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# Short Notes

## Microsatellite markers for *Bokermannohyla* species (Anura, Hylidae) from the Brazilian Cerrado and Atlantic Forest domains

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**Abstract.** We characterized 22 polymorphic microsatellite markers for the Brazilian treefrog *Bokermannohyla ibitiguara* and tested their cross-amplification in *B. alvarengai*, *B. circumdata* and *B. hylax*. Our focal species occurs in protected and disturbed Brazilian Cerrado landscapes, a highly threatened savanna in central Brazil. Fourteen markers successfully cross-amplified for at least one congener. These microsatellites will be useful for studies of mating systems, relatedness and landscape genetics of Cerrado populations under various deforestation levels. Moreover, variable markers for *B. circumdata* and *B. hylax* will also be useful for landscape genetic studies of taxa typical of the threatened Atlantic Forest domain.

*Keywords*: conservation, cross-amplification, Illumina MiSeq, marker identification, Neotropical frog, next-generation sequencing.

Landscape genetics explores the interaction between landscape variables and the evolutionary history of populations, thus quantifying the processes of migration and gene flow, genetic drift, and selection within and among populations (Manel et al., 2003; Holderegger and Wagner, 2008). Due to their high degree of polymorphism, microsatellite markers have been extensively used in landscape genetics for inference of recent demographic events, such as bottlenecks, reduced migration, and other genetic impacts of human activities (Pearse and Crandall, 2004). However, microsatellite marker specificity is typically high (Galbusera, Dongen and Matthysen, 2000; Duryea, Brasileiro and Zamudio, 2009) and for most non-model organisms, such as the frog genus *Bokermannohyla*, few microsatellite markers have been developed (Eterovick et al., 2012).

The hylid genus *Bokermannohyla* includes 33 species, distributed in both open and forested formations in central, south, southeastern, and northeastern Brazil (Faivovich et al., 2005; Frost, 2014). *Bokermannohyla ibitiguara* (Cardoso, 1983) is a stream-dwelling frog endemic to the Brazilian Cerrado, typical of the Serra da Canastra, State of Minas Gerais (MG), and surrounding mountainous areas, inhabiting gallery forests in both protected and anthropogenically altered landscapes (Haddad, Andrade and Cardoso, 1988; Nali and Prado, 2012, 2014). This species is classified as Data Deficient by the IUCN (Caramaschi and Eterovick, 2004), thus its true conservation status is unknown, precluding inference of its actual extinction risk (Morais et al., 2013). Very few studies have investigated the processes leading to genetic differentiation in frogs from open formations in the Neotropics (e.g., Maciel et al., 2010; Prado, Haddad and Zamudio, 2012). The Brazilian Cerrado is the most diverse and threatened savanna in the world (Silva and Bates, 2002); thus,

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this species is an excellent model for conservation genetic studies investigating the historical processes leading to diversification in the Cerrado and the genetic consequences of landscape fragmentation.

Here we characterize 22 microsatellite markers for *Bokermannohyla ibitiguara* and their cross-amplification in three closely related congeners: *B. alvarengai*, *B. circumdata*, and *B. hylax*. *Bokermannohyla alvarengai* also occurs in the Brazilian Cerrado, and the last two species occur in the Atlantic Forest, a highly diverse hotspot extremely threatened due to habitat loss (Ribeiro et al., 2009). For initial marker discovery, we created an enriched genomic library and used Illumina MiSeq next-generation sequencing to identify di-, tri- and tetra-repeats throughout the genome (Andres and Bogdanowicz, 2011). First we extracted whole genomic DNA from tissue of a single tadpole of *B. ibitiguara* with Qiagen DNeasy extraction columns. This individual was collected in the municipality of Sacramento, Minas Gerais state, Brazil, preserved in absolute ethanol, and deposited at Coleção de Anfíbios Célio F.B. Haddad, Departamento de Zoologia, I.B., Universidade Estadual Paulista, Rio Claro, São Paulo state, Brazil (tissue accession number: CFBH-T 11888). Genomic DNA (50-100 ng) was endonuclease-digested with AluI, RsaI, and Hpy166II, and pooled for subsequent adenylation with Klenow (exo-) and dATP. Restricted/adenylated DNA was then ligated to an Illumina Y-adaptor sequence using T4 DNA ligase in the presence of 1 mM ATP. Genomic fragments with repeats were captured by hybridization to biotinylated repeats and streptavidin-coated magnetic beads, and amplified/indexed with Platinum *Taq* polymerase and a pair of Illumina primers (one universal, one index primer). PCR products were quantified with a Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA), verified by electrophoresis on a 1.0% agarose gel, and size selected (300-600 bp) with AMPure beads (Beckman Coulter, Indianapolis, IN). We enabled the "design primers" function of msatcommander 1.0.3 software (Rozen and Skaletsky, 2000; Faircloth, 2008), also enabling the "repeats" and "primers" output files. Dimeric to tetrameric microsatellites with a product size range of 150-450 bp were selected.

