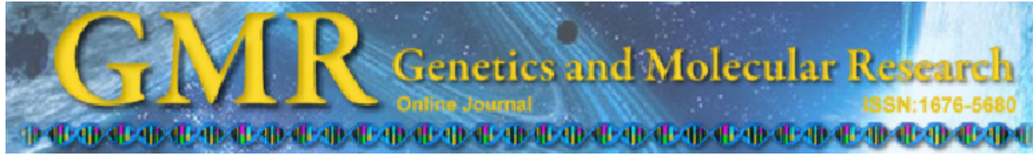

Research Note

Development and characterization of microsatellite markers for Brazilian four-eyed frogs (genus *Pleurodema*) endemic to the Caatinga biome

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ABSTRACT. We used pyrosequencing to develop microsatellite markers for the Brazilian four-eyed frog *Pleurodema diplolister* and tested the microsatellite markers for cross-amplification in its sister *Pleurodema alium*, which are both endemic species of the Caatinga biome in northeastern Brazil. We used multiplex sets to amplify and genotype 30 individuals of *P. diplolister* from three different populations and 10 individuals of *P. alium* from a single population. We successfully amplified 24 loci for *P. diplolister*, 13 of which we were able to amplify in *P. alium*. All loci were polymorphic. Significant deviations from the Hardy-Weinberg equilibrium and

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the presence of null alleles were only consistently detected at one locus (Pleu9). These markers will enable the study of geographic genetic diversity and evolutionary processes in these two Caatinga endemics, and the inclusion of genetic data for conservation planning of the Caatinga biome.

Key words: *Pleurodema diplolister*; *Pleurodema alium*; Caatinga; 454 shot-gun pyrosequencing

INTRODUCTION

The four-eyed frogs of the genus *Pleurodema* include 14 species occurring throughout the discontinuous dry environments of the Neotropics (Faivovich et al., 2012; Frost, 2013). Two species are endemic to the Caatinga biome in northeastern Brazil; the range of *Pleurodema diplolister* includes most of the biome, while the recently described *Pleurodema alium* shows a parapatric distribution to the south (Maciel and Nunes, 2010). A recent phylogenetic study revealed *P. diplolister* and *P. alium* to be sister species, geographically isolated from their closest relatives from the Andes and Llanos by the Amazon rainforests and the Cerrado savannas (Faivovich et al., 2012).

The Caatinga is a highly seasonal biome that harbors a very diverse and characteristic biota from both floristic and faunal perspectives (Sarmiento, 1975; Leal et al., 2005). Despite high levels of endemism, the biome remains poorly studied and precariously protected with less than 1% of the region sheltered in conservation units (Leal et al., 2005). While human occupation is rapidly growing in this biome, investigating the evolutionary processes behind the origin of endemic species is of special interest to create conservation strategies. Here, we provide a set of microsatellite markers developed for *P. diplolister* and tested for cross-amplification in *P. alium*. We hope these markers will be useful for mapping the genetic diversity of these two Caatinga endemics and for evolutionary studies using four-eyed frogs as biological models.

MATERIAL AND METHODS

For the microsatellite library construction, we used a pool of 10 individuals of *P. diplolister* from different populations across its range. We extracted total genomic DNA from liver samples preserved in 100% ethanol by digesting tissues with Proteinase K and purifying the DNA with DNeasy mini spin columns (DNeasy Blood & Tissue kit, Qiagen, Netherlands) according to manufacturer protocol. Construction and pyrosequencing of the microsatellite-enriched DNA library were performed by Genoscreen in France (www.genoscreen.fr) through 454 GS-FLX Titanium pyrosequencing of enriched DNA libraries, according to the procedures described by Malausa et al. (2011). Genoscreen obtained a total of 3482 microsatellite sequences and designed primers for 133 loci by using the QDD program (Meglécz et al., 2010). From these, we selected 56 primer pairs based on the diversity of motifs and number of repeats, favoring tetra and tri-repeat microsatellites. We evaluated all primer pairs for potential interactions, including primer-dimer and hairpin

formation, using the AutoDimer program (Vallone and Butler, 2004). For each locus, we 5'-labeled the forward primer with a fluorescent dye (6-FAM, VIC, NED, or PET). We arranged primer pairs in six multiplex reactions and performed polymerase chain reactions (PCRs) with 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), 3.5 µL RNase-free water, and 1 µL DNA template. We used the following cycling conditions: initial denaturation at 95°C; a touch-down program with 15 cycles of 95°C for 30 s, 65°C to 58°C for 1 min 30 s, decreasing 0.5°C each cycle, and 72°C for 45 s; 22 cycles of 95°C for 30 s, 58°C for 1 min 30 s, and 72°C for 30 s; 8 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s; and 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, Applied Biosystems, USA), and the markers were sized in an ABI prism 3130XL capillary sequencer (Applied Biosystems). We then scored and binned alleles using GeneMapper v3.7 (Applied Biosystems). For primer tests and polymorphism analyses, we sampled three localities for *P. diplolister* (Jussiape, Quixeramobim, and Nova Russas) and one locality for *P. alium* (Anagê). We genotyped 10 individuals from each locality (total, 40). We estimated the number of alleles, expected and observed heterozygosities, and deviation from Hardy-Weinberg equilibrium per population and locus by using ARLEQUIN v3.5.1.2 (Excoffier and Lischer, 2010) with the default values of the Markov chain parameters and permutations. We also tested for allele dropouts, stuttering, and the presence of null alleles using MICRO-CHECKER v2.2.3 (Van Oosterhout et al., 2004).

