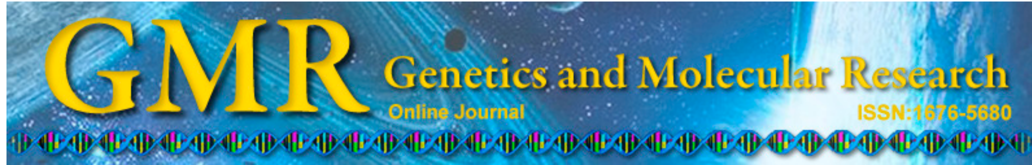

A suite of microsatellite markers for genetic management of captive cracids (Aves, Galliformes)

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ABSTRACT. Cracids are medium to large frugivorous birds that are endemic to the Neotropics. Because of deforestation and overhunting, many species are threatened. The conservation of several species has relied on captive breeding and reintroduction in the wild, but captive populations may be inbred. Microsatellite tools can permit the construction of genetic pedigrees to reduce inbreeding, but only a few loci are available for this group of birds. Here, we present 10 novel polymorphic microsatellite loci and the cross-amplification of these and of 10 additional loci available in the literature in a panel of 5 cracid species, including 3 species with high conservation concern. We provide the first polymorphic loci for the jacutinga, *Aburria jacutinga* (N = 8), and red-billed curassow, *Crax blumenbachii* (N = 9), and

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additional loci for bare-faced curassow, *C. fasciolata* (N = 8), Alagoas curassow, *Pauxi mitu* (N = 5), and razor-billed curassow, *P. tuberosa* (N = 5). The average number of alleles was 2.9 for *A. jacutinga*, 2.7 for *C. blumenbachii*, 3.5 for *C. fasciolata*, 2.6 for *P. mitu*, and 5.7 for *P. tuberosa*. The mean expected heterozygosities were 0.42, 0.40, 0.48, 0.37, and 0.59, respectively. The average probabilities that the set of loci would not exclude a pair of parents of an arbitrary offspring were 2.9% in *A. jacutinga*, 1% in *C. blumenbachii*, 0.5% in *C. fasciolata*, 0.4% in *P. mitu*, and 0.002% in *P. tuberosa* suggesting that these loci may be adequate for parentage analysis and to implement *ex situ* genetic management plans.

Key words: Galliformes; Endangered birds; Molecular markers; Parentage analysis; Genetic pedigrees

INTRODUCTION

Cracids (guans, chachalacas, and curassows) are medium to large frugivorous birds that are endemic to the Neotropics, where they play a significant role in tropical forest dynamics, especially through dispersing large-seeded plants (Sedaghatkish et al., 1999). Most species depends on large primary forest tracts, which make them highly vulnerable to habitat disturbance. For centuries, they also have been one of the major sources of proteins for subsistence hunters, and many populations were extirpated throughout their distributions. As a result, many species are vanishing, and 24 of the approximately 50 existing species are under some level of threat (Brooks and Fuller, 2006).

The conservation of a number of cracids has relied on captive breeding and reintroduction into the wild. Ongoing releasing programs of captive-bred individuals have reestablished populations of the endangered red-billed curassow, *Crax blumenbachii* (IBAMA, 2004; Bernardo et al., 2011), and of the jacutingas, *Aburria jacutinga* (Azeredo RA, unpublished results), in areas where they have become extinct. Other reintroduction programs have been planned to begin in the near future, and the most dramatic case is the Alagoas curassow, *Pauxi mitu*, which has been extinct in the wild since the late 1970s (Silveira et al., 2004).

Captive cracid populations were often started from a limited number of founders, either because they are difficult to capture alive or because there were no more individuals available in the wild (Silveira et al., 2004). This increases the risks that deleterious alleles become homozygous and lead to inbreeding depression, threatening the success of both captive breeding and reintroductions (Witzenberger and Hochkirch, 2011). The traditional approach to avoid inbreeding is minimizing the mating between closely related individuals following studbook data (Ballou and Foose, 1995). However, pedigree recordings are not available for the current captive cracid populations, although most of them were founded at least 2 decades ago. In these cases, molecular genetic analyses can provide accurate genetic pedigrees, as well as information on population genetic structure and variability (Witzenberger and Hochkirch, 2011). Thus, there is an urgent interest in implementing genetic monitoring programs for captive cracid populations that are intended to supply individuals for the ongoing and future

reintroduction programs.

Polymorphic microsatellite loci are reliable tools for investigating individual relationships in both captive and wild populations (Stoeckle et al., 2012), but only a few loci are available for cracids (Hughes and Larson, 2000; Gonçalves et al, 2010; Sousa et al., 2013). Here, we present a set of novel and heterologous microsatellite loci isolated from cracid genomic libraries and we tested their applicability for parentage analyses in a panel of 5 cracid species, 3 of which have a high conservation concern. These markers include the first polymorphic loci for the endangered *C. blumenbachii* and *A. jacutinga*, and additional loci for the extinct in the wild *P. mitu*.

