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PRIMER NOTE

Ten polymorphic tetranucleotide microsatellite markers isolated from the *Anolis roquet* series of Caribbean lizards

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Abstract

The *Anolis roquet* series of Caribbean lizards provides natural replicates with which to examine the role of historical contingency and ecological determinism in shaping evolutionary patterns. Here, we describe 10 polymorphic tetranucleotide microsatellites to facilitate studies on population differentiation and gene flow. All loci successfully amplified in several species from this series. Genotyping 96 individuals from two *A. roquet* populations demonstrated the markers' suitability as population genetic markers: genetic diversity was high (9–22 alleles per locus); there were no instances of linkage disequilibrium; and, with one exception, all genotypic frequencies conformed to Hardy–Weinberg equilibrium expectations.

Keywords: anole lizard, *Anolis roquet*, DNA, enrichment protocol, microsatellite

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The *Anolis roquet* series of tree lizard inhabits the southern Lesser Antillean Islands of the Caribbean. Geographical isolation over millions of years has resulted in inter-island speciation (Creer *et al*. 2001). Although most islands have only a solitary species and no island supports more than two, substantial intraspecies geographical variation in colour pattern has occurred within islands, driven by a combination of historical contingency and ecological determinism (Thorpe 2002; Thorpe & Stenson 2003). As such, the *A. roquet* series provides natural replicates with which to examine the role of historical divergence and selection in determining evolutionary patterns.

Polymorphic codominant markers additional to the eight existing microsatellites for *A. roquet* (Ogden *et al*. 2002) are now required to increase statistical power in analyses of population differentiation and gene flow within the species of this series. Here, we report the identification of 10 polymorphic microsatellite loci from the *A. roquet* series, each of which is amplifiable in multiple species from this group. Their suitability as population genetic markers is illustrated from *A. roquet* sample genotyping.

A genomic library was constructed and enriched for tetranucleotide microsatellite repeats for six *A. roquet* series species (*A. roquet* from Martinique, *A. bonairensis* from Bonaire, *A. trinitatis* from St Vincent, *A. richardii* and *A. aeneus* from Grenada, and *A. extremus* from Barbados). Genomic DNA was extracted from autotomized tail tips stored in 100% ethanol using a QIAGEN DNeasy Tissue Kit according to the manufacturer's instructions for purification from rodent tails. For each species, 5 ng DNA was pooled from equal amounts of DNA from eight lizards, which had been sampled from different locations spread across the species geographical range. We then followed a modified enrichment technique developed by Gardner *et al*. (1999), which is based on magnetic/biotin capture of repetitive sequences from restricted DNA, with minor modifications:

1 After the addition of the magnetic bead mixture to the prepared DNA fragments, the beads were washed eight times in 100 μL of 1 × SSC (0.15 M NaCl, 15 mM trisodium citrate) with 10 pmol of linker oligo A (S61: 5′-GGCCAGAGACCCCAAGCTTCG-3′). The first four washes were performed at 40 °C, the latter four at 50 °C.
2 Initially, four biotinylated oligos were used to enrich for AAAG, TCAG, TACA and TAGA repeat microsatellites in *A. bonairensis*, *A. trinitatis* and *A. roquet*. The success of isolating TAGA repeats in particular led to enrichment for this repeat only for the remaining species.
3 pCR2.1-TOPO vector and TOP10F′ competent cells were used for cloning, according to TOPO TA Cloning Kit instructions (Invitrogen).

