Characterization of 11 cross-amplifying microsatellites in *Bitis arietans* (Merrem, 1820)

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Abstract. *Bitis arietans* is a widespread and abundant African viper for which minimal genetic resources exist. We screened 88 microsatellite loci published for species within the clades Crotalinae and Viperinae. We identified 11 microsatellite loci that cross-amplified in *B. arietans* and were polymorphic. We characterized the number of alleles, observed and expected heterozygosities, and probability of identity for each locus by genotyping 87 adults. These loci provide a resource for researchers working with the genus *Bitis*.

Keywords: *Bitis arietans*, microsatellite loci, puff adder

Introduction

The puff adder, *Bitis arietans* (Merrem, 1820), is an abundant heavy-bodied, medium-sized viperid snake (ca. 700–900 mm snout–vent length; Fig. 1), which occurs in savannas and open woodlands throughout most of sub-Saharan Africa, including isolated populations within southwestern Morocco (Brito et al., 2011) and parts of the Arabian Peninsula (Barlow et al., 2013). While puff adder venom has been extensively studied (e.g., Strydom et al., 2016) the behavioural ecology of this iconic African snake species has only recently received attention, with most efforts focused on foraging ecology and crypsis (Miller et al., 2015; Glaudas and Alexander, 2017a,b).

To date, genetic studies of this group have focused on deep evolutionary history (Wittenberg et al., 2015), and genetic resources useful to studies of evolutionary ecology are minimal. While Barlow et al. (2012) identified five anonymous nuclear markers derived from a *B. arietans* genomic library, there remains a need for readily deployable genetic markers for which analytical pathways are well suited (e.g., microsatellite loci). We screened published microsatellite markers developed from species within the clades Viperinae and Crotalinae to identify microsatellites in support of a larger study designed to assess parentage within *B. arietans*.

Materials and Methods

We used a standard protocol (Appendix 1; Rice and Clark, unpublished: doi: 10.1101/060038) to extract genomic DNA from tissue samples consisting of scale and tail clips preserved in 95% ethanol. Primers for microsatellite loci were identified by a literature search focused on cross-amplifying primers within the clades Viperinae and Crotalinae (Villareal et al., 1996; Gibbs et al., 1998; Holycross et al., 2002; Carlsson et al., 2003; Goldberg et al., 2003; Oyler-McCance et al., 2005; Munguia-Vega et al., 2009; Ursenbacher et al., 2009; Anderson et al., 2010; Metzger et al., 2011; Castoe et al., 2012; Pozarowski et al., 2012; Geser et al., 2013).

Primers were screened for amplification and polymorphism following a three-step protocol (Table 1). We first screened primers using a single adult *B. arietans* across a range of buffers with varying magnesium chloride concentrations (100 mM Tris, 500 mM KCl, 10 mM-45 mM MgCl₂) and a touchdown polymerase chain reaction (PCR) protocol (Don et al., 1991). PCR products were visualized on 2% agarose gels. Primers that successfully amplified with clear single or double bands below 500 bp were screened with a temperature gradient protocol utilizing the buffers with the strongest...
amplification activity. Finally, primers that produced quality bands were screened for polymorphisms by labelling the forward primer and screening the locus across eight individuals to maximize the number of primers screened on each 96-well plate.

Polymorphic loci were then formed into multiplexes based on shared conditions and primer-primer interactions detected by the National Institute of Standards and Technology primer tools (2005). Alleles were independently called by two researchers using GeneMarker version 2.5.2. Loci were characterized using the Excel package GenAlEx version 6.5 (Peakall and Smouse, 2006, 2012).

Table 1. PCR protocol and reaction conditions for microsatellite screening. Time is displayed in parentheses as min:sec. All PCR reactions were carried out in 10 µl volumes. Template DNA was plated in volumes to yield ca. 10 ng per reaction and was allowed to air-dry before reaction. PCR reagents per reaction were 1 µl dNTPs (100 mM), 1 µl buffer (variable MgCl₂; see text), 0.1 µl 500U Taq polymerase, primers (0.5 µl, 20 mM) for touchdown and gradient PCRs, variable for genotyping PCR (see Table 2), and milli-q water to 10 µl.

