Molecular Ecology Notes (2004) doi: 10.1111/j.1471-8286.2004.00602.x

PRIMER NOTE

Isolation of polymorphic tetranucleotide microsatellite markers for the brown anole (*Anolis sagrei*)

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Abstract

Eleven polymorphic microsatellite markers have been developed for the brown anole, *Anolis sagrei*. The number of alleles range from five to 14 per locus with the observed heterozygosity ranging from 0.46 to 0.92. These markers will be useful for analysis of questions concerning population genetic structure and reproductive behaviour.

Keywords: *Anolis*, lizard, microsatellite, tetranucleotide

Received 26 November 2003; revision received 19 December 2003; accepted 19 December 2003

Anolis lizards in the Caribbean have undergone one of the most extensive vertebrate adaptive radiations yet studied in the wild (Williams 1983; Schluter 2000). Over 140 species of anole have radiated into six morphological classes termed 'ecomorphs' (*sensu* Williams 1983) that are differentiated by adaptations to habitat use, such as perching diameter and perching height (Losos 1990; Losos *et al*. 1998). Although much is known regarding Caribbean anoles (including their phylogenetic histories (Jackman *et al*. 1997) and morphological adaptations (Irschick *et al*. 1997), there is still a pressing need for finer scale genetic analysis using molecular markers for addressing questions of population genetic structure and reproductive behaviour. Here, we report on the development of 11 polymorphic microsatellite markers cloned from a Bahamian *Anolis sagrei*.

Microsatellite loci were isolated using a biotin capture method, as previously described (Bardeleben submitted) with some modifications. Briefly, genomic DNA was isolated from a toe clipping using standard proteinase K digestion followed by extraction with phenol/chloroform and precipitated with ethanol (Sambrook *et al*. 1989). Approximately 5 μg of genomic DNA was restricted with *Bst*YI, ligated to an adaptor generated by annealing together oligo A and oligo B (Refseth *et al*. 1997) and size fractionated on a 1% agarose/TAE gel. DNA in the 0.5–1.5 kilobase range was excised from the gel and the DNA purified by UltraClean (Mcfrugal). Approximately 1 μg of this material was used for subsequent hybridizations. Two separate hybridizations were carried out. Each hybridization was carried out with approximately 1 μg of the adaptor ligated size fractionated genomic DNA mixed with 50 nM biotin-labelled oligo probe (either 5′-$(AAGG)_6$GCA(Biotinyl-C)A-3′ or 5′-$(AAAG)_6$GCA(Biotinyl-C)A-3′ in 6X SSC (1M NaCl, 0.1 NaCitrate) overnight. Hybridization was carried out at 65 °C for the AAGG probe and 58 °C for the AAAG probe. Fifteen microliter aliquots of M280 strepavidin-coated magnetic beads (Dynal) were washed and equilibrated in 10 mM Tris-Cl pH 8, 1 mM EDTA, 1M NaCl, magnetically pelleted, resuspended with each hybridization reaction and incubated with occasional mixing at room temperature for 20 min. The beads were washed three times with 200 μL 1X SSC/0.1% SDS for 10 min at 10 °C below the annealing temperature to remove nonspecifically bound DNA. This was followed by two 30 second washes with 150 μL 2X SSC at room temperature to remove residual SDS. DNA was eluted from the beads in 40 μL H_2O at 95 °C for 10 min. Ten microliters of this fraction was used as template for a PCR amplification in a final volume of 30 μL containing 10 mM Tris-Cl pH 9, 2.5 mM $MgCl_2$, 50 mM KCl, 200 μM each dNTP, 50 pmol oligo A and 2.5 U *Taq* (Sigma). Amplifications were performed in an MWG Biotech thermal cycler: 3 min at 94 °C followed by 25 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s and extension at 72 °C for 3 min and a final extension step at 72 °C for 5 min. A second round of hybridization was carried out with 25 μL of this PCR-amplified DNA. Conditions were identical to those used for the first round selection except annealing was carried out in a final volume of 100 μL. Products from the PCR amplification of the

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Table 1 Characterization of 11 *Anolis sagrei* microsatellite loci

Locus	Primer sequences (5′–3′)	Repeat motif	T_a (°C)	Mg^{2+} (mM)	Size of cloned allele (bp)	Number of alleles	H_O	H_E	GenBank Accession number
AAGG-15	GCTTGACAGCCCAAAGC GCCACGGATAGACACCATC**	$(AAGG)_{11}$	54	1.5	109	9	0.46	0.84	AY444572
AAGG-38	GTTTATGAATCTGAATTGAAGAG TCCAGCCTTTATCTGAGTT**	$(AAGG)_7$	48	1.5	76	11	0.85	0.90	AY444573
AAAG-61	GCAACAGCTAATCCAGTGACATT GCTTACATTGAAGCCCATATTC**	imperfect*	52	2.0	324	5	0.69	0.70	AY444563
AAAG-63	CCTTTAGCCTTGCCAGAGTC** CCTAAGCACTAAACAGATGCC	$(AAAG)_{14}$	54	1.5	123	11	0.54	0.90	AY444564
AAAG-68	CTTCAGAGCAAGCGCAGGCAC TCCCTCCTTCTTTTCCCTCCGAG**	$(CTTT)_{18}$	52	1.5	120	11	0.69	0.90	AY444565
AAAG-70	GCAAGAGGTAGCCTCAGC GTTATCAGTACGAGGCACTG**	imperfect†	54	2.0	229	14	0.77	0.95	AY444566
AAAG-76	CGTTCTTGTTGCCATAAGAGG** AGGTGTTGCCCAGGTGTC	$(AAAG)_{13}$	54	1.5	130	8	0.60	0.89	AY444567
AAAG-77	GAGTAAAGGTCTGGGTCAGG GCAGTACAAATACCACAGAGC**	$(CTTT)_{16}$	54¶	1.5	128	10	0.85	0.84	AY444568
AAAG-91	CCTATTGGCTGTAGTTGTTG AATATCAGTCTCTCATCTATCC**	$(CTTT)_{15}$	54	2.0	110	10	0.92	0.91	AY444569
AAAG-94	GAAAATCCTGTGAATCCTGTG** GATACTAATCAAAGCCACTGT	imperfect‡	53	2.0	306	11	0.45	0.86	AY444570
AAAG-95	TTCACTTGGACAGCACTCAC** TCTGAGATGCACTGCTTAC	imperfect§	52	2.0	81	8	0.54	0.79	AY444571

