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Development of 22 Polymorphic Microsatellite Loci for the Critically Endangered Morato's Digger Toad, *Proceratophrys moratoï*

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Abstract: The Morato's digger toad (*Proceratophrys moratoï*) inhabits Brazilian moist savannas and is critically endangered due to its very limited geographic distribution, reduced number of isolated populations, and evidence of population decline and local extinctions. With the objective of providing tools for the genetic study of the species, 22 polymorphic microsatellite loci were isolated and screened using DNA extracted from samples of oral mucosa cells obtained from 113 individuals representing five remnant *P. moratoï* populations in the Brazilian state of São Paulo. These markers presented 2–18 alleles per locus, polymorphism information content (PIC) of 0.02–0.87, observed heterozygosity of 0.02–0.96 and expected heterozygosity of 0.02–0.87. Three of the loci deviated significantly from Hardy–Weinberg equilibrium in one of the populations, possibly due to the presence of null alleles. Significant linkage disequilibrium was also detected between three pairs of loci. The molecular markers developed in this study were able to discriminate each of the individuals sampled (identity analysis). This means that they will be extremely useful for future genetic studies applied to the conservation of *P. moratoï*, providing a baseline for estimating the levels of genetic diversity, pedigrees,

inbreeding, and population structure, which will be essential for the development of effective genetic management programs.

Keywords: *Proceratophrys moratoii*; endangered species; microsatellite; population genetics; Morato's digger toad; conservation genetics

1. Introduction

The class Amphibia has experienced a major global decline in recent decades, becoming more endangered than birds and mammals, due to a combination of factors [1]. Habitat destruction, climate change and infectious diseases are considered to be the primary cause of the decline of this group [2,3]. Ongoing anthropogenic impacts have contributed to the increasing deterioration of landscapes, which not only modifies aquatic and terrestrial habitats, but also reduces their connectivity, which are all factors that may affect amphibian populations adversely [4,5].

Proceratophrys moratoii is a digger toad of small size, typically with a snout-vent length of no more than 35 mm, which is endemic to the Cerrado savanna of the Brazilian state of São Paulo [6]. The species is found in *campo sujo* habitats (grassland dotted with small shrubs), invariably near the gallery forests associated with the headwaters of streams [6,7]. In São Paulo, the Cerrado biome has been modified intensively in recent decades, primarily for the planting of commercial crops such as sugarcane, but also for cattle ranching and urban development [8]. Currently, only about 6% of the original cover remains [9], which has drastically reduced the availability of potential habitat for the endemic *P. moratoii*. Due to its very restricted geographic distribution and the evidence of population decline and local extinctions [7,10], the species is currently listed as critically endangered by the International Union for Conservation of Nature [11], and is included in the official lists of endangered species of Brazil [12] and São Paulo [13].

The available genetic studies of *P. moratoii* include molecular analyses of mitochondrial and nuclear genes [14] and cytogenetics [15]. However, no population-level data—which may be essential for the development of effective management strategies—are available, due to the lack of appropriate molecular markers. In order to contribute to the development of these strategies, we have developed the first set of microsatellite markers for *P. moratoii*.

2. Results and Discussion

2.1. Characterization of the Enriched Microsatellite Library

A total of 384 clones were isolated and sequenced bidirectionally. The Codoncode Aligner 3.7.1 software (CodonCode Corporation: Centerville, MA, USA) revealed a redundancy of 17% in the library. Of the unique clones selected for analysis in Microsatellite Repeats Finder [16], 176 (46%) had at least one microsatellite. A predominance of dinucleotide repeats (56%) was found in the motifs that make up the library. The CA_N/GT_N repeats (130 motifs identified) were the most numerous, followed by CT_N/GA_N (48 motifs). This predominance of CA_N/GT_N repeats is typical of the eukaryote genome [17]. Considerable numbers of other types of motifs were also recorded, in particular the

dinucleotide AT_N/TA_N (36 motifs), the trinucleotides CAT_N/GTA_N (18 motifs), CTT_N/GAA_N (17), AAT_N/TTA_N and CTC_N/GAG_N (6 motifs each), and the tetranucleotides $CTAT_N/GATA_N$ (13 motifs) and $CATT_N/GTAA_N$ (6 motifs). These data provide a baseline that will support the development of additional probes for the isolation of new microsatellites in *P. moratoi*.