<table>
<thead>
<tr>
<th>PCR Step</th>
<th>Touchdown PCR</th>
<th>Gradient PCR</th>
<th>Genotyping PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denature</td>
<td>95°C (5:00)</td>
<td>94°C (3:00)</td>
<td>94°C (3:00)</td>
</tr>
<tr>
<td>Thermocycle Denature</td>
<td>20 Cycles 95°C (0:30)</td>
<td>20 Cycles 95°C (0:30)</td>
<td>20 Cycles 94°C (0:40)</td>
</tr>
<tr>
<td>Annealing</td>
<td>65°C, -0.5°C per cycle (0:30)</td>
<td>55°C (0:30)</td>
<td>55°C-65°C (0:40)</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C (0:30)</td>
<td>72°C (0:30)</td>
<td>72°C (0:45)</td>
</tr>
<tr>
<td>Final Extension</td>
<td>72°C (5:00)</td>
<td>72°C (5:00)</td>
<td>72°C (5:00)</td>
</tr>
</tbody>
</table>

Figure 1. Male and female *Bitis arietans* courtship. Male (top) courting female (beneath) and displaying the sexual dichromatism within the population at Dinokeng Game Reserve, Gauteng Province, South Africa. Photograph by Xavier Glaudas.
Results
We screened a total of 88 markers, of which 23 were screened for polymorphisms. We identified 11 polymorphic primers suitable for characterization (Brownstein et al., 1996). The magnesium chloride concentration in mM is listed under Buffer and the multiplex assignment is listed under Multiplex, wherein A and B denote separate reactions which were mixed post-PCR for fragment analysis. The sample size ($n$), number of alleles observed (K), size range, observed heterozygosity (Ho), expected heterozygosity (He), and probability of identity (POI) are listed for each primer.