MATERIAL AND METHODS

We collected blood samples from 27-30 captive individuals of each studied species (Table 1). These animals were available in 3 private breeding facilities: CRAX - Sociedade de Pesquisa do Manejo e da Reprodução da Fauna Silvestre, Criadouro Científico e Cultural de Poços de Caldas, and CESP/Paraibuna. DNA was extracted using a standard phenol:cloroform protocol. To isolate new microsatellite loci, we generated an enriched library by digesting a genomic DNA sample of *A. jacutinga* with a restriction enzyme (*Rsa*I; Promega, USA). DNA fragments were ligated to double-strand SNX linkers and hybridized with 8 biotinylated probes for dinucleotide repeats (Hamilton et al., 1999). Then, we selected DNA fragments containing potential microsatellites using magnetic beads (Streptavidin MagneSphere Paramagnetic Particles, Promega), and the enriched sample was cloned using TOPO TA Cloning Version P (Invitrogen Life Technologies). We sequenced 180 clones containing inserts in an ABI 3730 automated sequencer. Fluorescently labeled primers were designed for 22 microsatellite motifs consisting of at least 6 repeats using the Primer3 software program (Rozen and Skaletsky, 2000). We also designed primers for 15 new microsatellite motifs that were obtained from a genomic library previously developed for the razor-billed curassow, *P. tuberosa*, using the same methodology described above (see Sousa et al., 2013). We tested the broader use of these 37 loci and of an additional 10 loci available in the literature for *P. tuberosa* and *P. mitu* (Sousa et al., 2013). The loci were screened for polymorphism within and between the cloned species and in *C. blumenbachii*, bare-faced curassow, *C. fasciolata*, and *P. mitu*.

Polymerase chain reactions (PCRs) were performed in an Eppendorf MasterCycler Gradient thermal cycler in a 10-μL volume containing 150 ng DNA, 0.2 mM of each dNTP, 1X PCR buffer (200 mM Tris-HCl, pH 8.4, and 500 mM KCl), 0.2 μM of each primer, 3 mM $MgCl_2$, and 1 U *Taq*-polymerase. Amplification conditions were 94°C for 5 min; 30 cycles of 94°C for 30 s, 30 s at the annealing temperature specified in Table 1, and 30 s at 72°C; and a final extension of 72°C for 10 min. Amplified products were scored on an automated sequencer (ABI 3730).

We calculated the observed and expected heterozygosities, the probability of heterozygosity deficit, and linkage disequilibrium using GENEPOP 4.0 (Raymond and Rousset, 1995). Levels of significance for multiple tests were corrected through the sequential Bonferroni method (Rice, 1989). Evidence for null alleles was evaluated using Micro-Checker (Van Oosterhout et al., 2004). The suitability of the loci for parentage analysis was assessed

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for each species by calculating the average probability that the set of loci would not exclude a pair of candidate parents from parentage of an arbitrary offspring (PE), using the Cervus 3.0 software (Kalinowski et al., 2007).

Table 1. Primer sequences, repeat motifs, and annealing temperatures (Ta) of 10 novel polymorphic microsatellite loci isolated from the *Aburria jacutinga* and *Pauxi tuberosa* genomic libraries.

Locus	Primer sequence (5'-3')	Repeat motif	Ta (°C)
Aburria 21	GAGGATAAGCACGTCACAG		
	CAGCTAACTGGGTTTGAATCTG	$(CA)_{15}$	58.5
Aburria 22	ATGCGCACTAATGAACAGTC		
	TCAGCTAGCCTTCCATTCCCTCAC	$(AG)_5G(AG)_{10}$	58.5
Aburria 36	AAGTAGATCACCACATGAG		
	CATTAAGGTGGTAACTTCAG	$(GT)_5T(GT)_{15}$	46.4
Aburria 44	TCCCTAGCAGATTCTGAGAC		
	CACCACAGGAACAGTGAG	$(GT)_{10}$	55.8
Aburria 48	ATTGGTAAAATGTATGATCAG		
	TGCAAATAGTTTCATAGCATCAG	$(TG)_{14}$	48.2
Aburria 49	TTCCTTTGGAATAGTGTGAG		
	ACAAATGAACTGCAGAAGC	$(CA)_{20}$	46.4
Aburria 105	ACCATTTGCAGATTAGAC		
	ATCATCAGTGAAGAAACTG	$(TG)_8$	55.8
Pauxi 1-16	ATGAACAGCCATTGCATGAC		
	AGGCAGACATTAGCAGTG	$(GAAA)_5$	63.1
Pauxi 1-29	ACCATGTCTGGTTTCCT		
	ACTACATTGCTTCCTAACAG	$(GTTT)_3GG(GTTT)T(GTTT)_4$	58.5
Pauxi 1-36	ACGATTCTGCAGTGAG		
	GTTATATCAAGTGTCCTCAG	$(AAAG)_6$	55.8

