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Analysis of the genetic variability and breeding behaviour of wild populations of two Macaw species (Psittaciformes, Aves) by DNA fingerprinting

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RESUMO. **Análise da variabilidade genética e do comportamento reprodutivo de populações silvestres de duas espécies de araras (Psittaciformes, Aves) pela técnica DNA fingerprinting.** No presente trabalho, foram estudadas amostras de populações silvestres de duas espécies de araras, sendo uma de *Ara ararauna* (Parque Nacional das Emas/GO) e a outra de *Ara chloroptera* (Pantanal Mato-grossense/MS). Para estas populações, a variabilidade genética, a relação genética entre filhotes do mesmo ninho e a proporção sexual foram estimadas através da análise com as sondas de minissatélites humanos 33.15 e 33.6. A população de *A. chloroptera* apresentou maior variabilidade genética do que a população de *A. ararauna*, sendo que esta última apresentou índices de similaridade genética semelhantes aos observados para outras espécies de aves consideradas vulneráveis ou ameaçadas de extinção. Foi observado que a maioria dos filhotes de mesmo ninho apresentaram índices de similaridade genética próximos aos esperados entre indivíduos com parentesco de 1º grau em sistema monogâmico. No entanto, em um dos ninhos de *A. chloroptera*, os índices observados sugerem que os filhotes não pertecem ao mesmo casal. As duas populações estudadas não apresentaram desvios significativos nas proporções sexuais. Na população de *A. chloroptera* foi observada a presença de um polimorfismo relativo às bandas sexo-específicas. A identificação de populações que apresentam perda da variabilidade genética poderá fomentar a elaboração de estratégias de conservação.
Palavras-Chave: Psittacidae, *Ara*, DNA fingerprinting, variabilidade genética, comportamento reprodutivo, proporção sexual.

ABSTRACT. We used DNA fingerprinting to examine genetic variation in wild populations of two species of Macaw: the Blue and Yellow Macaw (*Ara ararauna*, in the Ema National Park, state of Goiás, Brazil) and the Green-winged Macaw (*A. chloroptera*, Pantanal, state of Mato Grosso do Sul/Brazil). Mean heterozygosity and genetic relationship between chicks from the same nest were estimated with the human multilocus minisatellite probes 33.6 and 33.15. The Green-winged Macaw has greater heterozygosity than the Blue-and-Yellow Macaw. The latter species showed a mean genetic similarity index similar to those in species considered vulnerable or endangered. Chicks from the same nest had genetic similarity indices close to those expected for first degree relatives in a monogamous species. In only one nest of the Green-winged Macaw did the index of similarity suggest that the chicks were from different parents. The sex ratio of both populations was close to 1:1. In the Green-winged Macaw population a sex-specific polymorphism was observed. Use of DNA fingerprinting can provide a tool to identify animal populations with low genetic variability, which can then lead to the elaboration of conservation programs.
Key Words: Psittacidae, *Ara*, DNA fingerprinting, genetic variability, breeding behaviour, sex ratio.

The Blue and Yellow Macaw (*Ara ararauna*) and the Green-winged Macaw (*Ara chloroptera*) are considered to have a broad geographic distribution, from Panama, Central America, through almost the whole Brazilian territory, the Southern limits being the State of São Paulo for the Blue and Yellow Macaw and Paraná for Green-winged Macaw (Sick 1997). Little is known about the current status of existing populations but both species are known to be strongly affected by habitat disturbance. Destruction and fragmentation of natural areas as well as the illegal trading of wild birds are considered as the main threats against both species that are already extinct in many localities within their original distribution and can be considered vulnerable in other. Thus, the Blue and Yellow Macaw, the most common Brazilian macaw, is considered as critically endangered in the State of São Paulo (São Paulo 1998), nearly extinct in the State of Rio de Janeiro (Bergallo *et al.* 1999) and vulnerable in the State of Minas Gerais (Machado *et al.* 1998).

