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TECHNICAL NOTE

Identification and characterization of nuclear microsatellite loci for multiple species of chorus frogs (*Pseudacris*) for population genetic analyses

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Abstract Microsatellites were developed from an enriched genomic library of *Pseudacris feriarum* and *P. nigrita*. For 85 *P. feriarum*, six loci averaged 59 alleles, with loci ranging from 42–71 alleles. Observed heterozygosity averaged 0.72/locus (range: 0.42–0.84). In 142 *P. maculata*, seven additional loci (plus two loci above) averaged 8 alleles, with loci ranging from 3–16 alleles. Observed heterozygosity averaged 0.60/locus (range: 0.35–0.81).

Keywords Chorus frogs · Hybrid zone · Microsatellites · *Pseudacris nigrita* · *Pseudacris feriarum* · *Pseudacris maculata*

Introduction

The North American chorus frogs (*Pseudacris*) have become the focus of phylogenetic and phylogeographic studies (Moriarty and Cannatella 2004; Lemmon et al. 2007a, b; Lemmon and Lemmon 2008) as well as recent work on speciation via reinforcement (Lemmon 2009; Lemmon and Lemmon 2010). The microsatellites in the present study were developed to examine natural hybridization rates between *P. feriarum* and *P. nigrita* (Lemmon and Juenger unpub.). Further, nine loci were optimized in *P. maculata* for a landscape genetic study (Murphy et al. in prep.). Markers were also tested in five other *Pseudacris* species.

Four microsatellite-enriched libraries were constructed from pooled genomic DNA of *P. feriarum*/*P. nigrita* by Genetic Identification Systems (as described by Matthews et al. 2007). Inserts from recombinant clones were sequenced with the M13(−24) forward and/or M13 reverse primers using a BigDye terminator Cycle Sequencing kit on an Applied Biosystems 3,100 PRISM DNA Sequencer. Of 269 clones sequenced, 234 contained microsatellites.

Primers were designed for 80 unique loci and tested in frogs from the following counties: *P. feriarum* (Macon:AL [n = 20], Liberty:FL [n = 4], Ann Arundel:MD [n = 4], Davie:NC [n = 3], Wake: NC [n = 7], and Cumberland:VA [n = 4]), and *P. nigrita* (Brevard:FL [n = 4], Holmes:FL [n = 6], Jefferson:FL [n = 3], Liberty:FL [n = 5], Watson:FL [n = 2], McIntosh:GA [n = 18], Harrison:MS [n = 2]). Twenty loci were tested for cross-species amplification in five additional species: *P. brachyphona* (Hocking:OH [n = 1], Harrison:WV [n = 1]), *P. clarkii* (Garfield:OK [n = 1], Cameron:TX [n = 1]), *P. fouquettei* (Beauregard:LA [n = 1], Lamar:TX [n = 1]), *P. kalmi* (Accomack:VA [n = 1], Northampton:VA [n = 1]), and *P. triseriata* (Ingham:MI [n = 1], Highland:OH [n = 1]). Twenty-three loci were tested for cross-species amplification in *P. maculata* (Park:CO [n = 4], Teton:CO [n = 3], Ward:ND [n = 1], Lawrence:SD [n = 1]).

Tissues were extracted using the Viogene Blood and Tissue Genomic DNA Extraction System. Unlabeled

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Table 1 Six microsatellite loci isolated from *Pseudacris feriarum* and *P. nigrita* and characterized in 85 individuals of *P. feriarum* from Macon Co., AL and nine microsatellite loci characterized in 142 individuals of *P. maculata* from five locations across Yellowstone National Park, WY

