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Available at: http://works.bepress.com/karenh_beard/1/
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Date received: ____________;

**Key words:** amphibian, invasive species, conservation genetics, population structure, primer, Puerto Rico

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**Running title:** *Eleutherodactylus coqui* microsatellites
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 3 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.

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 and 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). Phylogeographic 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 http://www.uga.edu/srel/DNA_Lab/protocols.htm for updates and probe mixes). Briefly, DNA was digested with restriction enzyme Rsal (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 v3.1 (Applied Biosystems) and an AB-3130xl sequencer. Sequences from both strands were assembled and edited in Sequencer 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: 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).

Sixty 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 (10 mM Tris pH 8.4, 50 mM KCl, 25.0 µg/ml BSA, 0.4 µM unlabeled primer, 0.08µM tag labeled primer, 0.36µM universal dye-labeled primer, 1.2-2 mM MgCl₂, 0.15 mM dNTPs, 0.5 units JumpStart Taq DNA Polymerase (Sigma), and 20-40 ng
DNA). Touchdown thermal cycling programs (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 AB-3130xl sequencer and sized with Naurox size standard prepared as described in DeWoody et al. (2004), except that unlabeled primers started with GTTT. Results were analyzed using GeneMapper version 4.0 (Applied Biosystems). Thirteen of these 60 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 analyzed individuals. Conditions and characteristics of the 13 loci are given in Table 1. One locus, Coq-19, showed evidence of duplication in three individuals (3 alleles), which were omitted from assessment of Hardy-Weinberg equilibrium and linkage disequilibrium. For remaining loci, we used CERVUS version 2.0 (Marshall et al. 1998) to estimate alleles per locus (k), observed and expected heterozygosity (H_o and H_e), polymorphic information content (PIC), 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 and 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.

Acknowledgements - This work was supported by National Science Foundation award DEB-0614208, Department of Energy award DE-FC09-07SR22506, the USDA/APHIS/WS/National Wildlife Research Center, Hilo Field Station, and NSF ADVANCE Collaborative Support Grant through Utah State University.

