



Research Note

Characterization of polymorphic microsatellite markers for the Neotropical leaf-frog *Phyllomedusa burmeisteri* and cross-species amplification

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ABSTRACT. Twelve polymorphic tetranucleotide microsatellite loci were isolated and characterized for the leaf-frog *Phyllomedusa burmeisteri*, an endemic species of the Brazilian Atlantic forest. These loci were screened in 25 individuals from two populations of the Minas Gerais State (Carangola and Juiz de Fora). The number of alleles per locus ranged from 3 to 16 (mean = 8). Observed and expected heterozygosities ranged from 0.25 to 0.92 and 0.56 to 0.92,

respectively. Evidence for both the presence of null alleles and Hardy-Weinberg equilibrium deviations were found in loci Phybu4, Phybu17, and Phybu21. Genotypic disequilibrium for each pair of loci across populations was not significant. Cross-species amplification was successful for 11 of the 12 developed loci for the sister-species, *P. bahiana*. These microsatellites will be important for future fine-scale population structure analyses.

Key words: Phyllomedusinae; Traditional enrichment method; Brazilian Atlantic forest; *Phyllomedusa burmeisteri* group; 454 Sequencing; *Phyllomedusa bahiana*

INTRODUCTION

The Brazilian Atlantic forest (BAF) is one of 25 biodiversity hotspots given priority for conservation (Myers et al., 2000). According to recent estimates (Morellato and Haddad, 2000; Ribeiro et al., 2009), only 7 to 11% of the original BAF remain today. Two of the most important factors causing this massive devastation are associated with the high level of industrialization and human population density (approximately 110 million people) present in this region. These factors have contributed to either the decline or the extinction of several organisms (Silvano and Segalla, 2005).

The leaf-frog *Phyllomedusa burmeisteri* together with *P. tetraploidea*, *P. bahiana*, *P. distincta*, and *P. iheringii* belong to the *P. burmeisteri* group, endemic to the Atlantic forest. In a recent study, Brunes et al. (2010) showed unexpected levels of mitochondrial divergence (5.3 to 10.2%) within this species group. Using a multilocus approach, these authors also revealed a deep phylogenetic break near the São Paulo State between two main geographically coherent clusters that likely diverged approximately 5 mya: the first comprising the northerly distributed species, *P. bahiana* and *P. burmeisteri*, and the other formed by the southerly distributed species, *P. distincta*, *P. tetraploidea* and *P. iheringii*. Additionally, Brunes et al. (2010) have uncovered high levels of intraspecific genetic variability in all species, particularly within *P. burmeisteri* (average = 1.83%). This may be explained by the wide distribution of *P. burmeisteri* (approximately 426,510 km²) combined with high levels of habitat fragmentation in the BAF (Pombal Jr. and Haddad, 1992). Herein, we used *P. burmeisteri* as the target species to develop variable markers for future fine-scale studies, in order to better understand the role of habitat degradation on the current patterns of population genetic structure and conservation status. Cross-species amplification was performed with the sister-species *P. bahiana*, which may be important for the future genetic study of the extensive phenotypic cline between these two species (Pombal and Haddad, 1992).

MATERIAL AND METHODS

We used two methodologies to construct microsatellite libraries: 1) we started with a traditional enrichment method following van de Vliet et al. (2009), and 2) we used the 454 GS-FLX Titanium pyrosequencing of enriched DNA libraries (GenoScreen, Lille, France; www.genoscreen.fr). For the 1st method, genomic DNA was isolated from a single leaf-frog using

a standard phenol-chloroform extraction protocol (Sambrook et al., 1989). Oligonucleotide repeat probes were 5'-biotinylated using biotin-16-ddUTP (Roche, Amadora, Portugal) and DNA fragments were hybridized to a single biotinylated (ATAG)₈ probe or were hybridized to a mixture of biotinylated probes: (CA)₁₅, (GA)₁₅, and (CAGA)₈. Finally, isolated DNA fragments were screened for microsatellites by oligonucleotide probes, which were labeled with the ECL3'-oligolabelling and detection system (Amersham Pharmacia, Amersham, UK).

For the 2nd method, DNA was isolated from a gene pool of 12 individuals using Qiagen (Hilden, Germany) DNeasy spin columns. Microsatellite libraries were developed through 454 GS-FLX Titanium pyrosequencing (GenoScreen) as described in Malausa et al. (2011). Briefly, 1 µg DNA was enriched for AG, AC, AAC, AAG, AGG, ACG, ACAT, and ATCT repeat motifs. Amplified polymerase chain reaction (PCR) products were purified, quantified, and then GsFLX libraries were developed following manufacturer protocols (Roche) and sequenced on a GsFLX-PTP. Finally, the redundant sequences were filtered in the QDD program (Megléczy et al., 2010), resulting in a final data set appropriate for primer design.