Locus name	Species	Primer sequence 5'-3' and Label	Multiplex	Repeat motif	Size (bp)	Prop. Genotyped	A_N	T_a (°C)	H_O	H_E	HW *P* value
D6	*P. feriarum*/ *P. nigrita*	F: CTGCTGTGATATTTTTGTG NED R: GGTGTCGTGAGCTAAGTGTT	Lemmon_1	$(TATC)_9(TA)_2$ $(TATC)_5TTTC(TATC)_3$	100–372	844/863	42	54	0.8000	0.9337	0.0022*
D_C08b	*P. feriarum*/ *P. nigrita*	F: CTTACACAGCTCCATAGAATATGACA HEX R: ACAAACCTACAGGAGCTGATAGAAT	Lemmon_1	$(TA)_3TGGGG(TC)_5$ $CCTCCGCTCCCTTC(CT)_6$ $ATACATGCATATAGAT(ATAG)_{16}$ $ATGTAGA(TA)_3$	227–445	845/863	70	54	0.8000	0.9433	0.0000*
D_D12b	*P. feriarum*/ *P. nigrita*	F: TATAACATGTAACTGGGCTAACA FAM R: AGGAGAAGAGCCATTTCCTG	Lemmon_1	$(TCTA)_{30}$	268–532	802/863	52	54	0.8353	0.8869	0.4316
D_D10	*P. feriarum*/ *P. nigrita*	F: CTCTACATACATTTACCTTCTACCTTC NED R: GCTGTCTACTGAATTTATACTGTAAGG	Lemmon_2	$(TCTA)_{22}$	153–398	842/863	46	54	0.4235	0.4821	0.0929
D_D10b	*P. feriarum*/ *P. nigrita*	F: AATAGGTCAGCACATCCAAA FAM R: CTGCATGATGGAATAAACCTAT	Lemmon_2	$(TAGA)_9T(TAGA)_2$ $CAGA(TAGA)_3$ $CAGATAGACAGATAAA(CAGA)_5$	148–484	823/863	71	54	0.7176	0.9696	0.0195
D_E10	*P. feriarum*/ *P. nigrita*	F: TAGATCATCAGTTTCTGAAATACC HEX R: AAACTGAAGGATGAGTATGCTG	Lemmon_2	$(AC)_4A(TATC)_6TAA(TATC)_3$ $TCA(TATC)_3TAA(TATC)_3TCA(TATC)_3$ $TAA(TATC)_3TCA(TATC)_5$	258–520	774/863	71	54	0.7470	0.9552	0.0000*
A8	*P. maculata*	F: AAAAAGGGTCAGTGATAGCC FAM R: GACCTAAGCTCAGCACACTG	Murphy_2	$(CA)3CG(CA)_{16}$	90–145	132/142	7	58	0.63	0.62	0.6734
D11	*P. maculata*	F: CTTAAGATTCATACCAGGTGAAATAGG VIC R: CAAGGGAACAGTTTTCTTCA	Murphy_2	$(TCTA)_{16}$	70–95	142/142	5	58	0.48	0.51	0.4123
D_A10b	*P. maculata*	F: ACTACTTTACATTGTAGCAGAGTGTCAT VIC R: CATAACTTCATTGTTCAACTGGATG	Murphy_1	$(TA)_3TCAA(TCTA)_{13}$ $TCTCATA(TCTA)_{14}$ $TCTGTCAG(TCTG)_4$	290–310	139/142	6	58	0.56	0.54	0.5436
D_C08b	*P. maculata*	F: CTTACACAGCTCCATAGAATATGACA FAM R: ACAAACCTACAGGAGCTGATAGAAT	Murphy_2	$(TA)_3TGGGG(TC)_5$ $CCTCCGCTCCCTTC(CT)_6$ $ATACATGCATATAGAT(ATAG)_{16}$ $ATGTAGA(TA)_3$	260–300	139/142	16	58	0.79	0.81	0.4217
D_D11b	*P. maculata*	F: GTATGACAGAACTGATCTCTGC NED R: GAGTCGTTCACTTTCATTTATAGTC	Murphy_2	$(TCTA)_9$	370–390	133/142	11	58	0.72	0.74	0.2142
D_E04	*P. maculata*	F: ATGAAGAGTCTGACGTGTCTTTCTT PET R: CCCACATGCCTTACTTTCATAG	Murphy_1	$(TCTA)_{17}$	200–220	142/142	3	58	0.41	0.45	0.1514
D_E06	*P. maculata*	F: ATTCACTGAGTGGCAGTATTAAACA PET R: AGCTTTCTATCAGCTTTAGTGAGTCTAT	Murphy_1	$(GATA)_7GAT(GATA)_2$	410–450	114/142	5	58	0.62	0.65	0.4352
D_E10	*P. maculata*	F: TAGATCATCAGTTTCTGAAATACC FAM R: AAACTGAAGGATGAGTATGCTG	Murphy_1	$(AC)_4A(TATC)_6TAA(TATC)_3$ $TCA(TATC)_3TAA(TATC)_3$ $TCA(TATC)_3TAA(TATC)_3TCA(TATC)_5$	350–410	125/142	4	58	0.35	0.40	0.1034