Habitat destruction may lead to population fragmentation resulting in small and isolated populations. This may lead to the reduction in heterozigozity levels and adverse effects of consanguinity. Such conditions are known to reduce general fitness and together with other stochastic demographic and environmental events, can drive natural populations to an extinction vortex (Gilpin and Soulé 1986).

Data on the reproductive biology and current status of wild populations for most of Psittacidae species are deficient and few genetic variability estimates have been performed. In Brazil, some studies have been performed with this focus on one population of the Hyacinth Macaw (*Anodorhynchus hyacinthinus*) from the state of Mato

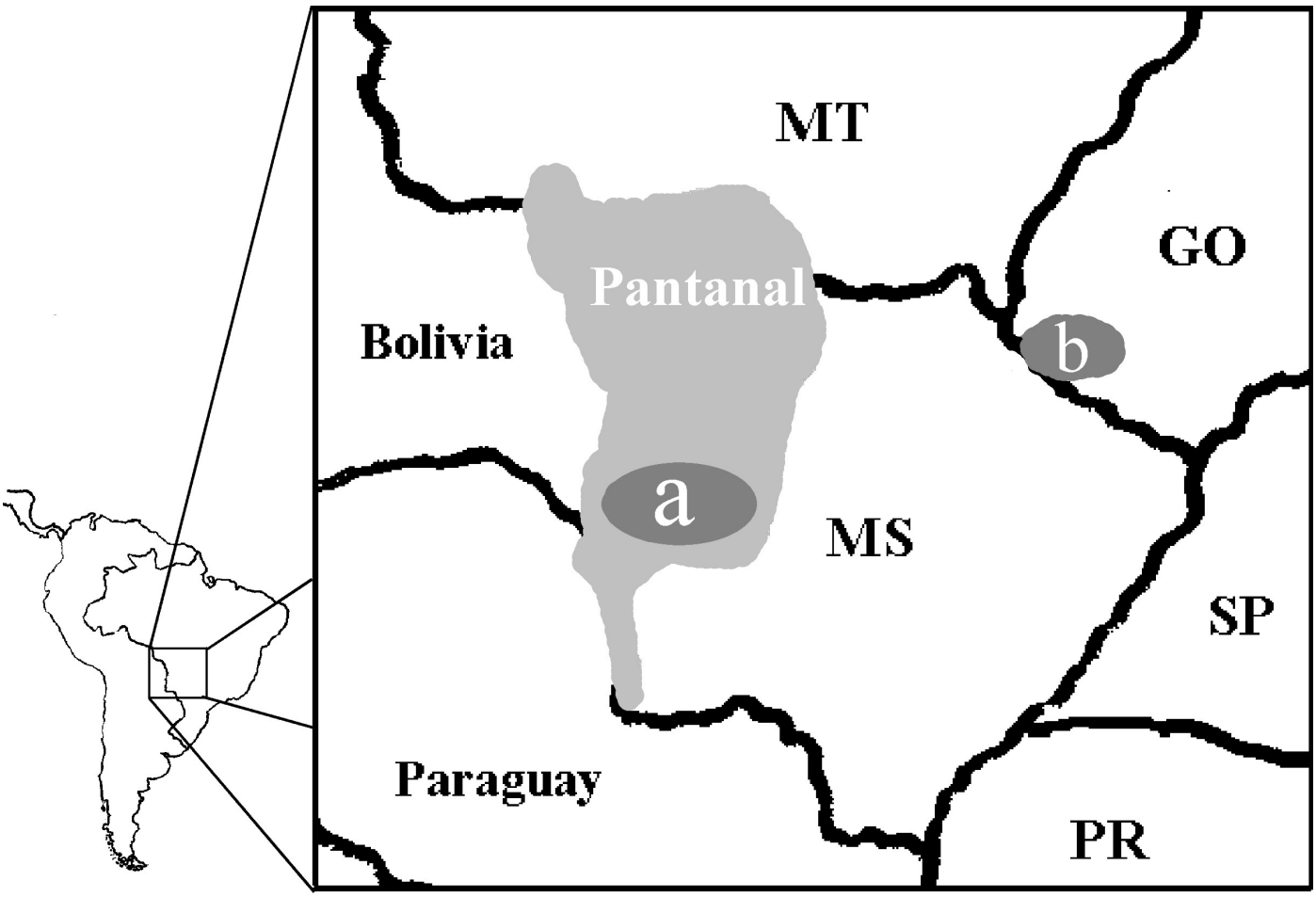

Figure 1. Regions where the wild populations' blood samples were collected. A) Green-winged Macaw (Pantanal Sul Mato-Grossense); B) Blue and Yellow Macaw (Parque Nacional das Emas).

