ORNITOLOGIA NEOTROPICAL 18: 439–444, 2007

CHARACTERIZATION OF MICROSATELLITE LOCI IN THREE SPECIES OF *AMAZONA* (PSITTACIFORMES) USING HETEROLOGOUS PRIMERS

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Caracterização de locos de microssatélite em três espécies de *Amazona* (Psittaciformes) por meio de iniciadores hetetólogos.

Key words: *Amazona,* Psittaciformes, parrot, microsatellite, heterologous primers, population genetics.

The genus *Amazona* presents 31 Neotropical species (Collar 1997) of which 16 are threatened at various levels (IUCN 2006). Among these, two endemic species of the Atlantic forest, the Red-tailed (*A. brasiliensis*) and the Red-spectacled (*A. pretrei*) amazons, are classified as vulnerable. Habitat destruction and pet trade are the most important threats to these species. Although the Blue-fronted Amazon (*A. aestiva*) is not listed as a threatened species, it is intensively captured in the wild for pet trade (Seixas & Mourão 2000).

Microsatellites have been widely used as markers in population genetics, parentage and conservation assessment studies as they present codominant inheritance and relatively high levels of polymorphism, and are easily analyzed using the polymerase chain reaction (PCR) (Bruford & Wayne 1993). However, the development of polymorphic microsatellite loci is usually time-consuming and expensive. Whenever cross-species amplification is possible, the cost of genotyping can reduce significantly and more population genetic and conservation studies can be done. This amplification is generally possible between closely related species as a consequence of the homology of the microsatellite flanking regions. The conservation of these regions has been detected in plants (e.g., Collevatti *et al.* 1999) and animals (e.g., Lau *et al.* 2004). Here we report the amplification of microsatellite loci in three *Amazona* species: *A. aestiva*, *A. pretrei* and *A. brasiliensis,* using heterologous primers designed for two other parrot species: *A. guildingii* and *Ara ararauna.*

METHODS

Total genomic DNA was extracted from

TABLE 1. Characterization of the 18 microsatellite loci for three *Amazona* species using heterologous primers.

Name	Repear motif[4]	*A. aestiva* (N = 22)			*A. pretrei* (N = 23)			*A. brasiliensis* (N = 18)		
		T_A	N_A	Length (bp)	T_A	N_A	Length (pb)	T_A	N_A	Length (bp)
UnaCT21[1]	$(GT)n(CTT)(GT)_n$	–	–	–	–	–	–	–	–	–
UnaCT32[1]	$(GT)_n$	–	–	–	–	–	–	60	1	248
UnaCT43[1]	$(GT)_n$	52	6	199 –211	52	5	186 –204	66	4	202 –208
UnaCT74[1]	$(GT)^n$	50	1	211	54	3	224 –228	60	2	212 –214
UnaGT55[1]	$(GT)n(AT)_n$	50	1	166	50	1	166	54	1	166
AgGT02[2]	$(GT)_n$	–	–	–	?	?	?	54	1	171
AgGT04[2]	$(GT)_n$	48	4	254 –262	?	?	?	54	4	252 –278
AgGT07[2]	(GT)n	50	12	257 –283	58 to 50	11	250 –288	50	8	261 –279
AgGT08[2]	$(GT)n(GCGT)_n$	52	1	304	50	1	302	50	2	322 –330
AgGT12[2]	$(GT)_n$	58 to 50	15	299 –333	52	7	296 –308	56	4	297 –307
AgGT17[2]	$(GT)_n$	58 to 50	1	419	58 to 50	5	420 –430	60	1	414
AgGT21[2]	$(GT)_n$	50	16	306 –340	58 to 50	5	299 –309	54	11	310 –340
AgGT22[3]	$(CA)_n$	59	5	190 –198	59	3	184 –190	60	7	186 –200
AgGT29[3]	$(CA)_n$	59	12	190 –224	59	9	193 –211	56	8	187 –221
AgGT72[3]	$(CA)_n$	50	14	270 –302	58 to 50	7	276 –288	50	6	272 –293
AgGT81[2]	$(GT)_n$	50	5	332 –344	?	?	?	52	7	330 –346
AgGT83[2]	$(GT)_n$	58 to 50	17	231 –275	54	18	233 –273	56	8	245 –269
AgGT90[3]	$(GT)_n$	58 to 50	13	196 –232	52	11	195 –227	50	7	192 –210