Table 2. Characterization of 11 polymorphic microsatellite markers. The primer name is given in the first column with its associated source and sequences. For screening, the forward primer (F) was labelled with a fluorophore whereas the reverse primer (R) was pig-tailed to promote adenylation (Brownstein et al., 1996). The magnesium chloride concentration in mM is listed under Buffer and the multiplex assignment is listed under Multiplex, wherein A and B denote separate reactions which were mixed post-PCR for fragment analysis. The sample size ($n$), number of alleles observed (K), size range, observed heterozygosity (Ho), expected heterozygosity (He), and probability of identity (POI) are listed for each primer.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Source</th>
<th>Sequence</th>
<th>Buffer</th>
<th>Volume/10 µl rxn</th>
<th>Multiplex</th>
<th>K</th>
<th>Size Range</th>
<th>Ho</th>
<th>He</th>
<th>POI</th>
</tr>
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<tbody>
<tr>
<td>CC227</td>
<td>Pozarowski et al., 2012</td>
<td>F: GTGATGGTCCTGAGTGTGATGC R: GTGATGGTCCTGAGTGTGATGC</td>
<td>15 mM</td>
<td>0.300 µl</td>
<td>1.B</td>
<td>73</td>
<td>18</td>
<td>197–272</td>
<td>0.890</td>
<td>0.894</td>
</tr>
<tr>
<td>CRT147</td>
<td>Munguia-Vega et al., 2009</td>
<td>F: CAGGTGAGGAGAATGCAGTTCG R: GTGATGGTCCTGAGTGTGATGC</td>
<td>15 mM</td>
<td>0.125 µl</td>
<td>1.B</td>
<td>75</td>
<td>6</td>
<td>141–154</td>
<td>0.307</td>
<td>0.337</td>
</tr>
<tr>
<td>CS2322</td>
<td>Pozarowski et al., 2012</td>
<td>F: CAGGTGAGGAGAATGCAGTTCG R: GTGATGGTCCTGAGTGTGATGC</td>
<td>15 mM</td>
<td>0.350 µl</td>
<td>1.A</td>
<td>64</td>
<td>10</td>
<td>376–412</td>
<td>0.703</td>
<td>0.854</td>
</tr>
<tr>
<td>CWB23</td>
<td>Holycross et al., 2002</td>
<td>F: CAGGTGAGGAGAATGCAGTTCG R: GTGATGGTCCTGAGTGTGATGC</td>
<td>25 mM</td>
<td>0.300 µl</td>
<td>3.B</td>
<td>64</td>
<td>2</td>
<td>177–185</td>
<td>0.172</td>
<td>0.207</td>
</tr>
<tr>
<td>MF105</td>
<td>Oyler-McCance et al., 2005</td>
<td>F: CAGGTGAGGAGAATGCAGTTCG R: GTGATGGTCCTGAGTGTGATGC</td>
<td>20 mM</td>
<td>0.150 µl</td>
<td>2.B</td>
<td>70</td>
<td>14</td>
<td>165–192</td>
<td>0.943</td>
<td>0.877</td>
</tr>
<tr>
<td>SCU17</td>
<td>Anderson et al., 2010</td>
<td>F: CAGGTGAGGAGAATGCAGTTCG R: GTGATGGTCCTGAGTGTGATGC</td>
<td>20 mM</td>
<td>0.250 µl</td>
<td>2.A</td>
<td>62</td>
<td>2</td>
<td>172–180</td>
<td>0.129</td>
<td>0.121</td>
</tr>
<tr>
<td>VaP29</td>
<td>Geser et al., 2013</td>
<td>F: CAGGTGAGGAGAATGCAGTTCG R: GTGATGGTCCTGAGTGTGATGC</td>
<td>20 mM</td>
<td>0.150 µl</td>
<td>2.B</td>
<td>61</td>
<td>8</td>
<td>142–171</td>
<td>0.836</td>
<td>0.832</td>
</tr>
<tr>
<td>Vb21</td>
<td>Carlsson et al., 2003</td>
<td>F: CAGGTGAGGAGAATGCAGTTCG R: GTGATGGTCCTGAGTGTGATGC</td>
<td>40 mM</td>
<td>0.250 µl</td>
<td>3.A</td>
<td>65</td>
<td>9</td>
<td>169–192</td>
<td>0.446</td>
<td>0.575</td>
</tr>
<tr>
<td>VbA8</td>
<td>Ursenbacher et al., 2009</td>
<td>F: CAGGTGAGGAGAATGCAGTTCG R: GTGATGGTCCTGAGTGTGATGC</td>
<td>20 mM</td>
<td>0.200 µl</td>
<td>2.B</td>
<td>66</td>
<td>13</td>
<td>152–187</td>
<td>0.742</td>
<td>0.791</td>
</tr>
<tr>
<td>VbD’13</td>
<td>Ursenbacher et al., 2009</td>
<td>F: CAGGTGAGGAGAATGCAGTTCG R: GTGATGGTCCTGAGTGTGATGC</td>
<td>15 mM</td>
<td>0.300 µl</td>
<td>1.B</td>
<td>74</td>
<td>2</td>
<td>164–181</td>
<td>0.932</td>
<td>0.498</td>
</tr>
<tr>
<td>Vu55</td>
<td>Metzger et al., 2011</td>
<td>F: CAGGTGAGGAGAATGCAGTTCG R: GTGATGGTCCTGAGTGTGATGC</td>
<td>25 mM</td>
<td>0.125 µl</td>
<td>3.B</td>
<td>50</td>
<td>3</td>
<td>149–157</td>
<td>0.080</td>
<td>0.436</td>
</tr>
</tbody>
</table>

Discussion
Surprisingly, we identified microsatellite markers that were informative across the deep phylogenetic divides between Old World and New World vipers. For the successful amplification of microsatellite loci, primers must be complementary sequences to the genomic regions flanking the locus. Mutations within these flanking regions can prevent primer binding and locus amplification, which can occur even in isolated populations of a single species (Jarne and Lagoda, 1996). Thus, our finding of cross-amplifying and polymorphic microsatellite loci indicates conservation of genomic regions across lineages separated by 40–50 million years (Wüster et al., 2008). This suggests that a broader screening of microsatellite loci across deeper evolutionary time and phylogenies is warranted for viperid species for which genetic resources may be lacking. We found that microsatellite loci from the subfamily Crotalinae were the most informative of the polymorphic markers and should be considered for initial screenings of other viperids, even across divergent clades. These loci are informative for parentage analyses and meet the minimum recommended number of loci utilized in such analyses (Harrison et al., 2013). However, we were unable to characterize loci with
respect to linkage disequilibrium or conformation to Hardy-Weinberg proportions due to a lack of knowledge regarding population delimitation within the species, paired with potential biases from Wahlund Effects (Wahlund, 1928; Sinnock, 1975). These loci will allow for analyses examining relatedness and relationships in wild-caught *B. arietans* and may be leveraged with additional loci in the future to conduct detailed population genetic analyses to elucidate the evolutionary ecology of the species.

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Accepted by Hinrich Kaiser


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