Grosso do Sul (Miyaki *et al.* 1995b, 1998) and a captive group of wild born Spix's Macaw (*Cyanopsitta spixii*, Caparroz *et al.* 2001).

In the present work, we estimated the mean heterozygozis, the genetic similarity between chicks sampled in the same nest and the sex ratio in one population of the Blue and Yellow Macaw and one of the Green-winged Macaw, using the human multilocus minisatellite probes 33.6 and 33.15 (Jeffreys *et al.* 1985a). Both populations studied have been monitored for many years (Green-winged Macaw by Neiva M. R.Guedes; and Blue and Yellow Macaw by Carlos A. Bianchi) and further data on their biology will be published in the future.

MATERIALS AND METHODS

Blood samples (0.1 ml) were collected by venipuncture of 9 Blue and Yellow Macaw nestling found in 6 wild nests at the Parque Nacional das Emas, state of Goiás (1997-1999). For the Green-winged Macaw, samples of 16 chicks from 11 nests and of one captive adult Green-winged Macaw were collected from southern Pantanal, Mato Grosso do Sul state (1995 - 1997) (figure 1). These samples were immediately transferred to microtubes with 0.5ml of absolute ethanol and stored at room temperature.

DNA was extracted by standard methodology and processed as described in Bruford *et al.* (1992). Briefly, approximately 5-6 μg of genomic DNA from each bird were digested overnight with the restriction enzyme *Hae* III at 37°C. The fragments were separated by electrophoresis through an 1% agarose gel (20 x 30 cm), during approximately 72 h at 40 V. All samples of the same population were loaded in the same gel.

In all gels, a molecular marker (λ Hind III) was loaded in first lane and DNA from the same bird was loaded in the second and in the last lanes in order to evaluate the degree of band distortion during electrophoretic migration. This allowed us to estimate more accurately the similarity indexes between any two birds in the same gel. DNA fragments were transferred onto a nylon membrane (Hybond, Nfp, Amersham) by capillary Southern blotting (Sambrook *et al.* 1989).

The human multilocus minisatellite probes 33.6 and 33.15 (Jeffreys *et al.* 1985a) were labelled by random priming with [α-^{32}P]dCTP, according to the manufacturer's recommendations (Life Technologies). Pre-hybridization

