Title: Characterization of five dinucleotide and six tetranucleotide polymorphic microsatellite loci for the squirrel treefrog (*Hyla squirella*)

Author's names: Tyler D. Hether, Eric A. Hoffman

Full postal addresses (for all authors):

University of Central Florida, Department of Biology, 4000 Central Florida Blvd., Orlando, Florida, 32816

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Corresponding author: Eric A. Hoffman, University of Central Florida, Department of Biology, 4000 Central Florida Blvd., Orlando, Florida, 32816, fax: 407-823-5769, email: eahoffma@mail.ucf.edu

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14 Abstract:

15 Herein we describe the cloning and characterization of five dinucleotide and six tetranucleotide
16 microsatellite loci for the squirrel treefrog (*Hyla squirella*). Loci were tested for significant deviations
17 from Hardy-Weinberg equilibrium and linkage equilibrium within a natural breeding population in
18 northern Florida, USA. In this sample of 32 frogs, polymorphism ranged from 2 to 21 alleles per locus
19 with expected heterozygosities ranging from 0.061 to 0.946. Additionally we found that 10 of the 11 loci
20 amplified in two congeners (*H. femoralis* and *H. cinerea*).

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22 Amphibians are declining at an unprecedented rate (reviewed in Storfer et al. 2009). Several
23 landscape features (e.g., cover type, roads, moisture, topographic relief) have been hypothesized to affect
24 gene flow among amphibian populations due to their susceptibility to a range of aquatic and terrestrial
25 environmental stressors (Spear et al. 2005). Because accurate classification of genetic diversity is critical
26 for conservation efforts (Storfer et al. 2009) it is useful to characterize diversity in a relatively abundant
27 species in order to determine (1) if any landscape features universally affect gene flow in amphibians and
28 (2) if these features are scale dependent. The squirrel treefrog (*Hyla squirella*) is an abundant hylid found
29 in a range of natural and human-modified habitats types throughout the United States' Coastal Plain
30 (Jensen et al. 2008). The microsatellites characterized herein will aid in studies that investigate the
31 evolutionary history and the influence of landscape features on gene flow and genetic structure in *H.*
32 *squirella*.
33 The microsatellite enrichment followed the procedures of Hoffman et al. (2003) with minor
34 modifications. Total genomic DNA from a single individual *H. squirella* was extracted using standard
35 phenol-chloroform methods (Sambrook & Russel 2001). The K6-MW primer (5'-
36 CCGAGGTACCNNNNNNATGTGG-3') (Macas et al. 1996) was used to generate DNA fragments of
37 varying size using the degenerate oligonucleotide-primer polymerase chain reaction (DOP-PCR)
38 procedure identical to Hoffman et al. (2003). Amplicons were hybridized with either a 5'-biotinylated,
39 3'-amino modified (hereafter 'biotinylated') $(CA)_{15}$ or biotinylated $(GATA)_8$ repeat motif bound to
40 streptavidin-coated particles (Promega) which allowed microsatellite enrichment using magnetic
41 separation. Hybridization conditions were identical to Ardren et al. (2002) with the following two
42 modifications. First, our temperature profile to hybridize the biotinylated $(CA)_{15}$ or $(GATA)_8$
43 oligonucleotides were 95°C for 5 min, then 52°C for 25 min (ramp speed 0.1 °C/sec). Second, the final
44 two washes were carried out at 72°C. Enriched products were made double stranded by a subsequent
45 DOP-PCR and cloned using TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Clones positive for $(CA)_n$
46 or $(GATA)_n$ microsatellites were identified using the screening procedure of Cabe & Marshall (2001)

47 whereby two PCR reactions were carried out: one included T3 and T7 primers while the other contained
48 an additional unbiotinylated $(CA)_{15}$ or $(GATA)_8$ oligonucleotide. Of the 672 clones screened 130 (19%)
49 appeared positive and were sent to Nevada Genomics Center (Reno, NV) for sequencing. Sequences
50 were aligned and edited with SEQUENCHER v. 4.1 (Gene Codes Corporation, Ann Arbor, MI) to check
51 for putative microsatellites and remove redundant sequences or those lacking adequate flanking regions
52 for primer design. Primers for all remaining sequences (35) were designed using Primer 3 (Rozen &
53 Skaletsky 2000) of which 11 (31%) were polymorphic and consistently gave PCR product.
54 For genotyping, these 11 loci (Table 1) were labeled with a M13 (-21) tail
55 (5'TGTAAACGACGGCCAGT-3') attached to the forward primer for fluorescent labeling of PCR
56 fragments (Schuelke 2000). All PCR reactions were performed in 10μL reactions and contained 40ng of
57 DNA template, 1X PCR buffer, 0.2μM each dNTP, 0.2 units of *Taq* polymerase, and locus-specific
58 $MgCl_2$ concentration (Table 1). Forward primer concentrations were 0.125μM (locus HSQ113) or 0.1μM
59 (all other loci); reverse primers and M13 tags, 0.5μM (HSQ113) or 0.4μM (all other loci). Loci HSQ126
60 and HSQ133 had identical reaction conditions and disparate allelic ranges to allow for multiplex PCR.
61 This multiplex panel was constructed with the same conditions as above except increased $MgCl_2$ and
62 dNTPs (1μM and 0.25μM each, respectively). Thermocycling conditions for locus HSQ131 were 94°C
63 for 4 min; 8 cycles of 94°C for 30 sec, 52°C for 35 sec, 70°C for 45 sec; 32 cycles of 94°C for 30 sec,
64 58°C for 35 sec, and 70°C for 45 sec; and a final extension of 72°C for 7 min. For all other loci the
65 thermocycling conditions were as follows: initial denaturation at 94°C for 4 min; 35 cycles of 94°C for 30
66 sec, locus specific annealing temperature (T_a; Table 1) for 35 sec, 72°C for 45 sec; and a final extension
67 of 72°C for 7 min. PCR Amplicons were sized using a CEQ 8000 genetic analysis system with provided
68 software (Beckman-Coulter, Fullerton, CA).
69 We amplified 32 individual *H. squirella* from a natural breeding population in Bradford County,
70 Florida to test for genetic variability and deviations from Hardy-Weinberg or linkage equilibria (via
71 Fisher's exact tests). Genetic analyses were carried out in Genepop v. 3.3 (Raymond & Rousset 1995).
72 Polymorphism ranged from 2 to 21 alleles per locus with expected heterozygosities ranging from 0.061 to

