaneously ligated to double-stranded SuperSNX linkers. Linker-ligated DNA was denatured and hybridized to biotinylated microsatellite oligonucleotide mixes, which were then captured on magnetic streptavidin beads (Dynal). Unhybridized DNA was washed away and remaining DNA was eluted from the beads, amplified in polymerase chain reactions (PCR) using the forward SuperSNX24 as a primer, and cloned with TOPO TA Cloning Kits (Invitrogen). A total of 192 plasmids were sequenced with M13 forward and reverse primers using BigDye version 3.1 (Applied Biosystems) and an ABI PRISM 3130xl sequencer. Sequences from both strands were assembled and edited in SEQUENCHER 4.1 (Gene Codes Corp.) and exported to MSATCOMMANDER version 0.4 (Faircloth 2007; available at http://code.google.com/p/msatcommander/) for microsatellite searching (note:

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Table 1 Characterization of 13 polymorphic microsatellite loci genotyped in 37 individuals of *Eleutherodactylus coqui* from two populations in Puerto Rico, El Yunque Forest Reserve (EYL) and Rio Abajo State Forest (RAL)

Locus GenBank Accession no.	Primer sequence 5′–3′	Repeat motif	*N* (EYL) (RAL)	T_a (°C)	$MgCl_2$ (mM)	Size (bp)	*k* (EYL) (RAL)	H_O (EYL) (RAL)	H_E (EYL) (RAL)	PIC
Coq-10	F: ^{Fam}GAGACTCATTCCAGATAAGT	$(AGAT)_{11}$	25			156–200	5	0.320	0.372	0.665
EF587703	R: TTTCTTTTTGACAATGAGTA		12	55	2.0		8	0.917	0.891	
Coq-19	F: TATTTGCTGCCATTTATAT	$(AGAT)_7$	21			119–263	24	0.762*	0.971	0.940
EF587705	R: ^{Fam}CTCTACATTTCCATCAGTCT		11	65	2.0		13	0.636*	0.948	
Coq-20	F: ^{Ned}ACATAAGCAACATAAATAATA	$(AGAT)_4$	25			214–236	10	0.440	0.452	0.518
EF587706	R: CTTGTCTGTCTGGTTTATAG		12	55	2.0		5	0.667	0.667	
Coq-27	F: GATGGGAGCGAATGAGAG	$(AG)_9$	25			153–174	9	0.480*	0.775	0.792
EF587716	R: ^{Fam}TACTCCCCTCCCTCTCTTAT		12	55	1.6		5	0.750	0.768	
Coq-28	F: ^{Ned}GTCAAGGTCTGGAAGTAGT	$(GAGT)_5$	25			267–283	6	0.520	0.614	0.651
EF587717	R: AGAACCTGTGATGATGTTAC		8	55	2.0		3	0.250	0.633	
Coq-31	F: ^{Ned}ACAGATTTCATTCTCCATAT	$(AATG)_7$	25			261–289	9	0.680	0.818	0.773
EF587718	R: ACCTGGACAGTAAAATGATA		12	55	2.0		5	0.750	0.699	
Coq-201	F: ACTCCCCCATCCATAATA	$(AGAT)_9$†	24			272–421	27	0.500*	0.970	0.963
EF587707	R: ^{Fam}ATACAACCGACCAATATGT		12	60	2.0		16	0.667*	0.971	
Coq-203	F: GTTCAAACAGCATGAGTATA	$(CTGT)_4$ ...	25			192–222	8	0.920	0.788	0.806
EF587708	R: ^{Fam}GAACCTGAGAACGAGATAC	$(CTGT)_5$	12	60	2.0		6	0.750	0.681	
Coq-208	F: GATCGGATGAATGGTGTAGTA	$(AC)_7$	25			115–123	2	0.480	0.372	0.453
EF587709	R: ^{Ned}CACAAACAGGTTCCAATCTATA		12	60	2.0		2	0.917	0.518	
Coq-211	F: TAAAAGCAATGGTCAAACTTAC	$(ATCT)_{14}$	23			107–213	13	0.565*	0.784	0.706
EF587711	R: ^{Vic}AACACCAAGGGAGTGAATACT		12	65	1.2		8	0.583	0.562	
Coq-219	F: ATGGTTGTCAAACACAGT	$(ACTG)_4$	25			164–218	23	0.640*	0.960	0.941
EF587712	R: ^{Vic}TCAGGACCTAAAGAATGTC		12	55	1.6		11	0.917	0.870	
Coq-221	F: ^{Vic}TGCAGCAACTGAAGAAATAT	$(ATCT)_6$ ...	25			192–252	26	0.920	0.969	0.949
EF587713	R: GAGTAGGCACGAAAAGTGTA	$(ATCT)_{12}$	12	60	2.0		15	0.667*	0.957	
Coq-224	F: TCCAGCCTCCAGATCACTA	$(ACAG)_5$	25			224–238	2	0.920	0.507	0.508
EF587714	R: ^{Vic}TATTGGCACCATTTTTTAGAT		12	55	1.6		4	0.917	0.736	