From the above enriched microsatellite library, we optimized a total of 22 loci for *B. ibitiguara* (table 1) that met the follow-

**Table 1.** Primer sequences (Forward – F and Reverse – R), annealing temperatures ($T_a$), marker sizes (bp), allelic diversity ($N_a$), observed ($H_o$) and expected ($H_e$) heterozygosity of 22 microsatellite loci genotyped across three *Bokermannohyla ibitiguara* populations. Forward primers had the following sequence added to the 5′ end: CGAGTTTTCCCAGTCACGAC (see text for details).

| Locus (Genbank #) | Primer (5′ – 3′) | Repeat | $T_a$ (°C) | Size (bp) | $N_a$ | $H_o/H_e$ |
|---|---|---|---|---|---|---|
| Bi1 (KF977107) | F: AGGTGCGCTTGTAAGTATGAAAG R: GTTTAATACAGGGCGGTTCAGG | $(AGAT)_{10}$ | 64.5 | 219-274 | 9 | NSF: 0.375/0.775 CM3: 0.571/0.813 R1: 0/0.7* |
| Bi94 (KF977108) | F: GATGATGAAACCTGTCACTGACC R: AATTCCCAAACCATCATTTCCAG | $(AGAT)_{14}$ | 61.5 | 179-220 | 11 | NSF: 1/0.917 CM3: 0.857/0.879 R1: 0.429/0.703 |
| Bi179 (KF977109) | F: ACAACCTGATGATGTTACCAACC R: ATAGTTTGGACATGAGGACCCTG | $(AG)_9$ | 59.5 | 216-235 | 8 | NSF: 0.75/0.692 CM3: 0.714/0.714 R1: 0.625/0.758 |
| Bi383 (KF977110) | F: GATATTACATCCAACAGAGGGCG R: GTTGGTATAAAGTCCTGCCTCAC | $(AC)_{13}$ | 59 | 208-234 | 8 | NSF: 0.143/0.736* CM3: 0.5/0.682 R1: 0.143/0.143 |
| Bi609 (KF977111) | F: CTACTGCAGAGCCATACAAACTG R: CAGATGTCCATGAACCAACTCAG | $(ACT)_7$ | 62 | 321-357 | 10 | NSF: 0.75/0.875 CM3: 0.427/0.846 R1: 0.5/0.864 |

(Continued.)