2.2. Development of Polymorphic Microsatellite Markers

A total of 29 pairs of primers were designed and optimized successfully for the PCR amplification of microsatellite loci (Table 1). Of the loci analyzed, *Pmoratoi*μ1 presented several nonspecific amplifications even after optimization (with varying concentrations of magnesium chloride and different annealing temperatures) and was excluded. Six loci—*Pmoratoi*μ2, *Pmoratoi*μ3, *Pmoratoi*μ4, *Pmoratoi*μ9, *Pmoratoi*μ20, and *Pmoratoi*μ22—were monomorphic. With the exception of *Pmoratoi*μ22, all these monomorphic loci represent interrupted or interrupted compound microsatellites characterized by a small number of repetitions, with predictably low polymorphism [18]. Twenty-two microsatellites were polymorphic (Table 2) in at least some populations (*Pmoratoi*μ7, *Pmoratoi*μ8, *Pmoratoi*μ10, *Pmoratoi*μ11, *Pmoratoi*μ14, *Pmoratoi*μ17, *Pmoratoi*μ18, and *Pmoratoi*μ21). The *Pmoratoi*μ5 locus was not amplified in some populations, possibly due to local mutations in the primer annealing site. The identity analysis calculated using Cervus 3.0.3 [19] detected four pairs of specimens with identical genotypes (exact match), suggesting the recapture of the same animal during fieldwork. In these cases, the duplicate genotype was excluded from the analyses.

Table 1. Microsatellites isolated in the present study with their respective primers and the optimal amplification conditions determined following visualization of the polymerase chain reaction (PCR) products in a polyacrylamide gel.

Genbank Accession n°	Locus	Repeat Motif	Primer Sequence (5'→3')	T _A	MgCl ₂ (mM)
JX441952	<i>Pmoratoi</i> μ1	(TTTC) ₉	Forward: GGTGAACATCCTTTTCGTAGC Reverse: CACTCCTTCCCTAATCCAGTTT	50 °C	0.6
JX441953	<i>Pmoratoi</i> μ2	(AC) ₄ AT(AC) ₇ (AC) ₄	Forward: ACACATCGTTCTGCACTACACAC Reverse: GCTCCCTTGTCTTGCTGTCT	63 °C	1.0
JX441954	<i>Pmoratoi</i> μ3	(TA) ₈ CACA CAT(AC) ₈	Forward: CTAACCGTCCAATAGCCTGTGT Reverse: CCTCTTTCCCTTGTGTGTCT	63 °C	0.6
JX441955	<i>Pmoratoi</i> μ4	(AC) ₆ G(CA) ₅	Forward: AAATGAGGTGGCTGTGCTAAAT Reverse: ATGCATTAGTGGTCATCACTGG	60 °C	3.5
JX441956	<i>Pmoratoi</i> μ5	(CA) ₈	Forward: TATCTGTATTGCCTGCTCCACAC Reverse: CCTAGTGAGCTAAAAGTTGTGCTTGT	68 °C	3.5
JX441957	<i>Pmoratoi</i> μ6	(ACAT) ₄ (AC) ₁₅	Forward: CTGACACCACCCCTTGAATAA Reverse: TGCACAGCAGGATCAATCTAAC	46 °C	0.8
JX441958	<i>Pmoratoi</i> μ7	(AC) ₈	Forward: ACTTCCAGGTGCCATATCTTCA Reverse: AATTCTTGGTCTGCCATACTGTG	51 °C	1.0
JX441959	<i>Pmoratoi</i> μ8	(AC) ₆	Forward: GCGAATAATTGGAAAGCACAG Reverse: GCCTGAGCCAGAGTTGAATAGTA	68 °C	3.5
JX441960	<i>Pmoratoi</i> μ9	(ATT) ₄ ...(TAT) ₄	Forward: GATAATTGACCGTTTCCGTCAT Reverse: CATGGAACAACTGAAGAGAACC	63 °C	4.0

Table 1. Cont.