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Table 1 Polymorphic microsatellite loci for the *Anolis roquet* series. Locus name prefix indicates the species of origin: Aae, *A. aeneus*; Abo, *A. bonairensis*; Aex, *A. extremus*; Ari, *A. richardii*; Aro, *A. roquet*; Atr, *A. trinitatis*. The repeat motif and GenBank Accession number of the sequenced clone are given. Primer sequences, annealing temperature (T_a in °C) and $MgCl_2$ concentration ($MgCl_2$ (mM)) for optimal PCR amplification in the species of origin are given for each locus, alongside the number (*N*) and range (in base pairs) of alleles found among six specimens of the species of origin sampled from different localities. Number of alleles, allele size range, and expected (H_E) and observed (H_O) heterozygosities per locus averaged over two sampled populations are described for the eight microsatellites that were polymorphic within *A. roquet* (*n* = 96 from two disparate populations from Martinique)

					Species of origin screening (*n* = 6)		*A. roquet* screening (*n* = 96)				
Locus	Repeat motif	Primer sequence (5′–3′) (F, forward; R, reverse)	T_a (°C)	$MgCl_2$ (mM)	*N*	Allele size range (bp)	*N*	Allele size range (bp)	H_E (± SD)	H_O (± SD)	GenBank Accession no.
AaeP2F9	$(CTAT)_{13}$	F: CAATGTTTTGCTCTTGCTATTT*	55	2.5	5	219–243	16	223–281	0.86 (± 0.00)	0.89 (± 0.03)	DQ379371
		R: GGCTGATTTGTCCTTTCTGG									
AaeP2F5	$(CTAT)_8$	F: GCAAAGGCAATAGGAAAAGG*	55	1.5	9	268–326	15	272–352	0.86 (± 0.00)	0.84 (0.03)	DQ379372
		R: GTTGGCGATGTCCCATAAAC									
AboP4A9	$(CTAT)_9$	F: GTGACTATGAAGGGGAATCTTG*	55	1.5	4	359–371	12	335–365	0.51 (± 0.24)	0.46 (0.23)	DQ379373
		R: GATGTAGGCTTTGCTGCTGT									
AexP2E3	$(CTAT)_{13}(AC)_8$	F: TCTTCCTCCCTTTCCCAGAT*	55	2.5	8	207–257	18	211–263	0.86 (± 0.02)	0.78 (0.13)	DQ379374
		R: TAGCTTCCCCTTTTGCTTTG									
AexP1H11	$(CTAT)_{11}$	F: GCTATCCATCCATCATTTCTATGT*	50	3.5	7	273–303	20	249–301	0.91 (± 0.01)	0.83 (0.08)	DQ379375
		R: AAACTGTAATTCCCAAGATTCCA									
AexP4H6	$(CT/CAT)_{17}$	F: TCTGGGTTTTCTGGAAGCTG*	53	3.5	7	167–217	22	171–231	0.90 (± 0.01)	0.74 (0.17)	DQ379376
		R: TCAAACCATGTAGGAACCTGTG									
AriP2D8	$(CT/CAT)_{24}$	F: GGAGCAGAAAGAGAAGAAACATC*	53	3.5	3	227–307	NA	NA	NA	NA	DQ379377
		R: TCAAACGGGAAAACAAGAAC									
AroHJ2	$(TAGA)_{10}$	F: ACATGAATGGTGGGAG*	60	1.5	4	218–226	9	210–242	0.77 (± 0.06)	0.70 (0.08)	DQ379378
		R: TTGACCACACTCTGATGTTGC									
AroHJ5	$(TAGA)_{11}$	F: TCTTGGAGAAAAGGCAGAAAG*	55	3	4	211–223	16	187–273	0.84 (± 0.00)	0.70 (0.05)	DQ379379
		R: CTGGAGGCCTACACTATGTCC									
AtrP16.55	$(CTAT)_6CAT$ $(CTAT)_{12}CGT(CTAT)_6$	F: GATAGTGGGCTGGGGAGAG*	50	3.5	11	97–149	NA	NA	NA	NA	DQ379380
		R: CCCGCTCCTGAGATAGATTG									

*Fluorescent dye-labelled primer (CY5 or CY5.5 dye).

