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Development and characterization of 15 polymorphic microsatellite loci isolated from Rafinesque's big-eared bat, *Corynorhinus rafinesquii*

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Abstract

We developed and characterized 15 microsatellite markers for Rafinesque's big-eared bat, *Corynorhinus rafinesquii*. In a population from Tennessee, the number of alleles per locus ranged from three to 13 and observed heterozygosities were 0.35 to 0.97 per locus. These loci will provide appropriate variability for estimation of population connectivity, demographic parameters, and genetic diversity for this species of concern.

Keywords: *Corynorhinus rafinesquii*, microsatellite, Rafinesque's big-eared bat

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Rafinesque's big-eared bat, *Corynorhinus rafinesquii* (Vespertilionidae), is an endemic, monotypic species from the southeastern USA (Piaggio & Perkins 2005). This bat is considered a federal species at risk (US Fish and Wildlife Service 1985), a species of concern in every state within its range, and is listed as endangered in Virginia. Currently, state and federal agencies are working towards developing an official conservation plan for this species, which includes critical research needs such as an evaluation of genetic diversity within populations of *C. rafinesquii*, estimation of population connectivity, and an assessment to determine if populations are experiencing expansion, contraction, or stability. The development of species-specific markers is crucial to accomplishing these goals. To this end, we have developed 15 autosomal microsatellite markers for this species. To assess the utility and variability of each marker, they were characterized using 31 *C. rafinesquii* individuals from southwestern Tennessee.

A *C. rafinesquii* individual from South Carolina was used for enrichment and development of a microsatellite library. This was achieved with an adapted method from Glenn & Schable (2005). Following this protocol, genomic DNA was digested with *Rsa*I and these fragments were ligated using double-stranded SNX-24 linkers. The resulting library was hybridized to 12 biotinylated microsatellite oligonucleotide probes with dinucleotide and trinucleotide repeats (e.g. CT, CA, AAG and GGA). Hybridized fragments were captured on streptavidin-coated Dynabeads (Dynal Biotech). These fragments were then amplified and cloned with the TOPO TA cloning kit (Invitrogen). Insert sequences from 96 colonies were obtained with M13 forward and reverse primers and run on an ABI 3730xl genetic analyzer (Applied Biosystems). Forty-seven clone sequences had recognizable microsatellite sequences, of which 87% (41) had enough or adequate flanking regions to design primers. Staden Package (Staden *et al*. 1998), TROLL (Castelo *et al*. 2002; Martins *et al*. 2006), and Primer 3 (Rozen & Skaletsky 2000) software packages were utilized to detect repeat regions and design primers within flanking regions. Parameters for Staden software were set according to step-by-step instructions included on the website for TROLL software. Forty-one primer pairs were designed and tested; 15 pairs amplified and were variable.

M13 primer sequences were added to the 5′ end of each forward primer. M13 primers were labelled with NED, FAM, or HEX. M13, forward and reverse primers were ordered from Applied Biosystems (ABI). Polymerase chain reactions (PCR) were carried out in a 10-μL reaction using 1.0 μL of genomic DNA, 0.5–0.9 μL each of 1 μM primer (Table 1), 1.0 μL of 10 mM dNTP (Promega), 0.2 μL of *Taq* DNA polymerase (Promega), 0.4 μL or 0.5 μL (Cora_G02_E04) 5 mg/mL BSA (Invitrogen), and locus-specific chemistry listed in Table 2. All PCRs were conducted using a Mastercycler Gradient thermacycler (Eppendorf, Germany). The thermal profile (Table 2) for all loci was an initial denaturation at 94 °C for 3–5 min followed by 35–40 cycles of 94 °C for 30 s,

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Table 1 Characteristics of the 15 microsatellite loci that were developed and optimized from *Corynorhinus rafinesquii*

Locus	Primer sequence (5′–3′); F, forward; R, reverse	Repeat motif	Size range (bp)	N_A total	H_O	H_E
Cora_B07_H12	F: TTAGACAAATGAGGGAGGATTG R: CATCAAAGAATGCCAAACTAAAG	$(AG)_{19}$	271–313	11	0.97	0.89
Cora_F_{11}_C04	F: AAGCTCAGAGACTGCTCCTTC R: ATCCATTATGTTTGCTGATGTTC	$(TG)_8$ ... $(TG)_{17}$	186–220	9	0.84	0.81
Cora_D12_D12	F: CATAAAGGAGACAACAAATCAATG R: CAGAGAGAGAAAGAAAGGGAAAG	$(TC)_7$	110–114	3	0.35	0.40
Cora_C05_E01	F: GCCTCTCTCATATCAATGTGTGT R: GCAGTTAGAGGAAAACAGGCT	$(TG)_6$	287–293	3	0.63	0.54
Cora_G02_E04	F: ACTCCTGAAAGTGCTGATGTTAT R: TGTTTCTCTCTCATTGATGTTTC	$(CA)_{11}$	320–326	4	0.52	0.52
Cora_E07_E07	F: TTACTAAAGGTTTGGGTAGGGAA R: GTGAAGTAGCCTGGCCTAAGA	$(GC)_9$ ... $(CA)_{12}$	163–179	9	0.84	0.83
Cora_F02_F02	F: GTCACTGGCTACAAAGAATGAAG R: GAAACACAGCAGAATTGTCTCTC	$(TC)_{24}$ ... $(CA)_{25}$	201–263	13	0.94	0.92
Cora_G04_F04	F: GAACATGCCACACAGAGACTTAC R: CTAGACCTTCCCGTGTGTAAAG	$(GT)_{23}$	255–300	13	0.71	0.85
Cora_C07_G01	F: CATTGGCTTTGTCTTAACAATTT R: TTTGTTTCAGTTTCTCTCTCTCC	$(AG)_{14}$	191–213	11	0.90	0.87
Cora_E10_G03	F: ACTTTTCATTCTTTCCCATTCT R: AAACCAACGAGTGCTAAATCTAC	$(TG)_{26}$	333–357	12	0.87	0.89
Cora_G10_A08	F: TTACAGTAGATACGGTTGTGCCT R: TTTTAGGACTGGTTTTAGGGAAG	$(GT)_{21}$	259–277	7	0.84	0.73
Cora_D08_H02	F: TTCCCACTTTATTTCCTTTATCT R: AAATCTATTTTCTCTACAACCAGAG	$(TC)_{15}$	332–354	11	0.71	0.83
Cora_G11_H04	F: GAGATACAACTGGAAAGAGCTGA R: TCGAAGACTTAAAAGCAATTTGA	$(GA)_9$ ... $(AG)_6$ ... $(AG)_6$ ... $(AG)_6$ ... $(AG)_9$	202–222	9	0.60	0.85*
Cora_H07_C05	F: TGATATGCACCTGAATTTCCTAC R: GGAGATGCTTCTATGACTTGCTA	$(AC)_{22}$	335–347	7	0.48	0.73*
Cora_H09_H09	F: ATTTATTCAGATGGAAATCAGCC R: GAGTATCACCTACAGCCTCCTTT	$(AC)_{12}$ ... $(AC)_{11}$	110–132	8	0.87	0.85