Genbank Accession n°	Locus	Repeat Motif	Primer Sequence (5'→3')	T _A	MgCl ₂ (mM)
JX441961	<i>Pmoratoiu</i> 10	(TA) ₄ (CA) ₇	Forward: CTAATAAAGTGGCCGGTGAGTG Reverse: ATAGGACTACATTGTGCCCTTGA	50 °C	0.8
JX441962	<i>Pmoratoiu</i> 11	(CA) ₈	Forward: TCCAAAGTTCTAGCCTTGTTAG Reverse: CGCTACACATACCTTGAGAAA	57 °C	4.0
JX441963	<i>Pmoratoiu</i> 12	(ATCT) ₇ (CA) ₅ ...(CA) ₄	Forward: CCTTCCCACCTTCCCTCTC Reverse: CGATCAACCTCCTCTTCTGTCTAC	66 °C	2.5
JX441964	<i>Pmoratoiu</i> 13	(CA) ₇	Forward: CTGTTTGGACTGCGATTCTT Reverse: GCATTTGTGTGTGAGAGTGAA	50 °C	1.0
JX441965	<i>Pmoratoiu</i> 14	(ACAT) ₈	Forward: GTCAAATGAGGCGGCTGTG Reverse: GCCATTATTGCTTGTATTGCTTCAG	63 °C	1.5
JX441966	<i>Pmoratoiu</i> 15	(GATA) ₁₂ (CA) ₈	Forward: CTTTAGGGCAGTCCAAGATTA Reverse: TGAAGGGGACACATTTTAAG	50 °C	1.5
JX441967	<i>Pmoratoiu</i> 16	(TCA) ₈	Forward: CTACACTAAAACGTCTCAATCAATG Reverse: ATGATGAAGAACTGGAGGAAGA	66 °C	2.5
JX441968	<i>Pmoratoiu</i> 17	(CAC) ₇	Forward: CCCAAAGAGTGCCAAGAAAATA Reverse: GGTAACAAACAACAAACCAGTATCAAC	60 °C	0.8
JX441969	<i>Pmoratoiu</i> 18	(CA) ₇	Forward: GTGTAATCCTGGGGTTCAGGTA Reverse: TCCCACCTTGATCAGATATTGT	57 °C	1.0
JX441970	<i>Pmoratoiu</i> 19	(CA) ₈	Forward: TATAGTCCAGGCAGCCCTTTA Reverse: GTCCGTGAGTGACGCAAAGT	68 °C	1.0
JX441971	<i>Pmoratoiu</i> 20	(CA) ₅ AG(CA) ₆	Forward: GATTCCCAGCAGAACATCAC Reverse: GGACTATGGAGCAATGAAAGAA	63 °C	0.8
JX441972	<i>Pmoratoiu</i> 21	(CA) ₄ ...(CA) ₄ ... (CA) ₅ ...(CA) ₇	Forward: GGGGCACAGTGTATATGTCAGT Reverse: TTGAGCTGGTGAGGCAGTT	66 °C	3.0
JX441973	<i>Pmoratoiu</i> 22	(TTTC) ₁₇	Forward: AAAATTCCGCTCAGTCATTA Reverse: ACTCCTTCCCTAATCCAGTTT	42 °C	4.0
JX441974	<i>Pmoratoiu</i> 23	(TA) ₄ (CA) ₁₁	Forward: ACCTGGTCTAACCCTTTGGAAAT Reverse: CAGCGTTACCAGACATTTTATGTTC	70 °C	3.0
JX441975	<i>Pmoratoiu</i> 24	(AT) ₇	Forward: GCTATTTGTCTACCTATCTATCTTTCAT Reverse: CAATAAAACTCTGGACCTTGAAC	40 °C	4.0
JX441976	<i>Pmoratoiu</i> 25	(ACT) ₁₁	Forward: TCTAATGTCCACACTGCTACTACT Reverse: GCTAATGGCCGAGTTATTG	70 °C	4.0
JX441977	<i>Pmoratoiu</i> 26	(CA) ₇	Forward: ATTTGGCTGTCTGACCTGTCTTA Reverse: CCCATATTAGTTCGGATCACAAG	63 °C	4.0
JX441978	<i>Pmoratoiu</i> 27	(TCTA) ₁₇	Forward: CTCTATCTAACCCTTTCATA Reverse: AAGATGGATAGATGTGAGA	57 °C	2.0
JX441979	<i>Pmoratoiu</i> 28	(CA) ₈	Forward: GAAATGAGAGGCGTGAGAGAT Reverse: GCTGTCCGTCAATGGGTAT	51 °C	1.0
JX441980	<i>Pmoratoiu</i> 29	(CA) ₁₆	Forward: GAGGAAAAGTCAAGGAATAAATGTC Reverse: ACAGTCTTCTCAATCTGCATGTCT	46 °C	0.8