RESULTS AND DISCUSSION

We successfully amplified 24 microsatellite loci for *P. diplolister*, 13 of which were also amplified in *P. alium* (Table 1). All loci showed allele polymorphism (with the exception of Pleu53, which was monomorphic only in *P. diplolister*). Some loci were monomorphic when considering populations separately. In *P. diplolister*, the number of alleles per population ranged from 1 to 10, and the expected heterozygozity ranged from 0.100 to 0.911. In *P. alium*, the number of alleles ranged from 2 to 10, and the expected heterozygozity ranged from 0.111 to 0.895. We detected significant deviations from the Hardy-Weinberg equilibrium ($P < 0.05$) at several loci for both species, but only locus Pleu9 consistently showed significant disequilibrium across all populations and species. We did not find evidence of large allele dropouts or stuttering, but we inferred the presence of null alleles for loci Pleu9 (all populations) and Pleu20 (Jussiape) in *P. diplolister*, and Pleu1, Pleu2, Pleu9, and Pleu52 in *P. alium*.

The sample size per population is relatively small and may have caused some of the Hardy-Weinberg disequilibria inferred in this study. However, the results obtained for Pleu9 were recurrent and suggest that this locus should be avoided or used with caution. There are no studies of Caatinga-endemic organisms using microsatellite markers in the literature, but Caetano et al. (2008) were able to detect genetic structure in *Astronium urundeuva*, a tree that also occurs in the biome, using only five microsatellite markers. Therefore, the number of loci obtained for both species should permit the unveiling of the genetic structure behind these Caatinga four-eyed frogs.

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Table 1. Primer sequences and characterization of 24 microsatellite loci isolated from *Pleurodema diplolister*.