73 0.946. No loci showed departures from Hardy-Weinberg equilibrium or linkage equilibrium after
74 sequential Bonferroni correction (Table 1, Rice 1989).
75 Using the same PCR and cycling conditions, we tested these primer sets for cross-species
76 amplification in two congeners, *H. femoralis* and *H. cinerea*. Visualization on a 2% agarose gel
77 demonstrated successful amplification for ten loci in *H. femoralis* and nine in *H. cinerea* (Table 1). These
78 loci will enable high-resolution genetic studies in these tree frog species.

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Table 1. Basic information for 11 microsatellite loci used in this study. Symbols next to Locus name denotes $MgCl_2$ concentration used in all PCR reactions: § = 1.85mM, † = 1.56mM. Asterisks (*) denote primer labeled with the M13 (-21) tail. GenBank accession number, repeat motif in clone, primer sequence, annealing temperature (T_a), allelic range, number of diploid individuals scored (N), number of alleles (A), observed (H_O) and expected (H_E) heterozygosities are provided. P-values for HWE testing (P) are reported; Bonferroni corrected P-value was 0.0045. Locus names labeled in boldface or italics showed PCR product in *H. femoralis* or *H. cinerea*, respectively.

Locus	GeneBank accession no.	Repeat motif in clone	Primer sequence (5'-3')	T_a	Allelic range	N	A	H_O	H_E	P
HSQ103†	GQ438807	$(CA)_7$-$(CA)_8$-$(CA)_7$	*TGGTAGCAAGTTCCATACATTAGG	58°C	232-246	32	7	0.688	0.753	0.55
			AAACTGTCACTCCGCAGACC							
HSQ106§	GQ438808	$(GT)_{10}$	*GTGGAGGAAGGGGAGGAG	56°C	228-232	28	3	0.107	0.104	1.00
			CCCAGCTTGTGGATTTTTG							
HSQ107†	GQ438809	$(CA)_5AT(CA)_3$ imperfect	*AGTGTGCGACGTGTATGGAG	59°C	194-196	32	2	0.344	0.288	0.56
			AGCAGAGCAGAATGTAGCAGAG							
HSQ111§	GQ438810	$(GT)_8$	*TTCATGTGGTCGTGTTAGTGG	53°C	233-237	32	2	0.063	0.061	1.00
			CAAAAATATAACCCGCATGAGC							
HSQ113§	GQ438811	$(CA)_{16}$	*CATGAAGACAACCCCTGTCC	56°C	180-213	29	16	0.724	0.911	0.01
			GCGCTCTTAGATTATGTGAACC							
HSQ116†	GQ438812	$(GACA)_5(GATA)_{10}$ imperfect	*TTTGGTGTGAATTTGTGAAGC	57°C	228-356	32	21	0.875	0.946	0.12
			TTTTGGCATTATTTGCTTGG							
HSQ126†	GQ438813	$(GATA)_7$	*TGAAGTTTTAGGTTTCCTTCTGC	59°C	233-333	27	15	0.926	0.88	0.25
			GAATGATTGGTCAGCACATCC							
HSQ131§	GQ438814	$(CTAT)_7$-$(CTAT)_2$-$(CTAT)_{10}$ imperfect	*AAGTCTTTGGTGCAATTTTGG	58°C	188-264	31	15	0.774	0.808	0.09
			AGTTGCTGCTGCCTATTTGC							
HSQ133†	GQ438815	$(CTAT)_{11}$	*ACAAACCCAGCCAGAAATGG	59°C	380-430	27	13	0.778	0.91	0.05
			CTTTCGCAAGGCACTGTAGG							
HSQ135†	GQ438816	$(GATA)_9$	*TACCCTCTCCCCCTATGACC	59°C	214-238	31	7	0.935	0.856	0.18

			GTGAAGCCAGTCCATTGAGG							
HSQ136†	GQ438817	$(GATA)_5$-$(GATA)_6$	*TTACACGGTGCTGCTCTCC	59°C	174-206	31	6	0.452	0.64	0.01
			CAGCTTTTACCCCAACAAGG							