T_A: annealing temperature; MgCl₂: magnesium chloride.

Table 2. Descriptive analysis of the genetic diversity in 22 polymorphic microsatellite loci obtained from five populations of *P. moratoi*. Significant deviations ($p < 0.002$) from Hardy–Weinberg Equilibrium (HWE) following the Bonferroni correction are indicated by an asterisk (*). Heterozygosity and HWE were not estimated for the populations with small sample size (Avaré and Lençóis Paulista).

Population Locus	São Carlos ($n = 41$)			Bauru ($n = 27$)			Brotas ($n = 41$)			Avaré ($n = 1$)	LP ($n = 3$)	Total		
	N_A	H_O	H_E	N_A	H_O	H_E	N_A	H_O	H_E	N_A	N_A	S	N_A	PIC
<i>Pmoratoi</i> μ5	5	0.29	0.35	-	-	-	3	0.06	0.06	-	-	201–239	6	0.47
<i>Pmoratoi</i> μ6	8	0.70	0.71	5	0.62	0.68	9	0.59	0.68	2	1	208–240	10	0.78
<i>Pmoratoi</i> μ7	1	0.00	0.00	1	0.00	0.00	2	0.46	0.48	1	1	185–187	2	0.28
<i>Pmoratoi</i> μ8	2	0.17	0.16	1	0.00	0.00	2	0.02	0.02	1	1	209–211	2	0.06
<i>Pmoratoi</i> μ10	3	0.30	0.31	1	0.00	0.00	3	0.12	0.12	1	1	109–113	3	0.15
<i>Pmoratoi</i> μ11	2	0.05	0.05	1	0.00	0.00	3	0.12	0.14	1	1	125–129	3	0.07
<i>Pmoratoi</i> μ12	6	0.80	0.77	8	0.52	0.64	13	0.85	0.87	3	2	144–196	14	0.82
<i>Pmoratoi</i> μ13	2	0.12	0.16	3	0.42	0.59	2	0.05	0.05	2	1	162–168	4	0.34
<i>Pmoratoi</i> μ14	7	0.75	0.76	1	0.00	0.00	5	0.68	0.71	1	2	190–218	8	0.71
<i>Pmoratoi</i> μ15	10	0.67	0.80	9	0.83	0.79	12	0.73	0.85	2	2	177–241	18	0.84
<i>Pmoratoi</i> μ16	3	0.62	0.55	3	0.75	0.64	2	0.10	0.10	2	1	146–158	4	0.48
<i>Pmoratoi</i> μ17	3	0.08	0.08	2	0.04	0.04	1	0.00	0.00	1	1	092–098	3	0.09
<i>Pmoratoi</i> μ18	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2	1	163–165	2	0.02
<i>Pmoratoi</i> μ19	2	0.50	0.51	2	0.46	0.49	2	0.36	0.43	2	1	167–169	2	0.40
<i>Pmoratoi</i> μ21	1	0.00	0.00	2	0.40	0.47	2	0.17	0.16	1	1	242–246	3	0.25
<i>Pmoratoi</i> μ23	9	0.72	0.86	4	0.29	0.61	7	0.76	0.81	3	1	225–251	12	0.87
<i>Pmoratoi</i> μ24	3	0.26	0.66 *	2	0.30	0.47	2	0.10	0.09	1	2	148–174	8	0.72
<i>Pmoratoi</i> μ25	3	0.60	0.55	3	0.57	0.58	2	0.54	0.51	1	2	246–252	3	0.53
<i>Pmoratoi</i> μ26	2	0.13	0.12	2	0.42	0.38	2	0.41	0.46	1	1	119–133	4	0.38
<i>Pmoratoi</i> μ27	8	0.45	0.77 *	8	0.87	0.84	9	0.69	0.85	4	2	196–244	12	0.86
<i>Pmoratoi</i> μ28	2	0.45	0.49	3	0.58	0.58	3	0.61	0.62	1	1	195–203	3	0.48
<i>Pmoratoi</i> μ29	8	0.90	0.84	7	0.96	0.77	6	0.47	0.64 *	3	1	154–198	11	0.80

