

Polymorphic microsatellite loci for the Puerto Rican crested anole (*Anolis cristatellus*) and their amplification in related Puerto Rican species

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Received: 31 January 2007 / Accepted: 9 February 2007 / Published online: 11 April 2007
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Abstract We isolate six highly polymorphic microsatellite loci for the Puerto Rican crested anole (*Anolis cristatellus*) from a genomic library enriched for CA repeats. The number of alleles per locus ranged from 14 to 19, with levels of observed heterozygosity ranging from 0.60 to 0.73. Most of these loci were successfully cross-amplified in other members of the *cristatellus* species group (*A. evermanni*, *A. gundlachi*, *A. krugi*, *A. stratulus*), but levels of polymorphism were lower.

Keywords *Anolis* lizards · Microsatellite · Puerto Rico

Two recent developments highlight the importance of molecular markers to the conservation biology of *Anolis* lizards. First, informed conservation of anole diversity requires the use of molecular markers to delimit and to diagnose cryptic species that occur within many widespread species complexes (Jackman et al. 2002; Glor et al. 2003, 2004, 2005). Second, anoles have established themselves as introduced or invasive species at a global scale, and molecular markers permit tracking the history and success of these introductions (Kolbe et al. 2004).

Because previous work on both topics is based primarily or exclusively on mitochondrial DNA, development of additional molecular markers is warranted.

Polymorphic microsatellite loci are available for three species: *Anolis sagrei* (Bardeleben et al. 2004), *Anolis roquet* (Ogden et al. 2002), and *Anolis oculatus* (Stenson et al. 2000). The polymorphic microsatellite markers reported here are the only ones available for Puerto Rican anoles. The Puerto Rican crested anole (*Anolis cristatellus*) is an ideal candidate for microsatellite development because it is likely a complex of evolutionarily distinct lineages and has established introduced populations in Florida and elsewhere (Leal and Fleishman 2004; Guyer and Donnelly 2005). It is among the most abundant and conspicuous lizards on Puerto Rico and a model for ecological and evolutionary studies. Here we present a set of polymorphic microsatellite loci for this species and test the utility of these markers in related members of the *Anolis cristatellus* series (sensu Brandley and De Queiroz 2004).

Development of a library enriched for CA repeats was contracted through BC Research Inc. (Vancouver, BC, Canada; now at Genetics Inc.), where isolation of microsatellite loci was implemented following a protocol outlined in Khasa et al. (2000). Briefly, this protocol involved: (1) ligation of adaptors (containing Eco RI sites) to total genomic DNA digested with Hae III, Rsa I and Alu I, (2) selection with biotinylated oligonucleotide poly TG12 and streptavidin magnetic beads, (3) amplification with an adaptor-specific primer and cloning the products into the Eco RI site of pGEM -3z(+), and (4) transformation into *E. coli* and screening of bacterial colonies for microsatellite loci via hybridization with ³²P-labeled poly-CA. PCR amplification was performed directly on resulting glycerol stocks by placing a micropipette tip into the partially thawed stock solution and then swirling this

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tip into a 25 µl PCR solution composed of 0.2 µM of each commercially available vector primer, 0.2 mM of each dNTP, 2.5 mM MgCl₂, 0.25 µl of taq polymerase (Promega, Madison, WI, USA), 2.5 µl of Promega 10× buffer (500 mM KCl, 100 mM Tris-HCl, pH 9.0, 1.0% Triton[®] X-100), and 12.5 µl distilled H₂O. Amplification entailed 30 cycles of 30 s denaturation at 95°C, 30 s of annealing at 50°C, and 60 s of extension at 72°C. PCR products were sequenced using BigDye Terminator cycle-sequencing mix (Applied Biosystems) and an amplification profile consisting of: initial denaturation at 96°C for 120 s followed by 35 cycles of 96°C for 15 s, 50°C for 1 s, 60°C for 240 s, and a final extension after the last cycle at 30°C for 60 s. Sequencing reactions were run on an MJ Research BaseStation and analyzed via the Cartographer software package (MJ Research). Sequencing identified 134 unique clones containing CA repeats. Primers to amplify internal repeats were designed with Primer3 (http://www.broad.mit.edu/cgi-bin/primer/primer3_http://www.cgi).

Following elimination of sequences that were inappropriate for primer development because of size or occurrence of additional repetitive elements in flanking sequence, primer pairs were developed for 34 clones. Amplification of microsatellite loci using these primers involved 30 cycles of 95°C for 30 s, 50–63°C for 30 s, and 72°C for 60 s. PCR conditions were optimized via experimentation with six non-commercial buffers and a gradient thermal cycler (Tgradient, Biometra). Each buffer consisted of 20 mM Tris (pH ranging from 8.4 to 9.2; see Table 1), 50 mM KCl, and 2.5 mM MgCl₂. The PCR reaction mix contained 1 µl of the preferred buffer, 0.2 µM of each primer, 0.2 mM of each dNTP, 0.25 µl of Promega taq polymerase, and 6 µl of distilled H₂O, making a total reaction volume of 10 µl. Following elimination of an additional 19 loci because of poor amplification, multiple bands, or uninterpretable banding patterns, fluorescent dye-labeled primers were obtained for 15 loci. Six of these loci showed clean, interpretable bands and high levels of polymorphism (Table 1).