**Table 1.** (Continued.)

| Locus (Genbank #) | Primer (5′ – 3′) | Repeat | $T_a$ (°C) | Size (bp) | $N_a$ | $H_o/H_e$ |
|---|---|---|---|---|---|---|
| Bi639 (KF977112) | F: AGTGGCCGTATATTTGATATGCAG R: AACAAAGTCCAGATCTTCACCTG | $(AAT)_{13}$ | 62 | 281-307 | 10 | NSF: 1/0.868 CM3: 0.833/0.864 R1: 0.625/0.75 |
| Bi1032 (KF977113) | F: ATGTGGTAAGATCTCCCTAGTGG R: TTCCAGTCTTGAGAAATTCTTTGG | $(AAAC)_7$ | 59 | 327-353 | 7 | NSF: 0.5/0.893 CM3: 0.333/0.758 R1: 0.4/0.6 |
| Bi1050 (KF977114) | F: GGCAGGCATCAGAGAAACTATG R: AATGCGTAGGTGTCTTTCTGAAC | $(AAT)_{11}$ | 64.5 | 203-222 | 7 | NSF: 0.429/0.494 CM3: 0.286/0.560 R1: 0.333/0.561 |
| Bi1122 (KF977115) | F: CTCATATGATTCAGAGGGTGGAC R: CCCTTTGGTCTTTCTTGTTCTCC | $(AC)_{13}$ | 59.5 | 213-235 | 5 | NSF: 0.25/0.8* CM3: 0.143/0.363 R1: 0.143/0.143 |
| Bi1397 (KF977116) | F: CTGCTTGAATCCAGGTCTGAATG R: CCGTCCCTTTATTGTCTCATTGG | $(AGAT)_{16}$ | 61 | 182-244 | 15 | NSF: 0.429/0.857 CM3: 0.857/0.934 R1: 0.6/0.8 |
| Bi1521 (KF977117) | F: TCAAACCACTCTCCATACGAATG R: GTTGCTGTAGACACTCTCTCTTG | $(AAT)_{10}$ | 59 | 278-313 | 7 | NSF: 0.286/0.791* CM3: 0.667/0.712 R1: 0.375/0.342 |
| Bi2312 (KF977118) | F: TCCCAACCTAGAATGCAGAGATC R: TCTCCCTGTAAATCTTGACTTTCC | $(AG)_9$ | 61 | 184-222 | 15 | NSF: 0.625/0.858 CM3: 0.571/0.802 R1: 0.286/0.835* |
| Bi2686 (KF977119) | F: CCCTTCAGTACTGTGACATCATG R: ATTGCGGAGAATTATTGACTCCC | $(ACAG)_{13}$ | 64.5 | 187-273 | 9 | NSF: 0.625/0.75 CM3: 0.5/0.818 R1: 0.571/0.758 |
| Bi2761 (KF977120) | F: GCCTGAAAGTGGAAGATGAGATC R: GGGCATCAACATTAAATCTCAAGC | $(AGG)_8$ | 59 | 240-278 | 10 | NSF: 0.667/0.712 CM3: 0.5/0.848 R1: 0.875/0.867 |
| Bi3003 (KF977121) | F: CGGCATCTTGGACCATTTATAGG R: TATCTCACAAACTGTCTGTGTCC | $(AGAT)_9$ | 64.5 | 322-460 | 11 | NSF: 0.571/0.824 CM3: 0.571/0.824 R1: 0.75/0.808 |
| Bi3029 (KF977122) | F: TTTGTTGAAATTTGGCACCCTGG R: ACTGCATCTCTCCTCACTAACC | $(AG)_{15}$ | 64 | 244-267 | 9 | NSF: 0.75/0.783 CM3: 0.714/0.857 R1: 0.571/0.802 |
| Bi3202 (KF977123) | F: AGGTATCCTCTTAGTTCTTGCCC R: GTCCAGTAAATATCAACCTGCCC | $(AAAG)_{13}$ | 57.5 | 204-281 | 8 | NSF: 0.75/0.742 CM3: 0.714/0.714 R1: 0.875/0.733 |
| Bi3370 (KF977124) | F: GAGACCACTGCCATAGACCATG R: GGAAACGTTCTCCAGTCAACTAC | $(AAC)_{12}$ | 56.5 | 266-288 | 7 | NSF: 0.625/0.683 CM3: 0.285/0.439 R1: 0.5/0.733 |
| Bi3438 (KF977125) | F: GTTGTCTTAGTAGTGCACGTGTG R: GTGACTTAACCCTTCACGTTCTC | $(AC)_7$ | 59 | 207-231 | 4 | NSF: 0.375/0.458 CM3: 0.429/0.385 R1: 0.4/0.711 |
| Bi3629 (KF977126) | F: CTTCCTCAGGGTCCTCAATCATC R: ATGATGGTGTGTACAGAACGAAC | $(AT)_{10}$ | 59 | 217-353 | 13 | NSF: 0.625/0.925 CM3: 0.429/0.846 R1: 0.4/0.711 |
| Bi3836 (KF977127) | F: GAAATTGCAGAGGGTCCTACTTG R: TGGAAGCGCATGTATATCAGTTC | $(AC)_{10}$ | 59 | 299-320 | 11 | NSF: 0.5/0.917 CM3: 0.286/0.648 R1: 0.5/0.817 |
| Bi4144 (KF977128) | F: CATACATGTGCCAACTTTGCTTC R: TAGTTATGCTGCACTTGTTGAGG | $(ACAG)_{12}$ | 59.5 | 191-289 | 17 | NSF: 0.625/0.942 CM3: 1/0.956 R1: 1/0.875 |