LP: Lençóis Paulista; 0.00: monomorphic locus; -: locus not amplified; N_A : number of alleles; H_O : observed heterozygosity; H_E : expected heterozygosity; S: size range; PIC: polymorphic information content.

The total number of alleles per locus (N_A) varied between 2 and 18 (Table 2). Observed heterozygosity (H_O) ranged from 0.02 to 0.96, expected heterozygosity (H_E) from 0.02 to 0.87, and polymorphism information content (PIC) from 0.02 to 0.87. As might be expected from the relatively large number of markers developed for this study, evidence of linkage disequilibrium was found in three pairs of loci (*Pmoratoi*μ10–*Pmoratoi*μ25, *Pmoratoi*μ12–*Pmoratoi*μ15, and *Pmoratoi*μ15–*Pmoratoi*μ25) following Bonferroni correction ($p < 0.002$). Significant deviations from Hardy–Weinberg Equilibrium (HWE) were found in *Pmoratoi*μ24 and *Pmoratoi*μ27 from the São Carlos population and in *Pmoratoi*μ29 from Brotas, due to a deficit of heterozygotes. These deviations can be attributed to the presence of null alleles in these populations. The estimated null allele frequency for the *Pmoratoi*μ24 locus from São Carlos was 0.23, and that for *Pmoratoi*μ27 from this same population was 0.18. The estimated frequency for *Pmoratoi*μ29 from Brotas was 0.11.

In addition to the loci with deviations from HWE, null alleles were detected in the *Pmoratoi*μ13 (0.10), *Pmoratoi*μ23 (0.19), and *Pmoratoi*μ24 (0.10) loci from Bauru, and in *Pmoratoi*μ27 from Brotas (0.07). In all these cases, the evidence of the presence of null alleles was relatively weak and thus insufficient to confirm a significant departure from HWE following the Bonferroni correction. Micro-Checker 2.2.3 [20] did not detect small allele dominance, but found evidence of the presence of stutter bands in one locus, *Pmoratoi*μ13 from Bauru. The identity analysis indicated that the combination of all the loci would permit the individual identification of each of the specimens.

3. Experimental Section

3.1. Construction of Enriched Microsatellite Genomic Library

We constructed an enriched partial microsatellite genomic library using an approach based on the selective hybridization method of Kijas [21]. The library was constructed using DNA extracted from the muscle tissue of one specimen of *P. moratoi* using the procedure of Sambrook *et al.* [22] with modifications. Six micrograms of genomic DNA were digested with 50 units of *Afa* I (Invitrogen) and the fragments were then ligated to *Rsa* I linkers (*Rsa*21: 5'-CTCTTGCTTACGCGTGGACTA-3'/*Rsa*25: 5'-TAGTCCACGCGTAAGCAAGAGCACA-3') using 2 units of T4 DNA ligase (Promega). The fragments were then amplified by polymerase chain reaction (PCR) with a reduced number of cycles (20 cycles) using the primer *Rsa*21. The PCR products were purified, denatured and hybridized with biotinylated microsatellite probes (GT₈ and CT₈) at room temperature for 20 min. The hybrid mixtures containing microsatellites were then collected by streptavidin-coated magnetic beads (Promega). The selected fragments were amplified via PCR and the products were ligated into a pGEM-T easy cloning vector (Promega). *Escherichia coli* XL1-Blue cells (Stratagene) were transformed with recombinant plasmids by electroporation and grown overnight in solid Luria-Bertani agar medium containing ampicillin, IPTG and X-Gal. The positive colonies were selected and grown in liquid medium with 2YT HMFm containing ampicillin. After growing for 16 h, they were stored at −80 °C.