Primer sequences, repeat motif, annealing temperature (T_a), Mg^{2+} concentration (mM), length (bp) of cloned allele, number of alleles, observed heterozygosity (H_O), expected heterozygosity (H_E), and GenBank accession number are given for each locus.
*$(AAAG)_6(AG)_2(AAAG)_5$.
†$(AAAG)_{15}(AG)_2(AAG)_3(AG)_4(AAAG)(AG)_2(ACAG)_5(AAAG)(AG)_2(AAAG)_6$.
‡$(AAAG)_{11}(AAGG)_3(AAG)(ACAG)(AAAG)(ACAG)(AAAG)_2(ACAG)(AAAG)_{13}(AG)_2(AAAG)_6$.
§$(CTTT)_4(CTTC)(CTTT)_2(CCTT)(CTTT)$.
¶using AmpliTaqGold (Perkin-Elmer).
**indicates labelled primer.

second round of hybridization eluate (1 μL) was ligated into pCR4-TOPO vector following the manufacturer's protocol and transformed into TOP10 One Shot Chemically Competent *Escherichia coli* cells (Invitrogen).

The PCR-based method by Gardner *et al*. (1999) was used to screen the clones. Briefly, a small amount of each colony was incubated at 94 °C for 10 min in 40 μL 10 mM Tris-Cl pH 8, 0.1 mM EDTA to lyse the cells. One microliter of this lysate was used as template in two parallel PCR reactions, one containing vector primers M13(–20) forward primer and M13 reverse primer and the other containing, in addition, a third nonbiotinylated primer with the motif used for selection [e.g. $(AAGG)_6$]. If a clone contains a repetitive element, an additional band or smear is expected for the PCR products carried out with three primers relative to the two primer PCR reaction. A total of 96 colonies were screened. For clones that were positive for a repetitive element the PCR product from the PCR amplification carried out with the two vector primers was gel purified and sequenced using ABI PRISM BigDye Ready mix (Applied BioSystems) using either the M13(–20) forward or M13 reverse primer and run on an automated DNA Sequencer (ABI Prism 377, PE, Applied Biosystems).

Primers were designed from the flanking sequences of unique clones to amplify the repetitive element. PCR conditions were optimized for each primer set and each locus was evaluated for polymorphism and heterozygosity in a total of 13 individuals captured from various islands in the Bahamas, including Crooked Island (2 samples), Staniel Cay (1), Fowl Cay (2), South Bimini Island (3), South Andros (2), Marsh Harbor (1), Grand Bahama (1) and Chub Cay (1) (Table 1). One primer was labelled with γ[^{32}P]-ATP using T4 polynucleotide kinase. The conditions for PCR were 10 mM Tris pH 9, 1.5 mM $MgCl_2$, 50 mM KCl, 200 μM each dNTP, 0.4 μM each primer and 0.5 U *Taq* (Sigma) in a final reaction volume of 25 μL. The PCR program was: 94 °C for 3 min, 28 cycles of 94 °C for 45 s, T_m for 45 s, 72 °C for 30 s, and a final extension of 72 °C for 5 min.

PCR products were mixed with formamide and loading dye, heat denatured at 94 °C for 3 min and resolved on 6% or 8% denaturing polyacrylamide gel.

Eleven polymorphic loci were found. The number of alleles ranged from five to 14 per locus. Observed and expected heterozygosity, and linkage-disequilibrium were calculated using GENEPOP (Raymond & Rousset 1995). Observed heterozygosity ranged from 0.45 to 0.92. Five of the 11 loci: AAGG-15; AAAG-63; AAAG-76; AAAG-94; and AAAG-95, were determined to be significantly ($P < 0.01$) out of Hardy–Weinberg equilibrium. This result could indicate the presence of null alleles at these loci. However, another study (Calsbeek *et al*. Submitted) using eight of the 11 primers and amplifying nearly 400 individuals suggests that the apparent lack of Hardy–Weinberg equilibrium is more likely related to the relatively small sample size in this preliminary data set. None of the loci were found to be linked. These markers are currently being used in analyses of genetic structure of several anole populations.

Acknowledgements

We thank Jonathan Losos for providing samples. This work was supported by NSF grants DEB69982736 and DEB9977072.

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