Genotyping included 141 individuals of *Anolis cristatellus*, representing 17 populations sampled from across Puerto Rico (Table 1). Heterozygosities (observed and expected) and tests for Hardy-Weinberg equilibrium and linkage disequilibrium were conducted with Genepop (<http://wbiome.curtin.edu.au/genepop/>). Because previous molecular and morphological studies reveal extensive geographic variation across the range of *A. cristatellus* (Heatwole 1976; Leal and Fleishman 2002, 2004), tests were conducted on a pooled sample containing all individuals, as well as each population individually. Observed heterozygosity was lower than expected and Hardy-Weinberg equilibrium was rejected for all loci when populations were pooled into a single group (Table 1). Similarly,

Table 1 Polymorphic microsatellite loci for *Anolis cristatellus*

Locus	Primer sequences (5'-3')	T _a (°C)	Size of cloned allele (in nucleotides)	Size range (amplified allele)	PCR buffer (pH of Tris)	Individuals sampled	# of Alleles	H _o	H _E	HWE	GenBank accession
CRIS 22	F: CGCATACCATATGTACACTCAAGTCC R: AACCCGAAGCGTTTCTTCCT	63	194	165–207	Any (pH 8.4, 8.8, or 9.2)	121	14	0.64	0.86	P < 0.001	DQ644569
CRIS 92	F: CTCAGAGGAAGAACTGGCAGAAC R: ACGAGATGGAGTGGGATCAA	60	413	297–325	Any (pH 8.4, 8.8 or 9.2)	128	15	0.70	0.89	P < 0.001	DQ644570
CRIS 124	F: TTGTGGACTCTCCAACCTATAATTC R: TTCCAAGTTGTCTCTGTAGATTGT	62.5	247	166–198	H (pH 8.8), L (pH 9.2)	106	15	0.61	0.89	P < 0.001	DQ644571
CRIS 128	F: TCCAGCTCTGAAAAATTAT ACGAACATAC	58	403	145–173	D (pH 8.4)	102	14	0.72	0.89	P < 0.004	DQ644572
CRIS 136	R: CAGCAGTTAAAGGGCGGAAA F: TTCTGCGGGAAGGTAACGATG R: TTTTGGGTGAGATAAGAAACAAA	62.5	337	196–240	Any (pH 8.4, 8.8, or 9.2)	136	19	0.73	0.89	P < 0.001	DQ644573
CRIS 140	F: ATTTGGCTGGTGCTTAGT R: TGTTTGTGATGGGAGGAAAAGA	62.5	297	188–244	H (pH 8.8)	133	16	0.60	0.93	P < 0.001	DQ644574

These results are for all sampled populations combined. H_o and H_E are the observed and expected heterozygosities, respectively. HWE is the tail probability of getting by chance alone departures from HW equilibrium as large or greater than those observed

Table 2 Results from related Puerto Rican species

	<i>A. gundlachi</i> (n = 15)		<i>A. krugi</i> (n = 10)		<i>A. stratulus</i> (n = 8)		<i>A. evermanni</i> (n = 2)	
	Sample	Alleles	Sample	Alleles	Sample	Alleles	Sample	Alleles
CRIS 22	–	–	–	–	3	1	1	1
CRIS 92	14	7	9	3	8	2	2	1
CRIS 124	14	9	–	–	–	–	1	2
CRIS 128	13	4	6	1	8	2	2	2
CRIS 136	11	5	9	5	8	1	2	2
CRIS 140	7	8	2	1	6	3	3	2

significant linkage disequilibrium was detected among all loci in the pooled sample (Table 1). However, Hardy-Weinberg equilibrium could not be rejected in most cases when populations were considered individually. Significant linkage disequilibrium was detected between only two markers in the analysis of individual populations: CRIS136 and CRIS140.

Cross amplification was attempted in four additional species of Puerto Rican anoles: *A. krugi*, *A. gundlachi*, *A. evermanni*, and *A. stratulus*. All of these species belong to the *crisatellus* species group, which includes eight Puerto Rican species and two species endemic to small islands off the coast of Puerto Rico (Brandley and De Queiroz 2004). Although most loci were successfully amplified in these other species, few were polymorphic, and none approached the levels of polymorphism observed in *A. crisatellus* (Table 2). Thus, the loci developed here for *A. crisatellus* are likely to be of limited value in other Puerto Rican anoles. However, their use has yet to be evaluated in the four members of the *crisatellus* species group that are most closely related to *A. crisatellus*: *A. monenesis*, *A. scriptus*, *A. ernestwilliamsi*, and *A. cooki* (Brandley and De Queiroz 2004). The latter two species are phylogenetically nested within populations of *A. crisatellus* that exhibit high levels of polymorphism (R. E. Glor, unpublished data), suggesting the possibility that polymorphism in these markers may also be observed in *A. ernestwilliamsi* and *A. cooki*. It is particularly important to test these markers on *A. cooki*, a protected species needing urgent conservation measures (Genet 2002).

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