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PRIMER NOTE

Twelve novel microsatellite markers for the Great Plains toad, *Bufo cognatus*

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Abstract

Here I report on 12 microsatellite loci designed for populations of the Great Plains toad (*Bufo cognatus*) in the deserts of the southwestern USA. Diversity at these loci measured for 134 individuals from four breeding aggregations was relatively high with seven to 34 alleles per locus (mean = 17.8). Observed heterozygosities ranged from 0.444 to 0.949 and expected heterozygosities ranged from 0.597 to 0.951. These markers will be useful for studies of population genetic structure, parentage and relatedness in this explosively breeding amphibian.

Keywords: anuran, Bufonidae, desert, frog, population genetics, relatedness

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The Great Plains toad, *Bufo cognatus*, is a widespread North American amphibian that inhabits lowlands such as prairie and desert-scrub environments. Adults emerge from burrows following the onset of heavy rains in spring and summer (Bragg 1940; Bragg 1941) and they have an explosive aggregate mating system (Wells 1977) with individuals gathering in high numbers at ephemeral water bodies such as playas, ponds and ditches for short periods of courtship and egg-laying (Sullivan 1983; Krupa 1994). The sex-ratio of breeding aggregations is generally highly male-biased and males in some populations adopt one of two alternative reproductive tactics either vocalizing to attract females or waiting silently near vocalizing males to intercept females for amplexus (Sullivan 1982). Despite its large range and abundance at breeding aggregations, little is known about the population genetic structure or the genetic consequences of this mating system in *B. cognatus*. Both the mating system characteristics and the temporal and spatial patchiness of breeding sites, particularly in the arid southern portion of the range, lead to natural questions about population connectivity and reproductive success in this species. Here I report on the development of 12 novel microsatellite loci for studies of population structure, kinship and paternity in the Great Plains toad.

Whole genomic DNA was extracted from a toe clip of single individual preserved in 100% EtOH (voucher: CUMV 13406) using lysis buffer and proteinase K followed by organic clean-up with phenol–chloroform (Sambrook & Russell 2001). I digested 300 ng of DNA with the restriction enzymes *Alu*I and *Hae*III (New England Biolabs) and ligated SNX linkers to the fragmented DNA using T4 DNA ligase (Hamilton *et al*. 1999). I enriched the pool of ligated fragments by hybridization with biotin-labelled di-, tri- and tetranucleotide repeats in three reactions each with a 10-min 97 °C incubation followed by a 60-min incubation at 52, 60, or 65 °C. I recovered single-stranded fragments containing microsatellite repeats using streptavidin-coated magnetic beads (New England Biolabs) and used polymerase chain reaction (PCR) with the SNX primer to make fragments double-stranded. PCR products cut with *Nhe*I (New England Biolabs) and the digested product was electrophoresed on a 1% TAE agarose gel for size selection. I excised product from 300 to 900 bp in size and purified this using a QIAquick Gel Extraction Kit (QIAGEN). The digested, size-selected fragments were ligated to pUC19 plasmids and transformed into DH5α T1 phage-resistant competent cells. I plated cells on Luria-Bertani (LB) agar/ampicillin plates and transferred colonies to nylon membranes that were then hybridized to ^{32}P radiolabelled probes of the same repeat motifs used in the enrichment and visualized on autoradiographs (Sambrook & Russell 2001). Clones were cultured overnight in Luria broth and DNA was extracted with a QIAprep Spin Miniprep Kit (QIAGEN).

I sequenced 144 positive clones using the forward M13F (5′-GTTGTAAAACGACGGCCAGTG-3′) and the BigDye

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Table 1 Primer sequences, amplification conditions and diversity in 12 microsatellite loci for *Bufo cognatus*. Significant departures from Hardy–Weinberg equilibrium are indicated by an asterisk (*)

	Locus (GenBank no.)	Primer (5′–3′)	Repeat	T_a	N/N_a	Size	H_O/H_E
1	BC52.10 (DQ922776)	F: 6-FAM — AGCCCTTTACATGCCTACAGTG R: AAATGCTTGCGATTGGTGAAA	$(GATA)_3GAT(GATA)_{11}$	56	139/12	157–209	0.885/0.895
2	BC52.12 (DQ922778)	F: VIC — CAAAGAAACCTGACCGTGTGGAC R: ATCAATGGGGCAATCGTATGGA	$(GATA)_{23}(A)_5$	62	139/31	257–408	0.770/0.923*
3	BC60.35 (DQ922781)	F: NED — TCAGTAAACGGAATTCATCAAGTAGA R: GATTAGGGGGTTCTCTGTAGCAC	$(AC)_5(GC)_2AC(GC)_2(AC)_{20}$	57	137/34	211–289	0.949/0.951
4	BC60.37 (DQ922780)	F: 6-FAM — TCTTTAGATACATTGTAACCCTTTCCTTTTCATA R: GCTTGGCTATAGAACTATCAGTGACATTCAG	$(AG)_{27}$	60	139/22	318–372	0.935/0.898
5	bco39 (DQ922784)	U: PET — CGAGTTTTCCCAGTCACGAC F: UNIV — TTTATTCTACAGCCTTCACTTTCA R: GTTTAGCTCTCCGATTCACTGTAA	$(GT)_{22}$	60	139/12	106–140	0.892/0.840
6	BC52.03 (DQ922774)	F: VIC — TCAAAAATTTCCTGTTTTCCTCA R: CAGTACAATGCAGCAAGGACA	$(TAGA)_3TGGG(TAGA)_{15}$	60	139/22	350–528	0.914/0.915
7	BC52.04 (DQ922775)	F: 6-FAM — TTGAGAAAAGTGCCCATAAAGAGAAA R: GGCAGCAGAAAGGGTAGGTCCAC	$(AGAT)_{11}$	60	139/20	155–204	0.691/0.828*
8	BC52.11 (DQ922777)	F: 6-FAM — TTGCCATTGTGAAACCTAGAACCTG R: AGTGGCCTGGGTATACCTCATTA	$(ATAG)_{17}$	57	133/18	265–339	0.444/0.923*
9	BC60.20 (DQ922779)	F: VIC — GCTTCATTCTTTCCACCTCCGACTTA R: CTTTTTCTGGGTTGGCTGATGACAC	$(TC)_7TTT(CT)_{14}$	57	139/17	289–329	0.928/0.912
10	bco04 (DQ922782)	F: PET — CACAGCACTGATTGGCTCAT R: GTTTGGAAAATGGCGCTGTCTCTA	$(CA)_{10}$	58	139/7	147–155	0.691/0.714
11	bco15 (DQ922783)	F: NED — GGAGAACCTGCCATAATCACA R: GTTTGGAGACTTCAGCCTGTCTGG	$(TCTA)_4(TCCA)_6$	60	135/12	298–370	0.600/0.749*
12	bco40 (DQ922785)	F: NED — CACAGACTTGCTGAGGAGGA R: GTTTTCACCAGCAAGTGGCAGTAA	$(AC)_{18}GG(AC)_5$	62	139/7	133–165	0.597/0.597