* Showed deviation from HWE, considering a $P$ value corrected for multiple comparisons (Bonferroni $P < 0.00227$).

ing criteria: (1) polymorphic, (2) with reliable amplification, and (3) clean genotyping peaks in preliminary scoring. Loci were optimized across 23 individuals of *B. ibitiguara* from three independent populations from Minas Gerais state, Brazil (population NSF: 20.2434°S, 46.4466°W; population CM3: 20.2273°S, 46.6164°W; population R1: 20.2727°S, 47.0735°W; see Supplementary table S1 online). The abbreviations represent population names (in Portuguese): NSF = Nascente do São Francisco, CM3 = Capão de Mata 3, and R1 = Riacho 1. All individuals were euthanatized by spraying a solution of lidocaine 10% in the gular region (McDiarmid, 1994), and liver tissue samples were collected. To test cross-amplification (table 2) we additionally genotyped 15 samples of *B. alvarengai* and *B. hylax*, and 16 samples of *B. circumdata*, collected in southeastern and southern Brazil (table S1). We extracted DNA from all individuals with DNeasy extraction columns (Qiagen, Valencia, CA, USA), following manufacturer's protocols. PCR profiles consisted of an initial denaturation step (94°C, 5 min) followed by 35 cycles of 1 min at 94°C, 1 min at primer-specific annealing temperatures (table 1), and 1 min at 72°C, followed by a final extension (75°C, 5 min). We performed PCRs in 10 $\mu$l reaction volumes, with 1 $\mu$l of template DNA (1-10 ng), 1× buffer, 1.5 $\mu$M $MgCl_2$, 0.1 $\mu$g/$\mu$l bovine serum albumin, 0.4 mM dNTP, 0.1 $\mu$M of the forward and reverse primers, 0.3 $\mu$M of universal dye-labelled primer, and 0.25 U *Taq* polymerase. PCR products included a 20 bp tag on the 5′ end of the forward primer (table 1) and were co-amplified with a fluorescently tagged third 'universal' oligonucleotide that hybridized to that tag. After amplification, we combined 1 $\mu$l of individual PCR products from various loci, diluted with 18.85 $\mu$l Hi-Di formamide and 0.15 $\mu$l GeneScan-500 LIZ, and ran the pooled samples on a 3730 ABI Prism Genetic Analyzer (Applied Biosystems, Foster City, CA) at the Cornell Genomics facility. We used GeneMarker v. 2.4.0 (SoftGenetics LLC, State College, PA) to analyze genotyping profiles, Microchecker v. 2.2.3 (Van Oosterhout et al., 2004) to check the final genotype assignments for null alleles, and Arlequin v. 3.5 (Excoffier and Lischer, 2010) to estimate observed/expected heterozygosities for each locus and population and test for linkage disequilibrium across all pairs of loci.