RESULTS

Seven of the 22 loci isolated from *A. jacutinga*, and 3 of the 15 unpublished loci isolated from the *P. tuberosa* genomic library amplified and were polymorphic in at least 1 of the target species (Table 2). Of the 10 loci previously available in the literature for *P. tuberosa* and *P. mitu*, 9 presented polymorphism in other species. In total, we obtained 9 polymorphic loci for *A. jacutinga*, 9 for *C. blumenbachii* (the first described for these species), 10 additional loci for *C. fasciolata*, and 5 additional loci for *P. mitu* and *P. tuberosa*. We found significantly linked loci in *A. jacutinga* (*Pauxi* 1-4), *C. fasciolata* (*Pauxi* 1-37 and *Pauxi* 1-16), and *P. tuberosa* (*Pauxi* 1-37, *Pauxi* 2-7 and *Pauxi* 3-1), which were not used for parentage analysis.

The average number of alleles per polymorphic locus was 2.9 for *A. jacutinga*, 2.7 for *C. blumenbachii*, 3.5 for *C. fasciolata*, 2.6 for *P. mitu*, and 5.7 for *P. tuberosa*. The mean expected heterozygosities were 0.42, 0.40, 0.48, 0.37, and 0.59, respectively (Table 2). Micro-Checker indicated homozygote excess in loci *Pauxi* 3-4 for *A. jacutinga*; *Pauxi* 1-36 and *Pauxi* 3-4 for *C. blumenbachii*; *Pauxi* 1-16, *Pauxi* 1-29, and *Pauxi* 1-36 for *C. fasciolata*; and *Aburria* 21, *Aburria* 22, and *Aburria* 36 for *P. tuberosa*. However, we believe that heterozygote deficiencies in these loci may be due to inbreeding rather than null alleles. For instance, locus *Pauxi* 3-4 was fixed in 1 of 2 breeding facilities from which we obtained samples of *C. blumenbachii*, which represented 13 of the 30 analyzed individuals. Besides, individuals that failed to amplify completely (potentially homozygous for null alleles) were not found in any of the locus-species combinations. PE values were 2.9% for *A. jacutinga*, 1% for *C. blumenbachii*, 0.5% for *C. fasciolata*, 0.4% for *P. mitu*, and 0.002% for *P. tuberosa*.

Table 2. Number of alleles per locus (N_A), number of individuals screened (n), allelic size range, observed (H_O) and expected heterozygosities (H_E), and probability of heterozygote deficits (P) for 20 microsatellite loci cross-amplified among 5 cracid species.

