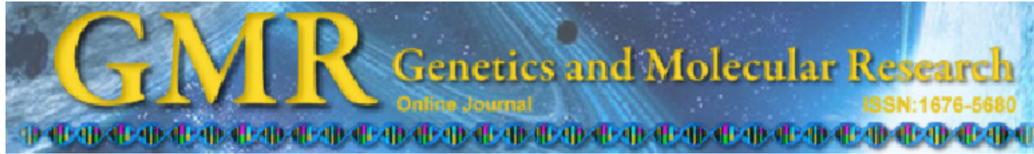

Methodology

Development of microsatellite markers for the Neotropical endemic Brazilian Guanabara frog, *Euparkerella brasiliensis*, through 454 shotgun pyrosequencing

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ABSTRACT. The new-generation 454 GS-FLX Titanium pyrosequencing was used to isolate microsatellite markers for the Brazilian Guanabara frog, *Euparkerella brasiliensis*, an Atlantic forest endemic species. Three multiplex polymerase chain reaction sets were optimized for genotyping of 11 polymorphic (di- and tetranucleotide) microsatellite markers. Genetic diversity was assessed in 21 individuals from a population (Reserva Ecológica de Guapiaçu, REGUA) located

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in the central region of the Rio de Janeiro State, in Brazil. The mean number of alleles per locus ranged from 3 to 12. Observed and expected heterozygosities ranged from 0.095 to 0.905 and from 0.094 to 0.904, respectively. After using the Bonferroni correction for multiple tests, there was no evidence of linkage disequilibrium between pairs of loci but deviations for Hardy-Weinberg equilibrium were found in 4 loci. We found no evidence for allele dropouts or stuttering, but we detected the presence of null alleles at loci Eb10 and Eb36. These markers will be useful for analyses of fine-scale population structure and determination of relative effects of habitat loss and fragmentation on population genetic variability within species.

Key words: Amphibians; Atlantic forest; *Euparkerella brasiliensis*; Microsatellites; 454 Sequencing

INTRODUCTION

The Brazilian Atlantic forest is one of the world's richest regions for biological diversity (Laurance, 2009). However, the biome has been historically submitted to high levels of destruction and fragmentation. At present, only about 11% of the original forest cover is remained, mostly distributed in sparse and small remnants (Ribeiro et al., 2009). This level of devastation has affected several organisms. For example, most reports of Brazilian amphibian declines come from the Atlantic forest, which also shows the highest number of threatened amphibian species amongst all Brazilian regions/biomes, including the Amazon (Silvano and Segalla, 2005). Unfortunately, the consequences of habitat loss and fragmentation for amphibian populations are still poorly understood due to a lack of studies of basic organismal biology and population ecology, absence of long-term monitoring programs, and, in particular, the fact that population genetic studies have been rarely undertaken.

The Brazilian Guanabara frog, *Euparkerella brasiliensis* (Parker, 1926), is a miniaturized species (adults ranging from 13 to 23 mm in snout-vent length) endemic to the Atlantic forest and restricted to the forests of the Serra dos Órgãos mountain range and adjacent forest remnants of the Rio de Janeiro State, found at elevations of near sea level to 1000 m (Izecksohn, 1988). It is a terrestrial breeder with slow walking locomotion, inhabiting the leaf-litter of primary and secondary forests. These morpho-ecological features, associated with the high level of habitat deterioration observed in the environment across its range, make this frog an interesting model to investigate the genetic consequences of habitat fragmentation on population structure. Here, we developed a set of microsatellite loci that will be useful to conduct fine-scale spatial genetic studies on *E. brasiliensis*.

MATERIAL AND METHODS

We developed a microsatellite library for *E. brasiliensis* from a pool of 8 individuals. We extracted DNA from toe-clipping or liver tissue samples preserved in 100% ethanol. Tissues were digested in lysis buffer solution with proteinase K. The whole genomic DNA was purified with DNeasy mini spin columns using DNeasy Blood & Tissue kit (QIAGEN, Hilden,

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Germany), following the manufacturer protocol. We used the same methodology for samples that were genotyped.

A microsatellite-enriched DNA library was constructed by next-generation 454 GS-FLX Titanium pyrosequencing carried out by Genoscreen (Lille, France), following the protocol of Malausa et al. (2011). Briefly, genomic DNA was digested with the restriction enzyme *Rsa*I, linked to standard adapters, purified, and enriched by hybridization with 8 microsatellite oligonucleotide probes of 2 dimers, 4 trimmers, and 2 tetramers. The enrichment was completed by capture with magnetic beads, and samples were amplified with primers corresponding to library adapters. The samples were prepared for sequencing by ligation with multiplex identifier adapters and single-stranded DNA library isolation. The single-strand template concentration was normalized by dilution and multiplexing in an equimolar mixture for the analysis of 8 samples on a 1/4 GS-FLX PicoTiter plate.