[1]Caparroz *et al.* (2003); [2]Russello *et al.* (2001); [3]Russello *et al.* (2005); [4]Repeat motif in the original species; T_A = the annealing temperature (°C) used in "touchdown" or standard PCRs; N_A = number of alleles; - = unsuccessful amplification; ? = not tested.

TABLE 2. Polymorphic 14 microsatellite loci characterized for three *Amazona* species using heterologous primers. The number of individuals genotyped for each species is showed between parentheses. Proportion of expected (H_E) and observed heterozygotes (H_O), paternity exclusion probability (Q), identity probability (I) are also showed. *Loci* with deviation from Hardy-Weinberg equilibrium ($P < 0.05$) are indicated as *. Unsuccessful amplification is indicated as -.

Name	*A. aestiva* (N = 22)				*A. pretrei* (N = 23)				*A. brasiliensis* (N = 18)			
	H_E	H_O	Q	I	H_E	H_O	Q	I	H_E	H_O	Q	I
UnaCT43	0.722	0.500*	0.493	0.206	0.638	0.478*	0.379	0.310	0.607	0.812	0.324	0.396
UnaCT74	-	-	-	-	0.124	0.130	0.062	0.780	-	-	-	-
AgGT04	-	-	-	-	-	-	-	-	0.583	0.500	0.305	0.410
AgGT07	0.838	0.810	0.687	0.079	0.803	0.565*	0.631	0.105	0.726	0.778	0.509	0.189
AgGT08	-	-	-	-	-	-	-	-	0.472	0.412	0.180	0.613
AgGT12	0.901	0.954	0.803	0.032	0.828	0.773	0.657	0.098	0.469	0.588	0.268	0.382
AgGT17	-	-	-	-	0.534	0.609	0.322	0.323	-	-	-	-
AgGT21	0.910	0.952	0.820	0.028	0.662	0.696	0.403	0.297	0.844	1.000	0.694	0.076
AgGT22	0.678	0.500*	0.449	0.223	0.643	0.435*	0.351	0.353	0.734	0.889	0.511	0.193
AgGT29	0.830	0.682	0.683	0.074	0.855	0.869	0.708	0.072	0.731	0.833	0.520	0.180
AgGT72	0.896	0.905	0.790	0.038	0.628	0.565	0.412	0.242	0.491	0.444	0.298	0.338
AgGT81	-	-	-	-	-	-	-	-	0.784	0.647*	0.591	0.132
AgGT83	0.915	0.952	0.828	0.026	0.925	1.000	0.849	0.020	0.797	0.823	0.601	0.132
AgGT90	0.830	0.818	0.687	0.072	0.855	0.304*	0.716	0.066	0.721	0.778	0.494	0.202
All loci			0.999	1.684×10^{-11}			0.999	1.908×10^{-9}			0.999	2.289×10^{-8}

blood samples of 22 *A. aestiva* (from Mato Grosso do Sul), 23 *A. pretrei* (from Rio Grande do Sul), and 18 *A. brasiliensis* (from Paraná), using standard proteinase K digestion followed by phenol: chloroform purification as described by Bruford *et al.* (1992) with modifications. The relatedness among all individuals analyzed was unkown.

