



Comparative analysis of microsatellite variability in five macaw species (Psittaciformes, Psittacidae): Application for conservation

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Abstract

Cross-amplification was tested and variability in microsatellite primers (designed for Neotropical parrots) compared, in five macaw species, viz., three endangered blue macaws (*Cyanopsitta spixii* [extinct in the wild], *Anodorhynchus leari* [endangered] and *Anodorhynchus hyacinthinus* [vulnerable]), and two unthreatened red macaws (*Ara chloropterus* and *Ara macao*). Among the primers tested, 84.6% successfully amplified products in *C. spixii*, 83.3% in *A. leari*, 76.4% in *A. hyacinthinus*, 78.6% in *A. chloropterus* and 71.4% in *A. macao*. The mean expected heterozygosity estimated for each species, and based on loci analyzed in all the five, ranged from 0.33 (*A. hyacinthinus*) to 0.85 (*A. macao*). As expected, the results revealed lower levels of genetic variability in threatened macaw species than in unthreatened. The low combined probability of genetic identity and the moderate to high potential for paternity exclusion, indicate the utility of the microsatellite loci set selected for each macaw species in kinship and population studies, thus constituting an aid in planning *in-situ* and *ex-situ* conservation.

Key words: conservation, cross-amplification, macaw, microsatellite, Psittaciformes.

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Around 30% of all parrot species are endangered or vulnerable due to habitat destruction and illegal trade (Collar, 1997). Conservation may include the establishment of *ex-situ* populations for reproduction programs, as well as *in-situ* management (e.g. translocation and reintroduction). Microsatellite data constitute a useful aid in planning the estimation of genetic relationships among individuals, demes and populations, as well as in the analysis of genetic variability and population dynamics (Jarne and Lagoda, 1996; Ellegren *et al.*, 1997; Li *et al.*, 2002). Moreover, these markers can be employed in guiding breeding decisions and improving the genetic management of captive populations, through the identification of potential couples with the lowest genetic similarities (Caballero and Toro, 2000; Jones *et al.*, 2002; Russello and Amato, 2004). However, the isolation of microsatellite loci is usually expensive and time-consuming. Furthermore, the avian genome contains about 10 times less microsatellite loci than is the case with humans (Primmer *et al.*, 1997). As an alternative, these

primers from closely related species can be cross-amplified in the focal organism (Primmer *et al.*, 2005).

Few microsatellite markers have been developed for parrots, although positive cross-amplification of some has been reported for several species (Hughes *et al.*, 1998; Robertson *et al.*, 2000; Russello *et al.*, 2001, 2007; Caparroz *et al.*, 2003, 2007; Sainsbury *et al.*, 2004; Chan *et al.*, 2005; Raisin *et al.*, 2009; Pillay *et al.*, 2009). Nevertheless, there is no available information regarding amplification efficiency or polymorphism levels in several macaw species.

The aim was to report the success in cross-species amplification of several microsatellite markers, and in the comparative analysis of polymorphism levels in five macaw species, three endangered (the Spix's macaw, *Cyanopsitta spixii*, extinct in the wild, the Lear's macaw, *Anodorhynchus leari*, endangered, and the hyacinth macaw, *Anodorhynchus hyacinthinus*, vulnerable), as well as two unthreatened species (the scarlet macaw, *Ara macao*, and the red-and-green macaw, *A. chloropterus*).

Blood samples were analyzed from 11 captive Spix's macaws (eight originally from the wild, and three non-siblings born in captivity), 21 captive Lear's macaws (although all wild birds, their geographical origin and rela-

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tionships were unknown), 30 wild hyacinth macaws from the Pantanal (state of Mato Grosso do Sul), 31 wild red-and-green macaws from the Pantanal, and 28 wild scarlet macaws from the southeast of the state of Pará, all in Brazil. Samples of the latter three species were obtained from nestlings. Only one chick per nest was studied. All the samples were preserved in absolute ethanol and stored frozen at the Laboratório de Genética e Evolução Molecular de Aves (LGEMA), Instituto de Biociências, Universidade de São Paulo. Total DNA was extracted from blood samples, according to standard protocol, with proteinase K digestion and phenol: chloroform (Bruford *et al.*, 1992).