3.2. Sequencing and Primer Design

Of the total of 596 clones obtained, 384 were sequenced bidirectionally in an ABI Prism 3100 automatic sequencer (Applied Biosystems: Foster City, CA, USA). The DNA sequences were exported into Codoncode Aligner 3.7.1 (CodonCode Corporation) which assembled the contigs and verified the redundancy of the library. The Bioedit program was used to check the quality of the sequences by chromatogram and to align them to form a consensus sequence. The repetitive elements were located using the Microsatellite Repeats Finder program [16]. After removal of the vector sequences, adapters, and restriction endonuclease sites by the Microsat software (version 1.0; CIRAD: Montpellier, France, 2005), the primers were designed using Primer 3 [23].

3.3. Genotyping

The polymorphic microsatellite markers were characterized by the amplification of the genomic DNA obtained from buccal epithelial cells (non-destructive method) following a modified version of

the procedure described by Pidancier *et al.* [24]. Samples were obtained from five remnant *P. moratoi* populations in the Brazilian state of São Paulo: 41 samples were collected in the municipality of São Carlos (22°01'00.5" S, 47°56'21.0" W), 41 in Brotas (22°12'53" S, 47°54'41" W), 27 in Bauru (22°20'48.46" S, 49°0'56" W), 3 in Avaré (22°53.227' S, 48°56.803' W), and 1 in Lençóis Paulista (22°49'13.17" S, 48°53'0.28" W). The PCRs were prepared in a final volume of 15 µL containing 10 ng of the DNA template, 1× reaction buffer, 0.3 mM dNTP, 0.6–4.0 mM MgCl₂ (Table 1), 0.6 µM of each primer, and 1 unit of Taq polymerase (Invitrogen). The reactions were conducted following the same cycling conditions: 5 min at 94 °C followed by 41 cycles of 30 s at 94 °C, 1 min at the locus-specific annealing temperature (Table 1), and 1 min at 72 °C, followed by a final extension of 30 min at 72 °C to minimize stutter bands. The PCR products were analyzed in a Dual Dedicated Height Sequencing Kit (CBS Scientific) vertical electrophoresis system in 6% denaturing polyacrylamide gel and stained with silver nitrate [25]. Allele size was estimated by comparison with a 10 bp DNA ladder (Invitrogen) and using the GelAnalyzer 2010a software [26].

3.4. Characterization of Polymorphic Markers

The levels of polymorphism of the microsatellites were evaluated as the number of alleles per locus (N_A), observed (H_O) and expected (H_E) heterozygosity calculated by Popgene 1.32 [27]. The polymorphism information content (PIC) was calculated with Cervus 3.0.3 [19], which was also used to conduct a test of individual discrimination (identity analysis). The Genepop 4.0.9 software [28] was used to detect an excess or deficiency of heterozygotes, linkage disequilibrium between pairs of loci, and deviations from the Hardy–Weinberg Equilibrium (HWE), for which significance levels were determined using the Markov chain algorithm [29], with 10,000 dememorization steps, 100 batches and 5000 iterations per batch. All significance levels were adjusted by the sequential Bonferroni correction for multiple tests [30]. Micro-Checker 2.2.3 software [20] was used to identify genotyping errors and verify the presence of null alleles using the Brookfield method [31].

4. Conclusions

These are the first microsatellite markers developed for Morato's digger toad, and in fact, the first for any member of the genus *Proceratophrys*. These markers constitute a powerful tool for the study of *P. moratoi* populations, allowing the identification of untagged individuals and providing a database for the development of kinship studies for future *ex situ* conservation programs. It will also be possible to analyze inbreeding, genetic diversity and structure, and gene flow in natural populations, which will be vital for the development of effective *in situ* conservation measures.

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