Preferably, primers were designed for tetranucleotide microsatellites with 1 unique repeat type in the DNA fragment. We favored this kind of repeat to reduce the presence of stutter bands and to improve the scoring process. A total of 36 primers (12 and 24 for the 1st and 2nd methods, respectively) were selected for further testing of amplification success and polymorphism. In addition, to prevent inconsistent amplifications in the multiplex PCR, primer pairs were tested for primer-dimer and intramolecular hairpin formation using the Autodimer software (Vallone and Butler, 2004).

We estimated polymorphism levels by genotyping a total of 25 individuals from 2 populations of the Minas Gerais State: Juiz de Fora (N = 12; S 21.7325°, W 43.3696°) and Carangola (N = 13; S 20.7986°, W 42.0836°) using 2X Qiagen Multiplex PCR Master Mix. For each locus, the forward primer was 5'-labeled with a fluorescent dye (VIC, PET, FAM, or NED). PCR amplifications were performed in a 10-µL reaction volume containing 5 µL Qiagen PCR Master Mix, 1 µL primer mix, (0.025 µM forward primer, 0.25 µM reverse primer, and fluorescent dye of each primer), 3 µL RNase-free water and approximately 100 ng DNA template.

PCR cycling conditions consisted of an initial denaturation at 95°C for 15 min; followed by a touchdown program with 13 cycles of 95°C for 30 s, 64°C to 58°C for 60 s, decreasing 0.5°C in each cycle, and 72°C for 40 s; followed by 19 cycles of 95°C for 30 s, 58°C for 1 min, and 72°C for 40 s, and by 8 cycles of 94°C for 30 s, 53°C for 1 min, and 72°C for 40 s; with a final extension at 60°C for 30 min. From the PCR-diluted products, we used 1 µL in combination with 10 µL deionized formamide and 0.2 µL internal size standard (Genescan-500 LIZ, ABI). Fragment size was determined on an ABI prism 3130XL capillary sequencer. Fragments were scored and binned using GeneMapper v3.7 (Applied Biosystems, Foster City, CA, USA).

We used MICROCHECKER v2.2.3 (Van Oosterhout et al., 2004) to check for the presence of null alleles at each locus (99% confidence level). GENEPOP on the web v3.4 (Raymond and Rousset, 1995) was used to test for Hardy-Weinberg and linkage disequilibria for each locus and across populations. We used the Markov chain method with 10,000 dememorization steps and 1000 batches of 10,000 interactions per batch. All results for multiple tests were adjusted using Bonferroni's correction (Rice, 1989). Cross-species amplification was performed in the sister-species *P. bahiana*, using 4 individuals from different populations. Multiplex PCR was conducted using the same conditions as those used for *P. burmeisteri*.

RESULTS AND DISCUSSION

Twenty-four (9 and 15 for the 1st and the 2nd methods, respectively) loci did not amplify, resulted in unexpected product sizes or ambiguous multiple bands using multiplex PCR. The remaining 12 were polymorphic loci, presented the correct product size and after several trials were arranged in 4 multiplex PCRs. Nine loci originated from the GenoScreen method (multiplex PCR: I, II, and III), and 3 originated from traditional enrichment (multiplex PCR: IV; Table 1).

Table 1. Polymorphism at 12 tetranucleotide microsatellite markers developed for *Phyllomedusa burmeisteri*.