Table 2 Cross-species amplification of 10 microsatellite primer pairs within the *Anolis roquet* series. Two samples per species were screened for PCR amplification of a well-defined band in the expected size range (+, presence; –, absence), using conditions listed in Table 1 and visualized on 2% agarose. Additional PCR optimization may recover loci not shown to amplify here

	Anoles						
Locus	*A. aeneus*	*A. bonairensis*	*A. extremus*	*A. griseus*	*A. richardii*	*A. roquet*	*A. trinitatis*
AaeP2F9	+	–	+	+	+	+	+
AaeP2F5	+	–	+	+	+	+	+
AaeP2E3	–	–	+	+	–	+	+
AboP4A9	–	+	–	+	+	+	–
AexP1H11	+	+	+	+	–	+	–
AexP4H6	+	+	+	+	+	+	–
AriP2D8	+	+	+	+	+	+*	+
AroHJ2	–	+	+	–	+	+	–
AroHJ5	+	+	+	+	+	+	+
AtrP16.55	+	+	+	+	+	–	+

*Some genotyped samples (see Table 1) have an ambiguous banding pattern.

4 For the detection of microsatellite-containing clones using polymerase chain reaction (PCR), we used Promega PCR buffer and *Taq* DNA polymerase with M13 forward (–20: 5′-GTAAAACGACGGCCAG-3′) and M13 reverse (5′-CAGGAAACAGCTATGAC-3′) primers, running the same PCR program used to amplify the captured DNA fragments.

Purified PCR products of 144 cloned inserts likely to contain a microsatellite were then sequenced by MWG-Biotech. Ninety-three of these contained a microsatellite (≥ 5 repeats). Some, however, were duplicates (n = 16) or had insufficient flanking region to enable primer design (n = 23). Fifty-five microsatellites were deemed unique and possessed adequate flanking regions for primer design, which was performed using PRIMER 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

Primers were tested on six individuals of the species from which the cloned insert was derived. Loci were amplified using 5 ng of template DNA, 1.5–3.5 mM $MgCl_2$ (Table 1), 0.2 mM of each dNTP, 0.5 μM of each primer (forward labelled with CY5 or CY5.5 dye), 0.5 U of *Taq* DNA polymerase and 1× buffer (Promega) in 10 μL. PCR was performed using a profile of denaturation for 2 min at 94 °C, followed by 30 thermal cycles (30 s at 94 °C, 30 s at a locus-specific annealing temperature [Table 1], and 30 s at 72 °C) and a final extension period of 5 min at 72 °C. A total of 10 loci yielded reproducible, easily interpreted polymorphic bands when analysed on a CEQ 8000 Genetic Analysis System (Beckman Coulter), with CEQ DNA Size Standard Kit-400 used as an internal size standard (Table 1).

To illustrate the utility of this microsatellite bank for *A. roquet* series population genetic studies, we screened eight *A. roquet* from three phylogenetic lineages (Thorpe & Stenson 2003). We then genotyped 96 *A. roquet* from two disparate populations at the eight loci that yielded reproducible, easily interpreted polymorphic bands for this species (Table 1). Genetic diversity was high: the number of alleles per locus ranged from nine to 22 (mean = 16; SD ± 4); gene diversity and observed heterozygosity per locus averaged over the two samples ranged from 0.51 to 0.91 (mean = 0.81; SD ± 0.13) and from 0.46 to 0.89 (mean = 0.74; SD ± 0.13), respectively. Tested in GENEPOP version 3.3 (Raymond & Rousset 1995), there were no significant genotypic linkage disequilibria among pairs of loci and all genotypic frequencies conformed to Hardy–Weinberg equilibrium expectations ($P > 0.05$, after Bonferroni correction), with the exception of a heterozygote deficit at locus AexP4H6 in population E1. F_{ST} estimates between the populations for each locus ranged from 0.01 and 0.45, and yielded a highly significant ($P < 10^{-5}$) global F_{ST} estimate of 0.10, suggesting that restricted gene flow and genetic drift are important determinants of genetic structure. Given the amplification of all 10 loci in multiple species from the *A. roquet* series (Table 2), this suite of markers serves not only to resolve patterns of gene flow within *A. roquet* but also enables the initiation of new genetic studies across the *A. roquet* series.

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