GenBank accession No.	Locus	Primer sequence (5'-3')[dye, multiplex set]	Motif	Size range (bp)	*Pleurodema diplolister*									*Pleurodema alium*		
					Nova Russas			Quixeramobim			Jussiape					
					N_A	H_O	H_E	N_A	H_O	H_E	N_A	H_O	H_E	N_A	H_O	H_E
KF819833	Pleu1	F: CTGCACTCCGGTCAGATACA[6-FAM, SET1] R: GTTTCCACCTGCTGTAATCCCTC	AGAT	273-293	4	0.500	0.679	3	0.200	0.358	3	0.600	0.653	2	0.000	0.442**
KF819834	Pleu2	F: CCTCAAGAGGCTGACTCCAT[6-FAM, SET1] R: GTTTGCCAGACCACCCTTTGACTATCC		109-127	3	0.600	0.468	1	-	-	2	0.100	0.100	4	0.100	0.563**
KF819835	Pleu5	F: GTGGTGGCAGTAGGATAGTAGG[NED, SET1] R: GTTTGCTATTAACTAAGAGGTTACAAGTCA	TGTA	251-266	3	0.600	0.668	4	0.900	0.726*	2	0.100	0.100	-	-	-
KF819836	Pleu9	F: TCCATGGGTCTATTCACAAAG[6-FAM, SET2] R: GTTTCCCAGACTTACAATATTGCCG	TAGA	232-283	5	0.400	0.774*	3	0.000	0.358**	6	0.100	0.795**	4	0.200	0.695**
KF819837	Pleu10	F: GGTGGACTGCAATCAGACAT[6-FAM, SET2] R: GTTTGGATCAACACTGTAGAATTAAAGG	ATAG	101-157	10	0.900	0.911	5	0.800	0.653	9	0.800	0.879	5	0.800	0.742
KF819838	Pleu12	F: AATGAGATTCTGAGTGGTGCC[VIC, SET2] R: GTTTCCCACGCACTGAACTATTGA	CTAT	114-150	8	1.000	0.884	4	0.900	0.763	7	0.800	0.832*	7	0.900	0.884
KF819839	Pleu16	F: GTTGATATGATGACCTGGGC[PET, SET2] R: GTTTCCATTTTGTTACATGTCCCTT	AGAT	108-136	6	0.800	0.763	5	0.600	0.568	5	0.500	0.768	-	-	-
KF819840	Pleu17	F: TGGGTTCAGTTTCATCATCCT[6-FAM, SET3] R: GTTTGGCGGTTAACATTGACAGT	TCTA	256-304	8	0.700	0.863	3	0.900	0.689	7	1.000	0.821	4	0.700	0.753
KF819841	Pleu18	F: ACCTGCCTAAAACCCTTGCT[6-FAM, SET3] R: GTTTCTTGGCCTGGACCTTATGT	AGAT	112-162	6	0.700	0.763	3	0.400	0.542	2	0.375	0.325	10	0.800	0.895
KF819842	Pleu19	F: GCGTCTAGAGGATTCTGGGA[VIC, SET3] R: GTTTCCCATACAACTTCTCCTTGTGC	TCTA	206-242	9	0.900	0.900	5	1.000	0.789*	7	0.700	0.642	-	-	-
KF819843	Pleu20	F: AAGGTGTTTAAAGGTGTTGTCCA[VIC, SET3] R: GTTTCTATCTGTCTGCCTACTCTATCTCA	TAGA	125-165	6	0.900	0.768	5	1.000	0.800	8	0.600	0.879**	-	-	-
KF819844	Pleu21	F: CTTTAGTACTGATCAGAGCCAGAAAA[NED, SET3] R: GTTTGGTTATTCTGTTAAGGTGACTGC	TCTA	214-298	9	0.700	0.905	5	0.800	0.679	6	0.600	0.637	-	-	-
KF819845	Pleu35	F: TTGACCTCTTCTGGCTCTACG[NED, SET4] R: GTTTGGCCAAGATGAAGTGGAAGA	CTT	123-132	3	0.500	0.532	2	0.000	0.189	2	0.400	0.337	3	0.200	0.195
KF819846	Pleu36	F: ACAGCAAACTTACAGAGCCCA[PET, SET4] R: GTTTGTTCAAGTTGGGAAACAAGG	AGAT	213-265	6	0.700	0.811	3	0.500	0.416	1	-	-	3	0.300	0.353
KF819847	Pleu38	F: TTGAGGTCAGGGATCAGAGG[VIC, SET4] R: GTTTGGAGAGATAGATAATGGATTGGTG	TCTA	123-163	9	0.800	0.905	5	1.000	0.795	6	0.700	0.637	-	-	-
KF819848	Pleu42	F: TGAATTGGTACTGGGCACTG[VIC, SET5] R: GTTTCTACACTGGCTGTGAACCGA	TAGA	117-169	5	0.800	0.742	3	1.000	0.653	6	0.900	0.863	7	0.600	0.737
KF819849	Pleu51	F: GTTCTGCCTTTGACTGTCCC[NED, SET5] R: GTTTCTTAAGACATGCCCTGGGTC1	TATG	181-185	2	0.300	0.479	2	0.500	0.395	2	0.900	0.521	-	-	-
KF819850	Pleu53	F: CTAACCTGGTGGAATGCAGG[6-FAM, SET5] R: GTTTGAGACAAGCAAGTGCAGC	GGA	182-185	1	-	-	1	-	-	1	-	-	2	0.000	0.189
KF819851	Pleu56	F: AAGTGCAGTTCATGGTTCCC[PET, SET5] R: GTTTACCAATCTACTGCTGATGGATG	ATCT	194-330	9	0.800	0.884	5	0.667	0.680	10	0.800	0.911	-	-	-
KF819852	Pleu26	F: GGGTCTTATACCTCCCAGCC[6-FAM, SET6] R: GTTTAGGGCAGTGAATGCAGAATC	TCTA	113-197	8	0.800	0.863	5	1.000	0.804**	8	0.800	0.868	-	-	-
KF819853	Pleu30	F: CAAGAGGGCACGCTAGGTTA[NED, SET6] R: GTTTAGACGAAGCCTTTTCAACCA	TCT	98-110	2	0.200	0.189	1	-	-	2	0.300	0.521*	2	0.111	0.111
KF819854	Pleu43	F: TTCATGTTCAGTGCCCTCAG[NED, SET6] R: GTTTCATCAAACAGTGACCATGCC	CTAT	135-171	6	0.700	0.805	3	0.111	0.569**	7	0.700	0.779	-	-	-
KF819855	Pleu46	F: TGGGTGTAGAGTGCCTGTTG[VIC, SET6] R: GTTTCCACTGTGGGATAGCATCTG	AGG	143-147	2	0.100	0.100	1	-	-	2	0.600	0.505	-	-	-
KF819856	Pleu52	F: CTTCTCTGGAGGCCATTCAC[PET, SET6] R: GTTTAGAACTGGAATGATGGGCA	TATC	173-217	6	0.900	0.826	3	0.889	0.699	5	0.400	0.621	6	0.444	0.732*

N_A = number of alleles; H_O = observed heterozygosity; H_E = expected heterozygosity. *Significant ($P < 0.05$) deviation from Hardy-Weinberg equilibrium. **Highly significant ($P < 0.01$) deviation from Hardy-Weinberg equilibrium. Forward primers were labeled with four different fluorescent tails (6-FAM, VIC, NED, and PET) to multiplex the reactions.

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