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TECHNICAL NOTE

Anonymous nuclear markers for the African adders (Serpentes: Viperidae: *Bitis*)

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Abstract We report five novel anonymous nuclear loci for the African viper genus *Bitis*, developed from a genomic library of a single individual puff adder (*Bitis arietans*). All loci amplify consistently and are variable among seven *B. arietans* sampled from disparate portions of the geographic range. Trials with nine congeneric species showed one locus amplifies across all species tested, three amplify in a subset of species and one only amplifies in *B. arietans*. Genetic divergences for these loci range from comparable, to more than double that of a commonly used nuclear protein-coding marker in *Bitis*. These loci will provide a valuable resource for future investigations of these snakes.

Keywords Anonymous nuclear loci · Phylogenetics · Phylogeography · Africa · *Bitis*

The use of multiple independent loci is now standard practise for phylogenetic and phylogeographic studies (Heled and Drummond 2010), however investigations at lower taxonomic levels may be hampered by a lack of availability of sufficiently variable nuclear markers (Brito and Edwards 2009). We report the development of five single-copy non-coding anonymous nuclear markers for the African viper genus *Bitis* to facilitate such studies.

The genus *Bitis*, commonly referred to as the African adders, represents Africa's most taxonomically diverse and geographically widespread genus of viperid snakes (Branch 1998), and includes well known and iconic large-bodied species such as the gaboon vipers (*B. gabonica*, *B. rhinoceros*) and medically significant puff adder (*B. arietans*). The genus also contains numerous smaller representatives, many of which have extremely restricted distributions within which several are threatened by habitat destruction and illegal collection for the pet trade (Branch 1999). Furthermore, two species (*B. inornata*, *B. schneideri*) are classified as vulnerable in the IUCN red list of threatened species (IUCN 2011).

We assembled a genomic library from a single *B. arietans* individual from Hartebeespoort, South Africa. Approximately 12 μg of genomic DNA was extracted from a large scale clipping using the high-salt method (Aljanabi and Martinez 1997) and randomly fragmented using the enzyme Rsa1. The resulting digest was visualised on a 1 % agarose gel and fragments within the range 0.5–1.5 kb excised and purified by gel extraction (Qiagen cat. no. 28704, eluted in 50 μL HPLC grade H_20). Size selected DNA was then centrifuged to achieve a final concentration >20 ng/μL and approximately 100 ng ligated into 25 ng of PCR Zero Blunt vector and transformed into competent One Shot TOP10 *Escherichia coli* cells (Invitrogen cat no. K280020), then grown overnight at 37 °C on agar plates containing kanamycin (50 μg/mL). Ninety-six individual colonies were selected and grown overnight at 37 °C with gentle shaking in 2 mL LB-kanamycin (50 μg/mL) liquid medium. Inserted fragments were then PCR amplified with standard M13 primers using 9.6 μL PCR Master Mix (Abgene, cat. no. AB-0575/LD/A), 0.27 μM of each primer and 0.5 μL of the culture medium. Forty-nine PCRs produced fragments >750 bp. These were cleaned using the enzymes exonuclease 1 and shrimp alkaline-phosphotase and sequenced by Macrogen Inc. (dna.macrogen.com).

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Table 1 PCR amplification success of anonymous nuclear loci for eleven representatives of the genus *Bitis*

Species, locality	Ba05[a]	Ba34[a]	Ba42[a]	Ba46[a]	Ba49[a]	PRLR[b]	ND2[c]
B. arietans, Morocco[d]	JX073336	JX073330	JX073319	JX073315	JX073313	JX073299	JX073288
B. arietans, South Africa[d]	JX073337	JX073329	JX073320	JX073316	JX073314	JX073300	JX073289
B. armata, South Africa	–	JX073324	–	–	–	JX073302	JX073291
B. atropos, South Africa	–	JX073325	–	–	JX073311	JX073298	JX073287
B. caudalis, South Africa	JX073333	JX073322	–	–	JX073312	JX073304	JX073293
B. gabonica, Congo	–	JX073328	–	JX073317	JX073309	JX073307	JX073296
B. parviocula, Ethiopia	JX073335	JX073327	–	JX073318	JX073310	JX073303	JX073292
B. rubida, South Africa	–	JX073323	–	–	–	JX073301	JX073290
B. schneideri, South Africa	JX073332	JX073321	–	–	–	JX073308	JX073297
B. worthingtoni, Kenya	–	JX073331	–	–	–	JX073306	JX073295
B. xeropaga, South Africa	JX073334	JX073326	–	–	–	JX073305	JX073294