Locus	Primer sequence (5'→3')	Repeat motif	Multiplex reaction	GenBank accession No.
Phybu3	F: VIC - CCTCAGGGCAGCGTAAAATA R: AACCCGACAGACTCCATTG	(TCTA) ₁₃	I	JQ890059
Phybu4	F: VIC - CCACCTCCATGAAGACTGC R: AGGTATATTAACCATGCAGGTAGC	(TAGA) ₁₄	II	JQ890060
Phybu5	F: NED - TCATGGATGTTACAGGCCAA R: GGCCTTTGGGGAAGTGTATC	(ATCT) ₁₄	II	JQ890061
Phybu6	F: NED - CTCCTCCCTTCTCAGCAGT R: TCGACCTTGACAACTATGTAAGTATG	(ATCT) ₁₅	I	JQ890062
Phybu7	F: PET - CTGCTGTGGAGACAAGCACT R: AGACCTGTCTATAGTGTAGGGTAAT	(CTAT) ₁₆	I	JQ890063
Phybu11	F: VIC - AACACGCCATGTGAACAAAC R: ATCTGGCCCTAACAGAATGC	(GATA) ₁₇	III	JQ890064
Phybu17	F: 6-FAM - TTCTGGCTCTGTGCAAATG R: TCGGCTTTAGGGGTTAATGA	(CTAT) ₁₂	III	JQ890065
Phybu18	F: 6-FAM - CTAAACATTTGAAACAGACATACAAG R: CACATGCTCCATAGTAGCAAAGA	(TAGA) ₁₃	II	JQ890066
Phybu21	F: NED - CTGAGCTGATACATTTCAATGGC R: TCTGACCCTAACAGAATGCCT	(GATA) ₁₄	III	JQ890067
Phybu26	F: 6-FAM - TCCAGAAGTGGAAACACCAAAACACA R: CAGAAAGACTCCCCATGCCCT	(GATA) ₉	IV	JQ890068
Phybu27	F: PET - CAGAACGAGGAGTTTCAAGGGC R: AACAAATGCATGGATGATTTAAAGGGA	(TCTA) ₁₇	IV	JQ890069
Phybu28	F: VIC - AGCTGTTATAGGAACGGGGTTGT R: TTGTGTGCTGCTGTTATTGTTATGTG	(ATCT) ₂₁	IV	JQ890070

For each locus, we list the primer pair with the respective tail, the repeat motif from the original sequence, the multiplex reaction, and the GenBank accession number. F = forward and R = reverse sequences.

Table 1 summarizes the characteristics of the 12 primer pairs of polymorphic loci derived from the leaf-frog *P. burmeisteri*. Evidence of null alleles was only statistically significant for loci Phybu4, Phybu17, and Phybu21 in the Carangola population. We found no evidence for large allele dropouts and stuttering. The number of alleles per locus ranged from 3 to 16 (mean = 8) and allele sizes ranged from 124 to 329 bp. Observed and expected heterozygosities ranged from 0.25 to 0.92 and 0.56 to 0.92, respectively (Table 2). Genotypic disequilibrium tests for each pair of loci in each population were not significant. The loci Phybu4, Phybu17, and Phybu21 were not in Hardy-Weinberg equilibrium in the Carangola population, which likely resulted from the reduced sample size associated with the presence of null alleles.

Eleven of the 12 developed loci were successfully amplified in *P. bahiana* with the expected size range, and 8 were polymorphic (Table 2). Due to the high cross-amplification success observed in this study, amplification of these markers should be further tested in other species of the *P. burmeisteri* group (*P. distincta*, *P. tetraploidea*, and *P. iheringii*). These microsatellite loci may be especially important for future analyses of fine-scale population structure in *P. burmeisteri* and also for genetic characterization of the extensive morphological cline between *P. burmeisteri* and *P. bahiana*.

Table 2. Polymorphism statistics at 12 tetranucleotide microsatellite markers in two populations of *Phyllomedusa burmeisteri* and cross-species amplification success in *Phyllomedusa bahiana*.

Locus	Size range (bp)	Carangola (N = 13)		Juiz de Fora (N = 12)		Cross-amplification success
		N_A	H_O/H_E	N_A	H_O/H_E	
Phybu3	229-266	10	0.833/0.854	5	0.750/0.739	+ #
Phybu4	141-193	11	0.500/0.843*	6	0.666/0.715	+ #
Phybu5	215-305	16	0.923/0.917	5	0.625/0.726	-
Phybu6	129-169	10	0.846/0.861	7	0.909/0.760	+ #
Phybu7	180-262	9	0.833/0.819	6	0.750/0.680	+
Phybu11	243-311	11	0.846/0.864	7	0.833/0.788	+ #
Phybu17	241-329	8	0.250/0.819*	7	0.818/0.810	+ #
Phybu18	124-168	9	0.923/0.775	3	0.636/0.558	+
Phybu21	202-270	10	0.250/0.871*	7	0.800/0.815	+ #
Phybu26	125-203	10	0.750/0.826	6	0.916/0.767	+
Phybu27	153-201	8	0.692/0.846	7	0.916/0.781	+ #
Phybu28	187-257	10	0.769/0.828	9	0.600/0.855	+ #

Allele size length in base pairs (bp) and N_A = number of alleles for each population and across all populations; H_O = observed and H_E = expected heterozygosities; *significant deviation from Hardy-Weinberg equilibrium; (+) amplification within the expected range; (-) no amplification; #polymorphic.

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