Table 1 continued

Locus name	Species	Primer sequence 5'-3' and Label	Multiplex	Repeat motif	Size (bp)	Prop. Genotyped	A_N	T_a (°C)	H_O	H_E	HW *P* value
D_G08b	*P. maculata*	F: CTAAGGGGAGACATTGTAAAGTTGT PET R: CCTATTTAACACTTGTCTGTTCACT	Murphy_1	$(TAC)_2(TG)_4(TA)_2$ $TCTA(TG)_2(TA)_3(GATA)_{14}$	245–275	142/142	11	58	0.81	0.84	0.3212

For each locus the primer sequence and fluorescent label, multiplex combinations, repeat pattern, allele size range, proportion of individuals tested that were successfully genotyped at each locus, total number of alleles (A_N), annealing temperature (T_a), genetic variability measures (H_O and H_E), and Hardy–Weinberg *P* values are provided (significant values after a Bonferroni correction are indicated with an asterisk). For the primer sequence, "*F*" and "*R*" indicate the forward and reverse primers, respectively. Note that the first six loci were optimized for *P. feriarum* and *P. nigrita* (Lemmon multiplexes), whereas the last nine loci were optimized for *P. maculata* (Murphy multiplexes). Note also that two loci (D_C08b and D_E10) were used in both studies

primers were designed using PRIMER3 and tested in 75 markers from a tetranucleotide library and 5 from a dinucleotide library. Microsatellites were amplified in 10 µl reactions of 4.4 µl H_20, 1.0 µl 10× NH4-based reaction buffer (Bioline), 0.8 µl 10 mM dNTPs, 0.3 µl 50 mM $MgCl_2$, 1.0 µl 1.5 µM forward primer, 1.0 µl 1.5 µM reverse primer, 0.02 µl Biolase DNA polymerase (Bioline), and 1.5ul of 10–50 ng/µl DNA on a MJ Research Tetrad 2 thermalcycler using the following PCR program: (1) 95°C for 15 min, (2) 94°C denaturation for 30 s, (3) 48° or 52°C annealing for 1:30 min, (4) 72°C extension for 1:30 min with 35 cycles of steps 2–4. PCR products were run on agarose gels.

Fluorescent-labeled primers were tested for eighteen markers that amplified in both *P. feriarum* and *P. nigrita* (D6, D_C08b, D_D10, D_10b, D_D11b, D_D12b, D_E04, D_E06, D_E08b, D_E10, D_F03, D_F10, D_G05, D_G08b, D_G10b, D_F07, A8, A11). Six markers (Table 1) were selected following the criteria: (1) consistent amplification of a single polymorphic locus in both species, (2) low allelic dropout and/or null alleles determined from a pedigree analysis of 16 F1 hybrid families (Supplementary Table 1). The six loci were amplified in 10 µl multiplexed reactions using the QIAGEN Multiplex PCR kit (Qiagen Inc.). Three primer pairs were added to each well, including fluorescently-labelled forward primers with Hex, 6-FAM (Integrated DNA Technologies), and NED (Applied Biosystems). Reactions included 5 µl QIAGEN Multiplex PCR Master Mix, 1ul 10× primer mix containing 2 µM of each primer, 3 µl RNAase free H_20, and 1 µl DNA (10–50 ng/µl). PCR amplification was conducted as above, except using a 54°C annealing temperature. Fragment analysis was conducted on an Applied Biosystems 3730 DNA sequencer, using GeneScan 500 ROX size standard. Loci were genotyped with GeneMapper 4.0 (Applied Biosystems).

Genotypic data for 85 individuals of *P. feriarum* from Macon Co., Alabama were analyzed using GENEPOP (Rousset 2008). This sample included multiple ponds in Tuskeegee National Forest. The null hypothesis of Hardy–Weinberg equilibrium was rejected for three of six loci in Table 1 following a Bonferroni correction, probably as a consequence of combining ponds. No linkage disequilibrium was observed (dememorization, 1,000; number of batches 100; number of iterations per batch 1,000) following a Bonferroni correction. Heterozygosity was less than expected at all loci but high allelic diversity (42–71 alleles/locus) was observed. The frequency of null alleles was estimated from a pedigree analysis of multiple families at 0–31%, with only one locus above 16% (Supplementary Table 1). A relatively high frequency of null alleles, such as in D_E10 (31%), could affect tests of Hardy–Weinberg equilibrium and estimates of population subdivision, thus