GenBank accession numbers for anonymous loci, nuclear (PRLR) and mitochondrial (ND2) gene sequences are also shown

Dashes indicate no amplification. Thermocycling conditions: [a] 2 min at 94 °C, 35–40X [30 s at 94 °C, 30 s at 58 °C, 1 min at 72 °C], 5 m at 72 °C; [b] 2 min at 94 °C, 40X [30 s at 94 °C, 1 m at 52 °C, 45 s at 72 °C], 5 m at 72 °C; [c] 2 min at 94 °C; 35X [30 s at 94 °C, 45 s at 55 °C, 90 s at 72 °C]; 5 m at 72 °C. [d] Represents the latitudinal range limits of *B. arietans*

Table 2 Primer sequences for anonymous nuclear loci and comparisons of variability with the nuclear PRLR and mitochondrial ND2 genes

Locus	Primer sequences (5′–3′)	B.p.	I.d.	V.s.	P.i.	Mean p-dist	Max. p-dist	p-dist/PRLR	P-dist/ND2
Ba05	F: CATGTGACCAACCCGATTTT	776	4	50	46	0.024	0.037	0.957	0.144
	R: CAAGTGACTGATCTGCCATGA								
Ba34	F: CGAGGAGTGATGGGAGTTGT	523	4	46	38	0.021	0.037	0.837	0.117
	R: CTAGCACCTCGTGGCAGAT								
Ba42	F: CAATTAAACAAAGTTGTCCTTTGG	597	0	3	1	0.003	0.005	1.120	0.043
	R: GAGTGAAAGGCAGGAGGTTG								
Ba46	F: TACCATGAGTCCCCCTTCAG	822	6	95	93	0.059	0.093	2.107	0.396
	R: GGTCCGTCAGGTGTTGAAAG								
Ba49	F: CACCAAGACTCACAGGCAGA	570	5	63	59	0.045	0.081	1.867	0.267
	R: TCCACGCTGCTTTAATGTTG								
PRLR	PRLR_f1[a]	525	1	46	43	0.025	0.040	–	0.140
	PRLR_r3[a]								
ND2	L4437b[b]	1014	0	434	305	0.175	0.228	7.000	–
	tRNA-trpR[c]								

Length of sequence alignment (b.p.); Number of indels (i.d.); variable sites (v.s.); parsimony-informative sites (p.s.). Divergence measures (mean p-dist, max. p-dist) represent mean and maximum uncorrected p-distances for the sequences in Table 1. For diploid nuclear sequences, divergence was calculated from the most probable haplotype-pairs estimated using PHASE v.2.1 (Stephens et al. 2001; Stephens and Scheet 2005). Note that mean and maximum divergence values are not comparable between loci as sequences were generated for different combinations of species. p-dist/PRLR and p-dist/ND2 show divergence relative to PRLR and ND2, calculated from mean values. For loci where sequences were generated for a subset of individuals (Table 1), only PRLR and ND2 sequences from the corresponding individuals were used to calculate relative divergence, so these values are comparable between loci. [a] Townsend et al. (2008); [b] Kumazawa et al. (1996); [c] Ashton and de Queiroz (2001)

Sequences were BLAST searched and translated, and any coding sequences or transposable elements discarded. Primers were designed for the remaining 27 potential loci using Primer 3 (Rozen and Skaletsky 2000) and tested using seven *B. arietans* individuals sampled from disparate portions of the range. PCRs were set up as described previously and subjected to a standard touchdown thermocycling regime: 2 min at 95 °C; 10X [35 s at 95 °C, 35 s at 63 °C (−0.5 °C/cycle), 1 min at 72 °C]; 10X (35 s at 95 °C, 35 s at 58 °C, 1 m at 72 °C); 15X (35 s at 95 °C, 35 s at 52 °C, 1 m at 72 °C); 10 m at 72 °C. Consistently amplifying loci were sequenced and five were found to be variable. These were tested in congeneric species representing all major clades of *Bitis* (Lenk et al. 1999). One

locus amplified consistently across all species, three amplified in a subset of species and one only amplified in *B. arietans* (Table 1).

Levels of genetic divergence among the resulting sequences were compared with those from the mitochondrial NADH dehydrogenase subunit 2 gene (ND2) and the nuclear prolactin-receptor gene (PRLR), representing one of the most variable nuclear protein-coding loci with widespread applicability among squamate reptiles (Townsend et al. 2008). Three anonymous loci (Ba05, Ba34, Ba42) provided levels of divergence comparable to PRLR and two (Ba46, Ba49) provided considerably greater levels of divergence (Table 2). These loci will provide a valuable resource for future systematic and population genetic investigations of the genus *Bitis*.

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