F, forward primer; R, reverse primer; U, universal primer. 'UNIV' corresponds to the sequence 5′-CGAGTTTTCCCAGTCACGAC-3′. T_a, annealing temperature; N, number of individuals scored; N_a, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity.

termination sequencing chemistry (PerkinElmer) on an ABI PRISM 377 DNA sequencer (Applied Biosystems). I trimmed the pUC19 vector and SNX linker from sequences and aligned all clone sequences containing microsatellites to identify duplicates. I found 60 unique sequences containing microsatellites and designed primer pairs using the programs PRIMER 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and PRIMERSELECT (DNASTAR).

DNA was extracted from a 1 mm³ piece of toe-clipped tissue by incubation at 55 °C for 180 min and 99 °C for 10 min in a 5% Chelex solution (Chelex-100; Bio-Rad) with 19 μg proteinase K. The resulting supernatant was used directly as DNA template for PCRs. PCRs were carried out at the total volume of 10 μL on a PTC-100 thermocycler (MJ Research). For the majority of loci, this reaction included: 1 μL Chelex-extracted DNA template, 1× buffer (10 mM Tris-HCl, 50 mM KCl; Roche), 1.5 mM $MgCl_2$, 0.4 μM dNTPs, 0.2 μM of each primer (forward and reverse), and 0.25 U *Taq* polymerase (Roche). Locus BC60.35 amplified best at 1.25 mM $MgCl_2$. For locus bco39, I used the three primer system described by Waldbieser *et al*. (2003) with 0.1 μM of the forward primer with a 5′-tail of CGAGTTT-TCCCAGTCACGAC, 0.4 μM of the reverse primer with a 5′-tail of GTTT, and 0.2 μM of the 'universal' primer (see Table 1). The forward primer of each cleanly amplifying primer pair and the universal primer for locus bco39 were labelled with a fluorescent tag (VIC, PET, 6-FAM, or NED) for visualization on an ABI 3100 Automated Capillary DNA Sequencer using the GeneScan-500 LIZ size standard (Applied Biosystems). Sequences of clones used for primer design are accessioned in GenBank (DQ922774–DQ922785).

Following optimization, 12 microsatellite loci produced clean peaks on the automated sequencer (Table 1). PCRs for all primer pairs consisted of an initial denaturation of 94 °C for 5 min followed by 35 cycles of 1 min at 94 °C, 1 min at a primer specific annealing temperature (see Table 1), and 1 min at 72 °C. A final extension for 5 min at 72 °C followed the last cycle. I pooled PCR product from these 12 loci and an additional previously published locus (ihhh, Gonzales *et al*. 2004) into two multiplex groups (loci 1–6 plus ihhh, loci 7–12, Table 1) and combined 1 μL of the pooled PCR product with 19 μL Hi-Di formamide and 0.1 μL GeneScan-500 LIZ for visualization on the ABI

3100 Automated Capillary DNA Sequencer (Applied Biosystems).

I chose a subset of 139 adult individuals from four breeding aggregations in southeastern Arizona to quantify variation at these loci. I tested for evidence of linkage disequilibrium and departures from Hardy–Weinberg equilibrium (HWE) using the software GENEPOP ON THE WEB version 3.4 (Raymond & Rousset 1995). A Markov chain method (Guo & Thompson 1992) with 2500 dememorization steps and 100 batches of 5000 iterations per batch was used to determine significance. Four loci (BC52.04, BC52.11, BC52.12 and bco15) showed significant deficiencies in heterozygotes in global test. Two of these loci (BC52.11 and BC52.12) continued to show significant departure from HWE in tests for each population individually, suggesting the possibility of a null allele at each of these loci. In overall tests of linkage disequilibrium, some pairs of loci revealed significant linkage in some but not all populations suggesting that demographic or population-level parameters, rather than true linkage may be responsible for this pattern.

These pilot data suggest that these microsatellite loci will be informative for population genetic studies and quantifying reproductive success and relatedness. Five other microsatellite loci previously designed for *Bufo cognatus* exhibited similar levels of diversity (Gonzales *et al*. 2004); combining these sets of markers may allow for particularly high levels of resolution in studies of the molecular ecology of this species.

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