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References


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). *N* is the number genotyped for each population; *T*₂₅ corresponds to highest annealing temperature for touchdown thermal cycling; MgCl₂ is an optimized concentration for magnesium chloride; size indicates the range of observed alleles in bp; *k* is number of alleles observed in each population; *H*₀ and *H*ₑ are observed and expected heterozygosity of each population, respectively, and PIC is polymorphic information content of each locus.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Genbank accession number</th>
<th>Primer Sequence 5' --&gt; 3'</th>
<th>Repeat Motif</th>
<th><em>N</em> (EYL) (RAL)</th>
<th><em>T</em>₂₅ (ºC)</th>
<th>MgCl₂ (mM)</th>
<th>Size (bp) (RAL)</th>
<th><em>k</em> (EYL) (RAL)</th>
<th><em>H</em>₀ (EYL) (RAL)</th>
<th><em>H</em>ₑ (EYL) (RAL)</th>
<th>PIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coq-10</td>
<td>EF587703</td>
<td>F: Fam®GAGACTCATTTCCAGATAAGT</td>
<td>(AGAT)₁₁</td>
<td>25</td>
<td>55</td>
<td>2.0</td>
<td>156-200</td>
<td>5</td>
<td>0.320</td>
<td>0.372</td>
<td>0.665</td>
</tr>
<tr>
<td>EF587705</td>
<td>R: TTTCTTTTTTGACAATGAGTA</td>
<td>(AGAT)₇</td>
<td>12</td>
<td>11</td>
<td>65</td>
<td>2.0</td>
<td>119-263</td>
<td>24</td>
<td>0.762*</td>
<td>0.971</td>
<td>0.940</td>
</tr>
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<td>Coq-20</td>
<td>EF587706</td>
<td>F: NedaACATAAGCACAATAATA</td>
<td>(AGAT)₄</td>
<td>25</td>
<td>55</td>
<td>2.0</td>
<td>214-236</td>
<td>5</td>
<td>0.667</td>
<td>0.667</td>
<td>0.518</td>
</tr>
<tr>
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<td>R: CTTGTCTGCTGTTATAG</td>
<td>(AG)₉</td>
<td>12</td>
<td>55</td>
<td>55</td>
<td>1.6</td>
<td>153-174</td>
<td>5</td>
<td>0.480*</td>
<td>0.775</td>
<td>0.792</td>
</tr>
<tr>
<td>EF587717</td>
<td>R: TACCTCCCCTCCTCTCTTTAT</td>
<td>(AGT)₅</td>
<td>25</td>
<td>55</td>
<td>55</td>
<td>1.6</td>
<td>267-283</td>
<td>6</td>
<td>0.520</td>
<td>0.614</td>
<td>0.651</td>
</tr>
<tr>
<td>Coq-31</td>
<td>EF587718</td>
<td>F: NedaACAGATTTACATCATAT</td>
<td>(AATG)₇</td>
<td>25</td>
<td>55</td>
<td>2.0</td>
<td>261-289</td>
<td>9</td>
<td>0.680</td>
<td>0.818</td>
<td>0.773</td>
</tr>
<tr>
<td>Coq-201</td>
<td>EF587707</td>
<td>R: Fam®AACAACGACAAATGTA</td>
<td>(AGAT)₉ ²</td>
<td>24</td>
<td>60</td>
<td>2.0</td>
<td>272-421</td>
<td>27</td>
<td>0.500*</td>
<td>0.970</td>
<td>0.963</td>
</tr>
<tr>
<td>Coq-203</td>
<td>EF587708</td>
<td>R: NedaGACCTGAAACAGATATA</td>
<td>(CTGT)₄⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻</td>
<td>12</td>
<td>60</td>
<td>2.0</td>
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<td>8</td>
<td>0.920</td>
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<tr>
<td>Coq-208</td>
<td>EF587709</td>
<td>F: GCAGTGGATATGGTTGAGTA</td>
<td>(AC)₇</td>
<td>25</td>
<td>60</td>
<td>2.0</td>
<td>115-123</td>
<td>2</td>
<td>0.480</td>
<td>0.372</td>
<td>0.453</td>
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<tr>
<td>Coq-211</td>
<td>EF587711</td>
<td>R: Vic®AACACAAGGGAGTGATAC</td>
<td>(ATCT)₁₄</td>
<td>23</td>
<td>65</td>
<td>1.2</td>
<td>107-213</td>
<td>13</td>
<td>0.565*</td>
<td>0.784</td>
<td>0.706</td>
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<tr>
<td>Coq-219</td>
<td>EF587712</td>
<td>R: Vic®TCAGACTCAAATAATGTC</td>
<td>(ACTG)₁₂</td>
<td>25</td>
<td>55</td>
<td>1.6</td>
<td>164-218</td>
<td>23</td>
<td>0.640*</td>
<td>0.960</td>
<td>0.941</td>
</tr>
<tr>
<td>Coq-221</td>
<td>EF587713</td>
<td>R: Vic®GACGGCAATGAAAATAT</td>
<td>(ATCT)₆⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻</td>
<td>25</td>
<td>60</td>
<td>2.0</td>
<td>192-252</td>
<td>26</td>
<td>0.920</td>
<td>0.969</td>
<td>0.949</td>
</tr>
<tr>
<td>Coq-224</td>
<td>EF587714</td>
<td>R: Vic®TATTGGCAACATTTTAGAT</td>
<td>(ACAG)₀₅</td>
<td>12</td>
<td>55</td>
<td>1.6</td>
<td>224-238</td>
<td>4</td>
<td>0.917</td>
<td>0.736</td>
<td>0.508</td>
</tr>
</tbody>
</table>
Significant deviations from Hardy-Weinberg equilibrium are indicated at $P = 0.0000, 0.0006, 0.0019, 0.0000, 0.0002, 0.0055, 0.0000, \text{ and } 0.0006$, respectively. Primers with CAG tag ($5'\text{- CAGTCGGGCGTCATCA-3'}$) 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 ($5'\text{-GGAACAGCTATGACCATG-3'}$) and fluorescently labeled with FAM.

‡ All repeats in clone: (AGAT)$_9$..(AGAT)$_6$..(AGAT)$_7$..(AGAT)$_7$