A total of 18 heterologous microsatellite primer pairs were tested (Table 1): 13 with *Amazona guildingii* (Russello *et al.* 2001, Russello *et al.* 2005), and 8 with *Ara ararauna* (Caparroz *et al.* 2003). PCR amplifications were carried out in a total reaction volume of 10 μl containing 50–20 ng of template DNA, 10mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 0.5 μM of each primer, and 0.5 unit of Taq polymerase (Phoneutria, BR) in a PE 9700 thermal cycler (PE Biosystems). PCR conditions for the majority of the primers were: initial denaturation at 95ºC for 10 min, 35 cycles of 95ºC for 1 min, annealing ranging from 48 to 66ºC for 1 min, and 72ºC for 1 min, followed by 72ºC for 20 min. Reaction conditions for some primers were optimized using the "touchdown" cycling program as described by Russello *et al.* (2001). For *A. brasiliensis*, the PCR products were fluorescently (FAM, HEX/TET or NED, Applied Biosystems) labeled using an M13 forward primer as described by Caparroz *et al.* (2003). For the other two species, we used fluorescent labeled forward primers.

Initially, PCR products for two individuals from each species were visualized in 1.5% TBE agarose gels stained with ethidium bromide. The loci that presented clear and reproducible products were amplified in all individuals and analyzed in 7% denaturing polyacrylamide gels in an ABI 377 DNA Sequencer using the GeneScan and Genotyper 2.1 (Applied Biosystems). For all positive amplifications, one homozygous individual was sequenced and the presence of microsatellites was confirmed.

The number of alleles per locus, the observed and expected heterozygosities, and the paternity exclusion and the genetic identity probabilities were estimated using Identity 1.0 (Wagner & Sefc 1999). Deviation from Hardy-Weinberg expectations and linkage disequilibrium were analysed using approaches as implemented in Genepop 3.4 (Raymond & Rousset 1996).

RESULTS AND DISCUSSION

Among the 18 primer pairs, 15 successfully amplified products in *A. aestiva* (11 polymorphic). In *A. pretrei* and *A. brasiliensis*, 13 and 17 primer pairs resulted in products (12 and 13 polymorphic), respectively (Table 2). Evidence of deviation from Hardy-Weinberg equilibrium was found for some loci (Table 2) probably due to the presence of null alleles. Null alleles are alleles that consistently do not amplify during PCR, such that many of the homozygotes are, in reality, heterozygotes (Pemberton *et al.* 1995). Nucleotide sequence variation in the primer annealing site flanking the microsatellite may be the main cause of the failure to amplify alleles by PCR (Callen *et al.* 1993). The presence of null alleles may result to homozygosity excess, leading to an underestimation of genetic diversity, population size and failure in determining genetic structure, migration rates, kinship and parentage (Marshall *et al.* 1998, Taberlet *et al.* 1999, Piggott & Taylor 2003, Chapuis & Estoup 2004, Dakin & Avis 2004). The loci AgGT07 and AgGT90 presented linkage disequilibrium ($P < 0.05$) in the three species. This was also observed between AgGT04 and AgGT21 in *A. brasiliensis*, and between AgGT12 and AgGT72 in *A. aestiva*. The number of loci developed for *A. guilinguii* that successfully resulted in amplification products was higher for *A. brasiliensis* than for the other species. This result may be due to the phylogenetic relationship among these species, since *A.*

guildinguii is phylogenetically more related to *A. brasiliensis* than the other two species (Russello & Amato 2004). The same three heterologous primer pairs from *A. ararauna* showed amplification in the three species, but in *A. brasiliensis* an additional pair resulted in products.

The use of these heterologous microsatellite primers will allow the collection of population genetic data for the development of conservation strategies for these species.

ACKNOWLEDGMENTS

We are grateful to Elenise A. B. Sipinski, Gláucia H. F. Seixas, Jaime Martinez and Nêmora Prestes for providing samples. This work was funded by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação o Boticário de Proteção à Natureza. Mike W. Bruford and one anonymous referee provide important corrections on the manuscript.

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Accepted 29 April 2007.