The QDD program (Meglécz et al., 2010) was used for microsatellite detection and primer design. The total number of microsatellite sequences obtained was 4908, of which we selected 36 on the basis of diversity of motifs and number of repeats. Primers designed by the QDD program were chosen to amplify the selected sequences. We used the AutoDimer program (Vallone and Butler, 2004) to screen hairpin and primer-dimer interactions of all primer pairs. For each locus, the forward primer was 5'-labeled with a fluorescent dye (6-FAM, VIC, NED, or PET). We were able to amplify 14 microsatellite loci that were subsequently tested for polymorphisms. Three of 14 loci were monomorphic, and thus, were discarded. The remaining 11 polymorphic loci were used to genotype 21 individuals of *E. brasiliensis* collected at the Reserva Ecológica de Guapiaçu (REGUA), Cachoeiras de Macacu, Rio de Janeiro State, Brazil (22°24'14" S; 42°44'16" W). We used 3 multiplex reactions (Table 1) and polymerase chain reaction (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 1.5 µL DNA template. PCR cycling conditions were as follows: initial denaturation at 95°C, a touch-down program with 15 cycles of 95°C for 30 s, 65° to 58°C for 90 s, decreasing 0.5°C each cycle, and 72°C for 45 s, followed by 22 cycles of 95°C for 30 s, 58°C for 90 s, and 72°C for 30 s and 8 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s, with a final extension at 60°C for 30 min. We mixed 1 µL PCR product with 10 µL formamide and 0.2 µL internal size standard (Genescan-500 120 LIZ, ABI). Fragments were separated using an ABI prism 3130XL capillary sequencer. Alleles were scored and binned using GeneMapper v. 3.7 (Applied Biosystems).

Levels of polymorphism, deviation from Hardy-Weinberg equilibrium, and linkage disequilibrium were estimated with GENEPOP on the web v. 4.0 (Raymond and Rousset, 1995), using the default values of the Markov chain parameters.

RESULTS AND DISCUSSION

The number of alleles per loci varied from 3 to 12 and observed heterozygosity ranged from 0.095 to 0.905 (Table 1). After applying the Bonferroni correction for multiple tests (Rice, 1989), there was no evidence of linkage disequilibrium between each pair of loci, but 4 loci deviated significantly from Hardy-Weinberg equilibrium (Eb10, Eb14, Eb19, and Eb36). No evidence for large allele dropouts, stuttering, and null alleles were detected at the 99% confidence level across all loci using MICRO-CHECKER v. 2.2.3 (Van Oosterhout et al., 2004), with the exception of loci Eb10 and Eb36, for which we inferred the presence of null alleles.

Table 1. Primer sequences and characteristics of 11 microsatellite loci isolated from *Euparkerella brasiliensis*.

Locus	Primer sequence (5'-3')	Repeat motif	N_A	Size range (bp)	H_O	H_E	HWE (P)	GenBank accession No.
Mix-1								
Eb2	F: TGTAAAACGACGGCCAGTACTGAGCCACTGTGCAGC$^{\text{6-FAM}}$ R: GTTTGCAGACAAATAAGACCTAGAACAGG	$(atct)_{18}$	12	120-168	0.905	0.904	0.159	JQ996233
Eb3	F: TAATACGACTCACTATAGGGCTTAAAACAGAGGCTGGGCA$^{\text{VIC}}$ R: GTTTGGAGTAGAGAATAGATGGCTGG	$(atag)_{16}$	11	218-262	0.762	0.891	0.237	JQ996234
Eb21	F: TTTCCCAGTCACGACGTTGCTGCTGTGTGCGAGCTGT$^{\text{NED}}$ R: GTTTCCAGAAGGACAGGCTGAAT	$(ct)_{10}$	6	219-229	0.714	0.772	0.674	JQ996240
Eb24	F: GATAACAATTTCACACAGGATGTTGTGGGAAAATGCACC$^{\text{PET}}$ R: GTTTGCCGAGAAAAGGAAGGGTAA	$(gata)_{11}$	10	115-151	0.810	0.885	0.930	JQ996241
Mix-2								
Eb10	F: TGTAAAACGACGGCCAGTCATTGTCACCTTCAACAGCAG$^{\text{6-FAM}}$ R: GTTTATGTGGTTTGTCAGTGTCGG	$(atag)_{11}$	10	154-194	0.524	0.886	*	JQ996236
Eb19	F: TAATACGACTCACTATAGGGATACCCCGCTATGGAGAGGA$^{\text{VIC}}$ R: GTTTAGGGTTAGTTGCAGCACCAT	$(ag)_9$	4	259-271	0.381	0.460	*	JQ996239
Eb33	F: TTTCCCAGTCACGACGTTGGATGTAATGCAATTCCAGCCT$^{\text{NED}}$ R: GTTTCATTCATATCACACTGATGCTGC	$(ag)_9$	3	163-169	0.095	0.094	1.000	JQ996242
Eb36	F: GATAACAATTTCACACAGGAGATCTGTGTTTTATGTGTATGGAGT$^{\text{PET}}$ R: GTTTCATGCATAGCTTTGCTCGAT	$(tg)_9$	4	129-141	0.333	0.619	*	JQ996243
Mix-3								
Eb4	F: TAATACGACTCACTATAGGGGGACCCTAAGAACAGCCAAAC$^{\text{VIC}}$ R: GTTTCAGTGTAGGAGTAAATAGCAGTCG	$(atct)_{12}$	9	143-183	0.810	0.884	0.391	JQ996235
Eb14	F: TTTCCCAGTCACGACGTTGAGGCTTTTGTGTCTCCTCACA$^{\text{NED}}$ R: GTTTAATGCCCGGCAGCTTTTA	$(ag)_{12}$	9	141-161	0.619	0.775	*	JQ996237
Eb15	F: GATAACAATTTCACACAGGAAATGCTGTGACACAATTCCA$^{\text{PET}}$ R: GTTTACACAGTGTGCTGCTGTGAA	$(ag)_8$	3	189-213	0.143	0.138	1.000	JQ996238

N_A = number of alleles; H_O = observed heterozygosity; H_E = expected heterozygosity. P values of the exact test for Hardy-Weinberg equilibrium (HWE) were based on 21 individuals from Reserva Ecológica de Guapiaçu (REGUA), Cachoeiras de Macacu, State of Rio de Janeiro, Brazil. Forward primers (F) were labeled with four different fluorescent tails (6-FAM, VIC, NED, and PET) for multiplex purpose. *HWE desiquilibrium. R = reverse primers.

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