Locus	*Aburria jacutinga*					*Pauxi tuberosa*					*Pauxi mitu*					*Crax blumenbachii*					*Crax fasciolata*				
	N_A/n	Size	H_O	H_E	P	N_A/n	Size	H_O	H_E	P	N_A/n	Size	H_O	H_E	P	N_A/n	Size	H_O	H_E	P	N_A/n	Size	H_O	H_E	P
Aburria 21	4/27	139-157	0.89	0.70	0.987	6/30	141-145	0.10	0.41	0.000	2/30	141-145	0.53	0.48	0.813	-	-	-	-	-	-	-	-	-	-
Aburria 22	2/27	303-305	0.52	0.50	0.714	8/30	296-326	0.47	0.74	0.000	3/30	308-326	0.63	0.53	0.926	-	-	-	-	-	-	-	-	-	-
Aburria 36	-	-	-	-	-	3/30	448-454	0.40	0.61	0.049	2/30	452-454	0.33	0.28	1.000	-	-	-	-	-	-	-	-	-	-
Aburria 44	3/27	129-139	0.07	0.07	1.000	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aburria 48	4/27	92-100	0.67	0.55	0.975	3/30	87-93	0.43	0.46	0.416	2/30	87-89	0.10	0.15	0.167	2/30	87-89	0.37	0.30	0.557	-	-	-	-	-
Aburria 49	-	-	-	-	-	4/30	175-205	0.53	0.57	0.019	2/30	181-205	0.13	0.13	1.000	-	-	-	-	-	-	-	-	-	-
Aburria 105	2/27	217-219	0.37	0.45	0.292	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
**Pauxi* 1-4	2/27	166-174	0.18	0.17	1.000	11/30	166-222	0.90	0.87	0.046	3/30	166-190	0.20	0.24	0.056	3/30	152-172	0.40	0.43	0.545	8/30	194-226	0.73	0.78	0.093
**Pauxi* 1-13	-	-	-	-	-	16/30	126-252	0.67	0.85	0.007	4/30	210-266	0.67	0.55	0.944	-	-	-	-	-	2/30	139-151	0.40	0.32	1.000
Pauxi 1-16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2/30	244-248	0.57	0.50	0.709	2/30	236-244	0.13	0.32	0.005
Pauxi 1-29	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2/30	119-123	0.07	0.18	0.013
**Pauxi* 1-30	-	-	-	-	-	5/30	294-310	0.47	0.59	0.067	3/30	300-306	0.10	0.10	1.000	2/30	298-302	0.53	0.50	1.000	2/30	294-302	0.58	0.50	0.859
Pauxi 1-36	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6/30	294-340	0.30	0.71	0.000	7/30	301-351	0.41	0.79	0.000
**Pauxi* 1-37	-	-	-	-	-	4/30	148-180	0.23	0.51	0.000	2/30	148-180	0.53	0.51	0.755	2/30	168-180	0.03	0.10	0.053	2/30	168-180	0.27	0.23	1.000
**Pauxi* 2-2	5/27	233-261	0.78	0.68	0.925	9/30	195-249	0.73	0.71	0.472	3/30	227-237	0.53	0.47	0.474	-	-	-	-	-	-	-	-	-	-
**Pauxi* 2-7	-	-	-	-	-	3/30	141-153	0.47	0.54	0.043	2/30	149-153	0.33	0.33	0.745	-	-	-	-	-	-	-	-	-	-
**Pauxi* 2-19	-	-	-	-	-	2/21	190-194	0.62	0.51	0.926	-	-	-	-	-	-	-	-	-	-	5/30	177-193	0.60	0.68	0.266
**Pauxi* 2-30	2/27	197-201	0.18	0.17	1.000	2/30	197-201	0.27	0.36	0.160	3/30	197-205	0.43	0.48	0.244	2/30	201-205	0.63	0.50	0.266	2/30	193-201	0.36	0.44	0.301
**Pauxi* 3-1	-	-	-	-	-	6/30	161-185	0.30	0.58	0.000	4/30	157-173	0.53	0.62	0.027	3/30	173-183	0.27	0.27	0.508	3/30	172-180	0.57	0.55	0.606
**Pauxi* 3-4	2/27	202-204	0.11	0.48	0.000	3/30	207-219	0.41	0.57	0.130	2/30	207-217	0.33	0.28	1.000	2/30	213-217	0.43	0.30	0.702	-	-	-	-	-

*Loci previously published by Sousa et al. (2013). Adjusted P values after Bonferroni's correction were 0.005 for *A. jacutinga*, 0.003 for *P. tuberosa*, 0.004 for *P. mitu*, 0.006 for *C. blumenbachii*, and 0.005 for *C. fasciolata*.

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DISCUSSION

Cross-species amplification seemed to be a plausible alternative among cracids, with 19 of the 20 polymorphic loci showing transferability, often across genera, reducing the costs and time investment for the generation of polymorphic markers. Six loci that were monomorphic in the original species (2 from *A. jacutinga* and 4 from *P. tuberosa*) amplified and were polymorphic in other species.

The PE values varied among the studied species and sets of loci. Although some studies can reach probabilities of non-exclusion around 0.1% (Faircloth et al., 2010; Cherel et al., 2011), the populations that we analyzed invariably had a limited number of founders, and individuals must be related, weakening the analysis power (Jones et al., 2010). For this reason, we considered the PEs of 1% or less satisfactory, as the loci must be effective to resolve most of the assignments. The non-exclusion of more than 1 pair of potential parents must be more frequent in *A. jacutinga*, indicating the need of additional loci for this species.

The most critically endangered cracid species are those endemic to the Brazilian Atlantic forest (*P. mitu*, *C. blumenbachii*, and *A. jacutinga*). The current population of *P. mitu* is around 60 individuals living in 2 Brazilian breeding facilities, this species is one of the most endangered birds on earth. Most living individuals of *C. blumenbachii* are also in captivity. In 2004, when the captive population was 637 individuals, it was estimated that no more than 250 animals were living in the wild (IBAMA, 2004). Since captive breeding was demonstrated to be a feasible strategy to preserve cracids, Brazilian governmental authorities started to assist private captive breeding facilities and created official conservation action plans for these birds. The microsatellite markers presented here will contribute to the improvement of these official captive breeding programs by constructing genetic pedigrees, indicating individuals to be translocated among breeding facilities and allowing the selection of individuals for reintroduction in the wild based on their levels of heterozygosity and allelic diversity.

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