GenBank Accession nos FJ469632–FJ469646; N_A, mean number of alleles per locus; H_O, observed and H_E, expected heterozygosities. The following loci had evidence of null alleles, null allele frequencies (in parentheses) are based on Brookfield1 estimates from Micro-Checker software; Cora_D12_D12 (0.03), Cora_G04_F04 (0.08), Cora_D08_H02 (0.06), Cora_G11_H04 (0.13), Cora_H07_C05 (0.14). *denotes violation of Hardy–Weinberg equilibrium and significant evidence of null alleles; these loci should not be used for population analyses in these populations but may be useful to test in other populations or species.

52–54 °C for 30 s, and 72 °C for 45 s. Cycling was followed with either a 30-min extension at 60 °C or a 45-min extension at 72 °C (Cora_C05_E01). For characterization of the markers, tissue samples were collected through wing biopsies (Wilmer & Barratt 1996) from 31 *C. rafinesquii* from southwestern Tennessee. Samples were preserved in a 20% dimethyl sulfoxide, 0.25 M EDTA, saturated with NaCl, pH 8.0 solution (Seutin *et al*. 1991). Genomic DNA was extracted from half of the biopsy using a DNeasy Tissue Extraction Kit (QIAGEN Inc.) following the manufacturer's protocol. Samples were genotyped on an ABI 3130 automated genetic analyzer and analyzed with ABI GeneMapper software.

Each locus was tested for null alleles with Micro-Checker (van Oosterhout *et al*. 2004). Hardy–Weinberg equilibrium tests and number of alleles were obtained from Arlequin 3.1 (Excoffier *et al*. 2005). Tests for genotypic disequilibrium between pairs of loci were performed using FSTAT 2.9.3 (Goudet 2001). There was evidence of null alleles in five loci at moderate rates (Table 1). However, only two loci demonstrated significant deviations from Hardy–Weinberg equilibrium (Table 1) after sequential Bonferroni correction (Rice 1989). These two loci, Cora_G11_H04 and Cora_H07F_C05R, should not be used in population-level analyses of these samples under the assumption that they suffer from a prevalence of null alleles. However, they may be useful for testing in other populations or species. The number of alleles per locus in the remaining loci ranged from three to 13 and observed heterozygosity ranged from 0.35 to 0.97 per locus (Table 1). There was no linkage disequilibrium between any pair of loci. The development of these markers provides a valuable tool for researchers to gain knowledge of *C. rafinesquii* population demographics. This is particularly

Table 2 PCR conditions for each primer pair

Marker	Primer quantity for PCR using 1 μM primer F,R/M13 (μL)	Invitrogen 5× buffer C (μL)	Initial denaturation time (min)	Annealing temperature (°C)	Cycles
Cora_B07_H12	0.60/0.60	3.3	3	52	40
Cora_F_{11}_C04	0.90/0.90	3.3	3	52	35
Cora_D12_D12	0.50/0.50	3.3	5	52	40
Cora_C05_E01	0.90/0.90	3.4	5	52	40
Cora_G02_E04	0.90/0.80	3.0	5	54	35
Cora_E07_E07	0.50/0.50	3.3	3	52	35
Cora_F02_F02	0.90/0.90	3.4	5	52	40
Cora_G04_F04	0.90/0.90	3.1	3	53	35
Cora_C07_G01	0.80/0.80	3.4	3	52	35
Cora_E10_G03	0.90/0.90	3.3	3	52	35
Cora_G10_A08	0.80/0.80	3.3	3	52	35
Cora_D08_H02	0.90/0.90	3.3	3	52	35
Cora_G11_H04	0.90/0.90	3.3	3	52	35
Cora_H07_C05	0.60/0.60	3.3	5	52	40
Cora_H09_H09	0.50/0.50	3.3	5	52	35

useful for this species because there is tremendous concern about declining populations across its range.

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