N is the number genotyped for each population; T_a corresponds to highest annealing temperature for touchdown thermal cycling; $MgCl_2$ is an optimized concentration for magnesium chloride; size indicates the range of observed alleles in base pair; *k* is number of alleles observed in each population; H_O and H_E are observed and expected heterozygosities of each population, respectively, and PIC is polymorphic information content of each locus. *Significant deviations from Hardy–Weinberg equilibrium are indicated at $P = 0.0000$, 0.0006, 0.0019, 0.0000, 0.0002, 0.0055, 0.0000 and 0.0006, respectively. Primers with CAG tag (5′- CAGTCGGGCGTCATCA-3′) are indicated with superscript FAM, NED, or VIC (except see below), which was used as the fluorescent dye for genotyping. Three primers (Coq-27, Coq-201, Coq-203) were tagged with M13R tag (5′-GGAAACAGCTATGACCATG-3′) and fluorescently labelled with FAM. †All repeats in clone: $(AGAT)_9$ ... $(AGAT)_6$ ... $(AGAT)_7$ ... $(AGAT)_7$.

version 0.4 did not include primer design). PCR primers were designed using OLIGO 6.67 (Molecular Biology Insights). One primer in each pair was modified on the 5′ end with an engineered sequence (CAG tag or M13R tag; see Schable *et al*. 2002).

Fifty five primer pairs were tested for amplification and polymorphism using a subset of seven samples from Puerto Rico: El Yunque Forest Reserve (EYL) (eastern $n = 4$) and Rio Abajo State Forest (RAL) (western $n = 3$). PCR amplifications were performed on an Applied Biosystems 9700 using 12.5 μL reactions [10 mM Tris pH 8.4, 50 mM KCl, 25.0 μg/mL BSA, 0.4 μM unlabelled primer, 0.08 μM tag-labelled primer, 0.36 μM universal dye-labelled primer, 1.2–2 mM $MgCl_2$, 0.15 mM dNTPs, 0.5 U JumpStart *Taq* DNA Polymerase (Sigma), and 20–40 ng DNA]. Touchdown thermal cycling programmes (Don *et al*. 1991) encompassing annealing temperatures of 65–55 °C, 60–50 °C or 55–45 °C were used for the amplification (Table 1). Cycling parameters were 21 cycles of 96 °C for 20 s, highest annealing temperature (decreased 0.5 °C per cycle) for 20 s, and 72 °C for 30 s; and 15 cycles of 96 °C for 20 s, lowest annealing temperature for 20 s, and 72 °C for 30 s. PCR products were run on an ABI PRISM 3130xl sequencer and sized with Naurox size standard prepared as described in DeWoody *et al*. (2004), except that unlabelled primers started with GTTT. Results were analysed using GENEMAPPER version 4.0 (Applied Biosystems). Thirteen of these 55 primer pairs amplified high quality PCR product showing polymorphism across seven individuals.

Polymorphism in these 13 loci was further assessed on 30 additional coqui frogs (EYL $n = 21$; RAL $n = 9$), yielding a total of 37 analysed individuals. Conditions and characteristics of the 13 loci are given in Table 1. One locus, Coq-19, showed evidence of duplication in three individuals (three alleles), which were omitted from assessment of Hardy–Weinberg equilibrium and linkage disequilibrium. For the remaining loci, we used CERVUS version 2.0 (Marshall *et al*. 1998) to estimate alleles per locus (k), observed and expected heterozygosities, polymorphic information content, and frequency of null alleles. All loci had estimated null allele frequencies ≤ 0.27. Deviations from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium were determined using GENEPOP version 3.4 (Raymond & Rousset 1995). In Coq-19 and Coq-201, both populations deviated significantly from HWE and, in Coq-27, Coq-211, Coq-219 and Coq-221, one population deviated significantly from HWE after Bonferroni correction. Deviations from HWE are heterozygote deficiencies which may be a result of short allele dominance (Wattier *et al*. 1998) rather than null alleles since estimated frequencies of null alleles for the deviant loci were low (0.12–0.27). No linkage disequilibrium was detected among 66 paired loci comparisons for either population.

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