**Table 2.** Microsatellite amplification and number of alleles for *Bokermannohyla ibitiguara* and congeners (*B. alvarengai*, *B. circumdata* and *B. hylax*). Dashes represent non-genotyped loci due to poor or failed PCR amplifications. Sample sizes ($n$) are reported for each species, and numbers of successfully amplified congeners are shown in parentheses for loci with less successful genotyping.

| Locus | *B. ibitiguara* $n = 23$ | *B. alvarengai* $n = 15$ | *B. circumdata* $n = 16$ | *B. hylax* $n = 15$ |
|---|---|---|---|---|
| Bi1 | 9 | – | 10 | 7 |
| Bi94 | 11 | 7 | – | – |
| Bi179 | 8 | 10 | 15 | 7 |
| Bi383 | 8 | – | 1 | 1 |
| Bi609 | 10 | 5 | 6 | 2 |
| Bi639 | 10 | 6 | 9 | 9 |
| Bi1050 | 7 | – | – | 1 |
| Bi1397 | 15 | 16 | 13 | 8 |
| Bi2312 | 15 | – | 8 | 6 |
| Bi2761 | 10 | – | 4 (10) | 3 |
| Bi3029 | 9 | 3 (11) | 4 | 6 |
| Bi3202 | 8 | 2 (7) | 4 | 6 |
| Bi3370 | 7 | 1 | 6 | 4 |
| Bi3438 | 4 | 1 | – | 4 |

For *B. ibitiguara*, the 22 polymorphic loci ranged in allele numbers from 4-17 across all individuals (table 1). We found evidence for linkage disequilibrium between 12 pairs of loci (Bi3370/Bi1, Bi3003/Bi2312, Bi 3370/Bi2312, Bi2312/Bi3836, Bi1/Bi1397, Bi1032/Bi1397, Bi3029/Bi609, Bi2312/Bi2761, Bi1397/Bi2761, Bi1397/Bi639, Bi3836/Bi383 and Bi1397/Bi383) across all populations. For the CM3 population, all loci were under Hardy-Weinberg Equilibrium (HWE), but two loci showed possible null alleles (Bi609 and Bi3629). For the R1 population, two loci showed deviation from HWE (Bi1 and Bi2312), both possibly with null alleles. The NSF population showed nine loci with possibility of null alleles (Bi1, Bi1032, Bi4144, Bi3836, Bi3629, Bi1397, Bi1122, Bi1521 and Bi383), the last three loci

showing deviation from HWE. Null alleles can cause excess homozygosity, which in turn can cause linkage disequilibrium between markers (Sabatti and Risch, 2002). However, excess homozygosity may also be caused by other factors such as inbreeding within populations, or recent bottlenecks (Falush, Stephens and Pritchard, 2007). We plan further studies with larger sample sizes to investigate population-specific factors mediating genetic structure.

For the three *Bokermannohyla* congeners, 14 of the 22 loci showed visible PCR amplicons of the appropriate size (table 2). For *B. alvarengai*, seven loci were polymorphic, although two of these genotyped poorly, and two were monomorphic. *Bokermannohyla circumdata* individuals were genotyped at one monomorphic and ten polymorphic loci, although one of the latter genotyped poorly. For the *B. hylax* samples, eleven loci were polymorphic and two were monomorphic. We found no relationship between cross-amplification success and phylogenetic distance among species; *B. alvarengai*, which is closely related to *B. ibitiguara* (Faivovich et al., 2005), showed less successful amplification than the other two more distantly-related species. Although loci varied from monomorphic to highly polymorphic for the three congeners (1-16 alleles), *B. alvarengai* and *B. circumdata* had loci with the highest numbers of alleles (16 and 15, respectively) when compared to *B. hylax* (9 alleles at most).

The microsatellite markers designed and optimized in this study will be useful for future landscape genetic and mating system studies in *B. ibitiguara*, as well as in the three additional *Bokermannohyla* species. Combined, these species have dominant ranges that include the threatened domains of the Brazilian Cerrado and Atlantic Forest. Successful cross-amplification of the optimized markers demonstrates the utility of this technique, and increases the availability of genetic resources for this poorly known Neotropical frog genus.

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