Primer pairs were tested for 19 di- and tetranucleotide microsatellite loci. Seven of these were designed for *Ara ararauna* (UnaCT21, UnaCT32, UnaCT43, UnaCT74 and UnaGT55; Caparroz *et al.*, 2003; UnaCT41int; Gebhardt and Waits, 2008; UnaCT35 F 5'TCTATCCCTTTTGTGTCAGC3' and UnaCT35 R 5'TAGCTAGATTTTCTTCTCTG3'; R. Caparroz, unpublished), eight for *Amazona guildingui* (AgGT07, AgGT08, AgGT12, AgGT17, AgGT21, AgGT81, AgGT19 and AgGT32; Russello *et al.*, 2001, 2005), two for *Anodorhynchus hyacinthinus* (Scott K. Davis, unpublished; MAC 436 F 5'GCACCAAACA CAACATCTTATTC3' and MAC 436 R 5'TTGGGACAC CAATGTAATTTG3', and HYA 1172 F 5'GATCCTTTGCTTAAGACAGATGTC3' and HYA 1172 R 5'GAGTCAAATACACATTCAGCTTCTG3'), and two for *Psittacus erithacus* (Peeμ11 and Peeμ16; Taylor and Parkin, 2007). In each primer pair, the forward one had an additional 5' M13 sequence tail (5'-TGAAAACGACGCCAGT-3') (Schuelke, 2000), so as to enable applying the universal dye-labelling method (Boutin-Ganache *et al.*, 2001).

We initially tested the potential cross-amplification of each locus (Table 1) with two to four samples from each species. PCR was carried out in a total volume of 12 μL with 20-50 ng of template DNA, 10 mM of Tris-HCl, 50 mM of KCl, 1.5 mM of MgCl₂, 200 μM of each dNTP, 0.2 μM of a M13 fluorescent primer (FAM, HEX or NED, Applied Biosystems, CA), 0.1 μM of an M13 tailed forward primer, 0.3 μM of a reverse primer and 0.5 unit of *Taq* polymerase (Pharmacia). PCR conditions were: initial denaturation at 95 °C for 10 min; followed by 35 cycles of 95 °C for 1 min, 52-58 °C (see Table 1) for 40 s and, 72 °C for 40 s; and a final extension of 72 °C for 7 min. Amplification products were visualized in 1.5% agarose gel and fragments sized by comparison with the 1 kb Plus DNA ladder (Invitrogen). Any amplification that produced fragments with similar sizes to those observed in source species was considered successful. Allele sizes were determined on an ABI 377 DNA sequencer (Applied Biosystems) for blue macaws, and a MegaBACE 1000 (GE Healthcare) for red macaws, using a weight standard (TAMRA 500C or GeneScan -500 ROX STANDARD, Applied Biosystem) in

Table 1 - Number of alleles (A), allele size ranges (in base pairs) and annealing temperature (T °C) of microsatellite loci in five macaw species. In parenthesis are the numbers of individuals analyzed in each species.