Table 2 Pattern of cross-species amplification in thirteen microsatellites tested in seven *Pseudacris* species and twenty-three microsatellites tested in *P. maculata*

Locus name	*P. brachyphona*	*P. clarkii*	*P. fouquettei*	*P. kalmi*	*P. maculata*	*P. triseriata*	*P. feriarum*	*P. nigrita*
D6	2/2	2/2	2/2	0/2	0/2	2/2	2/2	2/2
D_C08b	2/2	2/2	2/2	2/2	0/2	2/2	2/2	2/2
D_D12b	n/a	n/a	n/a	n/a	n/a	n/a	2/2	2/2
D_D10	2/2	2/2	2/2	1/2	0/2	0/2	2/2	2/2
D_D10b	1/2	0/2	0/2	0/2	0/2	0/2	2/2	2/2
D_E10	0/2	2/2	2/2	1/2	2/2	1/2	2/2	2/2
D_G08b	1/2	1/2	2/2	2/2	2/2	0/2	2/2	1/2
D_E06	2/2	2/2	0/2	2/2	2/2	2/2	2/2	1/2
D_A10b	0/2	0/2	1/2	2/2	1/2	0/2	0/2	0/2
D_D11b	0/2	0/2	2/2	1/2	2/2	0/2	2/2	2/2
A8	n/a	n/a	n/a	n/a	2/2	n/a	n/a	n/a
D11	1/2	2/2	0/2	2/2	2/2	1/2	1/2	2/2
D_E04	0/2	0/2	2/2	2/2	2/2	0/2	1/2	2/2
A2	n/a	n/a	n/a	n/a	2/2	n/a	n/a	n/a
D_C12	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2
D_A08b	0/2	0/2	0/2	0/2	1/2	1/2	1/2	1/2
D_E09b	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2
D_B09	n/a	n/a	n/a	n/a	0/2	n/a	n/a	n/a
D_F07	2/2	2/2	2/2	0/2	2/2	2/2	2/2	2/2
D_F10	0/2	0/2	1/2	0/2	2/2	0/2	2/2	2/2
D_G05	2/2	2/2	2/2	0/2	2/2	2/2	2/2	2/2
D8	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2
D5	1/2	2/2	2/2	2/2	2/2	2/2	0/2	1/2
D_B10b	0/2	2/2	2/2	2/2	2/2	1/2	1/2	1/2

At least two individuals were tested for each species for each locus. Ratios indicate the proportion of individuals for which the locus showed amplification. An "*n/a*" indicates species that were not tested for a particular locus

reducing the utility of this marker. In the cross-species amplification tests, most loci amplified in most of the eight species, although no locus amplified in all species (Table 2).

After screening twenty-three loci in *P. maculata*, twenty of which amplified a band, fluorescent-labeled primers were tested for nine markers (A8, D11, D_A10b, D_C08b, D_D11b, D_E04, D_E06, D_E10, D_G08b; Table 1) on 142 individuals (methods from Murphy et al. 2010). Markers were selected based on: (1) consistent amplification of a single polymorphic locus, (2) ability to multiplex loci, and (3) low null alleles as determined from 47 trial samples using Microchecker (Van Oosterhout et al. 2006). Loci were amplified in two multiplexed reactions using the QIAGEN Multiplex PCR kit (Table 1). Multiplexes consisted of 3 µl Qiagen multiplex mix, 1.6 µl Q-solution, primer mix containing 0.09–0.17 µl 10 µM each primer, 1ul DNA ($\sim$5–50 ng/µL) and RNAase free H_20 to volume (8 µl). PCR was conducted on a Bio-Rad thermalcycler using the following program: (1) 95°C for 15 min, (2) 94°C denaturation for 30 s, (3) 58°C annealing for 1:30 min, (4) 72°C extension for 1:30 min, (5) 35–45 cycles of steps 2–4, (6) 72°C for 1 h, and (7) hold at 4°C. Fragment analysis was conducted on an Applied Biosystems 3100 DNA sequencer, using the GeneScan 500 LIZ size standard, and loci were genotyped with GeneMapper 3.8 (Applied Biosystems). Data were analyzed for heterozygosity, allelic diversity, Hardy–Weinberg and linkage disequilibrium using GENEPOP (Rousset 2008; Table 1). No violations of Hardy–Weinberg equilibrium or linkage disequilibrium were observed after Bonferroni correction. We now hope to employ the microsatellites developed in this study for various population genetic studies within the genus *Pseudacris*.

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