Loci	Spix's macaw (11)			Lear's macaw (21)			Hyacinth macaw (30)			Red-and-green macaw (31)			Scarlet macaw (28)		
	A	Size	T °C	A	Size	T °C	A	Size	T °C	A	Size	T °C	A	Size	T °C
UnaCT21	5	237-245	58	3	239-245	58	5	243-271	58	12	247-287	60-54*	14	243-273	60-54*
UnaCT32	2	265-267	56	1	265	56	2	261-265	58	-	-	58	-	-	58
UnaCT35	4	127-135	55	2	127-129	54	-	-	55	-	NT	NT	NT	NT	NT
UnaCT43	5	205-219	60	3	203-229	60	4	203-231	53	5	199-207	60-54*	7	191-215	60-54*
UnaCT74	5	239-253	58	5	239-251	58	5	247-257	56	5	253-261	60-54*	12	245-267	60-54*
UnaGT55	2	188-190	58	6	192-210	58	4	184-208	56	2	182-184	60-54*	-	-	56
UnaCT41int	NT	NT	NT	NT	NT	NT	7	143-173	60-56*	4	143-159	60-54*	5	151-165	60-54*
AgGT07	-	-	59-51*	-	-	59-51*	-	-	59-61*	NT	NT	NT	NT	NT	NT
AgGT08	1	324	60	1	324	60	1	324	60	NT	NT	NT	NT	NT	NT
AgGT12	1	286	58	NT	NT	NT	-	-	59-61*	NT	NT	NT	NT	NT	NT
AgGT17	NT	NT	NT	NT	NT	NT	2	222-224	60-54*	2	222-224	60-54*	11	222-246	60-54*
AgGT19	NT	NT	NT	NT	NT	NT	3	190-194	60-56*	8	188-218	60-54*	7	190-202	60-54*
AgGT21	1	309	57	1	309	60	1	311	58	3	313-317	60-54*	15	299-333	60-54*
AgGT32	NT	NT	NT	NT	NT	NT	NT	NT	NT	1	249	60-54*	9	241-267	60-54*
AgGT81	-	-	57/55	-	-	57/55	-	-	57/55	NT	NT	NT	NT	NT	NT
MAC436	1	154	58	5	164-172	56	6	154-174	56	-	-	56	-	-	56
HYA1172	1	153	58	1	157	56	3	153-175	56	-	-	56	-	-	56
Peeμ11	NT	NT	NT	NT	NT	NT	5	337-357	60-56*	6	325-345	60-54*	6	325-345	60-54*
Peeμ16	NT	NT	NT	NT	NT	NT	3	255-267	60-56*	9	261-305	60-54*	9	261-293	60-54*

NT - not tested, - no amplification, * - touch down PCR method (decreasing 0.5 °C per cycle).

each sample lane. Profile analysis was undertaken with GeneScan and Genotyper 2.1 (Applied Biosystems), or MegaBACE Genetic Profiler Software Suite v2.2 (GE Healthcare). A fragment from one homozygous individual from each species was also sequenced, using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems), to check for the presence of microsatellite loci.

The number of alleles per locus, observed and expected heterozygosities (Nei, 1978), paternity exclusion (Q, Weir, 1996) and genetic identity probabilities (Paetkau *et al.*, 1995) were estimated using Identity 1.0 (Wagner and Sefc, 1999). Deviation from Hardy-Weinberg expectation and linkage equilibrium were analyzed with Genepop 4.0 (Raymond and Rousset, 1995), and by applying the Bonferroni correction to account for multiple comparisons. Genotyping errors, due to null alleles, stutter bands or allele dropouts, were analyzed using Micro-checker 2.2.3 (van Oosterhout *et al.*, 2004).

Among the heterologous primer pairs tested, eleven of thirteen (84.6%) amplified products in the Spix's macaw, 10 of 12 (83.3%) in the Lear's, 13 of 17 (76.4%) in the hyacinth, 11 of 14 (78.6%) in the red-and-green and 10 of 14 (71.4%) in the scarlet macaw (Table 1). Sequencing results showed that the repeat units are the same as those in source species. Among these successfully amplified loci, six were polymorphic in the Spix's (54.5%) and Lear's macaws (60%), eleven in the hyacinth (84.3%), and ten in the red-and-green (90.9%) and scarlet (100%). The number of alleles per polymorphic locus ranged from two to five in the Spix's macaw, two to six in the Lear's macaw, two to seven in the hyacinth, two to 12 in the red-and-green, and five to 15 in the scarlet (Table 1).

All the pairs of polymorphic loci were in linkage equilibrium in all the species studied. Sporadic cases of departure from Hardy-Weinberg equilibrium (HWE, $p < 0.01$) were found in the blue macaw species: locus UnaCT43 in the Spix's; UnaCT35 and MAC436 in the Lear's; and MAC436, UnaCT41_{int}, and PEEP11 in the hyacinth macaw (Table 2). Analysis with Micro-Checker software revealed null alleles at all these loci. Expected heterozygosity across all the loci in Hardy Weinberg equilibrium were 0.55 in the Spix's macaw, 0.62 in the Lear's, 0.40 in the hyacinth, 0.55 in the red-and-green, and 0.74 in the scarlet (Table 2).

Threatened species, usually present in small populations, are more vulnerable to loss of genetic diversity through processes such as genetic drift and inbreeding. Thus, it was expected that levels of genetic variability in the threatened species studied would be lower than in the unthreatened. This proved to be so, results showing lower levels of genetic variability in the threatened blue macaws than in the unthreatened red. On considering only the four loci UnaCT21, UnaCT43, UnaCT74 and AgGT21, which successfully amplified in all the species, expected mean heterozygosity in the three blue macaws proved to be lower than in the two red (Table 2). Furthermore, monomorphic

Table 2 - Expected (H_E) and observed heterozygosities (H_O), paternity-exclusion probability (Q) and probability of genetic identity (I) of microsatellite loci in five macaw species. In parenthesis are the numbers of individuals analyzed in each species.

Loci	Spix's macaw (11)			Lear's macaw (21)			Hyacinth macaw (30)			Red-and-green macaw (31)			Scarlet macaw (28)			
	H_E	H_O	I	H_E	H_O	Q	H_E	H_O	Q	H_E	H_O	Q	H_E	H_O	Q	I
UnaCT21	0.77	0.82	0.50	0.54	0.62	0.26	0.67	0.73	0.41	0.16	0.94	0.74	0.02	0.82	0.80	0.02
UnaCT32	0.45	0.45	0.17	M	M	M	0.18	0.20	0.08	0.69	NT	NT	NT	NT	NT	NT
UnaCT35	0.45	0.18	0.23	0.45*	0.06*	0.17	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
UnaCT43	0.62*	0.18*	0.36	0.66	0.62	0.35	0.13	0.10	0.06	0.76	0.45	0.28	0.30	0.86	0.54	0.09
UnaCT74	0.67	0.82	0.38	0.62	0.62	0.34	0.52	0.43	0.29	0.28	0.74	0.51	0.11	0.88	0.77	0.02
UnaCT75	0.37	0.45	0.14	0.68	0.67	0.44	0.58	0.60	0.28	0.27	NT	NT	NT	NT	NT	NT
UnaCT14 _{int}	NT	NT	NT	NT	NT	NT	0.64*	0.46*	0.40	0.18	0.19	0.12	0.60	0.57	0.42	0.16
AgGT08	M	M	M	M	M	M	M	M	M	M	NT	NT	NT	NT	NT	NT
AgGT12	M	M	M	NT	NT	NT	M	M	M	M	NT	NT	NT	NT	NT	NT
AgGT17	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.17	0.08	0.70	0.86	0.75	0.03
AgGT19	NT	NT	NT	NT	NT	NT	0.28	0.32	0.13	0.54	0.82	0.65	0.06	0.71	0.75	0.11
AgGT21	M	M	M	M	M	M	M	M	M	M	0.42	0.18	0.41	0.86	0.73	0.03
AgGT32	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	M	M	M	0.62	0.63	0.16
MAC436	M	M	M	0.75*	0.25*	0.51	0.67*	0.34*	0.41	0.17	NT	NT	NT	NT	NT	NT
HYA1172	M	M	M	M	M	M	0.31	0.24	0.14	0.52	NT	NT	NT	NT	NT	NT
PEEP11	NT	NT	NT	NT	NT	NT	0.72*	0.63*	0.45	0.14	0.66	0.40	0.17	0.74	0.75	0.11
PEEP16	NT	NT	NT	NT	NT	NT	0.33	0.33	0.15	0.50	0.76	0.55	0.09	0.81	0.64	0.06
All loci [§]	0.55	0.48	0.89	0.62	0.63	0.82	0.40	0.38	0.90	1.68x10 ⁻⁴	0.55	0.54	0.99	0.74	0.77	0.99
Shared loci [§]	0.51	0.45		0.46	0.46		0.33	0.32			0.63	0.62		0.85	0.86	0.64x10 ⁻¹²

M- monomorphic, NT - not tested, * Loci in Hardy-Weinberg disequilibrium ($p < 0.01$), [§]Only loci in Hardy-Weinberg equilibrium, & (loci UnaCT21, UnaCT43, UnaCT74, and AgGT21).

loci were more frequently observed in the former. This is in accordance with results from DNA fingerprinting, showing a certain correlation between genetic similarity among pairs of individuals and the level of threat. In the extinct Spix's macaw, the similarity is 64% (Caparroz *et al.*, 2001b), in the vulnerable hyacinth, 34% (Miyaki C. Y., unpublished results), and in the unthreatened red-and-green, 27% (Caparroz *et al.*, 2001a). Moreover, 21 single locus minisatellite markers also showed higher variability in red-and-green macaws than in the hyacinth (Faria and Miyaki, 2006).

The Spix's macaw, already rare since its discovery, is now extinct in the wild (Ridgely, 1981, Sick, 1981). Although most of the Spix's samples analyzed were from wild individuals, given the rarity of the species, they are all possibly related, a possible reflection of its historically small wild population.

In 2003, the population of Lear's macaws was estimated to be around 450 individuals (Menezes *et al.*, 2006). This species occurs in a small area in the state of Bahia (Brazil). As most likely all the individuals analyzed here came from this very same, small wild population, the low genetic variability levels observed here could be thus related. However, as the relationships among these individuals are unknown, it is possible that genetic variability levels are biased, due to possible kinship among some.

The population of the hyacinth is the largest among the blue macaws. It is estimated to be around 6,500 individuals, with 5,000 in the Pantanal alone (Birdlife International, 2010). However, its mean expected heterozygosity has proved to be relatively low, compared to that estimated for each of the other two more threatened blue species (Table 2). All the individuals analyzed, although possibly not closely related (only one chick per nest was studied), were sampled in the same area, most likely from the same population. Thus, this sampling appears to be a reliable representation of the wild population in the Pantanal. Even so, the analysis of individuals from other localities is essential for characterizing the genetic variability of the species, as a whole.

The total population sizes of both red macaws studied here is unknown, but it is not believed to approach the thresholds of vulnerability under the population-size-criterion of the International Union for Conservation of Nature (< 10,000 mature individuals with a continuing decline estimated to be > 10% in ten years or three generations, or with a specified population structure; Birdlife International, 2000). Thus, it becomes essential to expand their geographical sampling, in order to evaluate the variability levels in the two species.

On considering all the loci, the potential of paternity/maternity exclusion was greater than 78% in all the macaw species studied (Table 2). It is noteworthy that in the case of red macaws the potential was more than 99%, thus indicating the suitability of these loci for parentage testing.

This study placed in evidence that, in the macaw species analyzed, the cross-amplification of previously developed microsatellite loci can increase the availability of markers to address both ecological and population questions. The low combined-probabilities of genetic identity, and the moderate to high probabilities of paternity exclusion (Table 2), indicate the utility of these microsatellite sets in studying parentage and population differentiation, a valuable aid in planning *in-situ* and *ex-situ* conservation action.

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