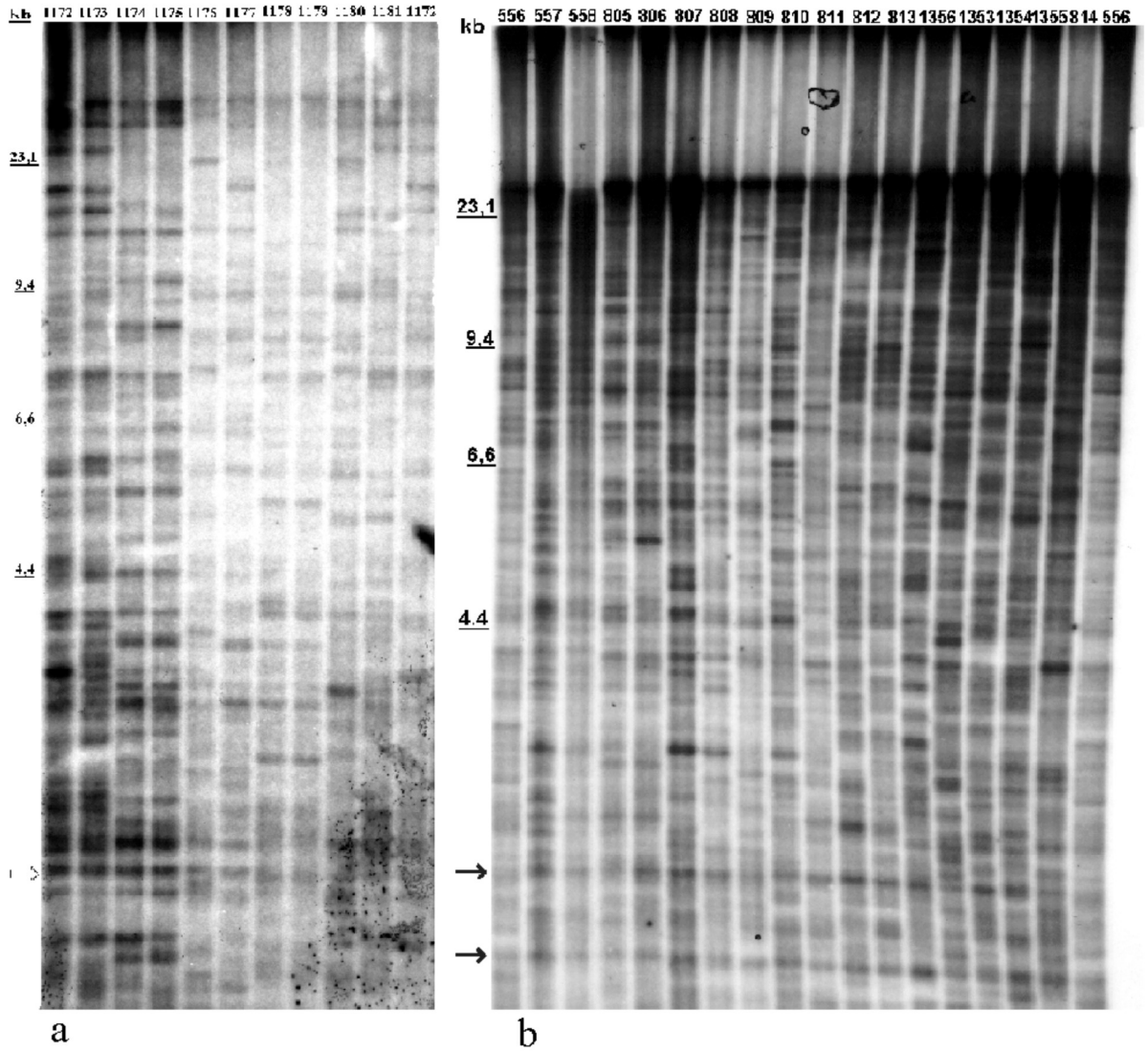

Figure 2. Band profiles of birds from two wild Macaw populations obtained by hybridization using human multilocus minisatellite probe 33.6. a) Blue and Yellow Macaw ; b) Green-winged Macaw. The values of the molecular size marker are showed in the left side of each autoradiograph. The white arrow shows the band present in eight of nine birds studied and the black arrows show the bands present in all studied birds. Note that the bird in the first lane is repeated in the last lane in both.

was undertaken in a solution of 0.263M Na_2HPO_4, 1mM EDTA, 1% BSA and 7% SDS at 65°C. After 2 to 4 h, one probe was added to the solution and left overnight at the same temperature. The membrane was washed in low stringency solutions and exposed to an x-ray film with one or two intensifying screens, at –70°C for two to seven days. Then, the membrane was dehibridized with a solution of 0.25M NaOH for 10 minutes and 0.1xSSC/1%SDS for 30 minutes at 45°C. After this the other probe was used as described above.

Only the bands between 4.0 and 23.0 kb were considered for analysis and marked on acetate overlays as described by Westneat (1990). The band sharing coefficient (index of similarity) between the individuals was calculated using the formula: $x = 2N_{AB}/(N_A+N_B)$; where N_{AB} is the number of bands shared between the individuals A and B. N_A and N_B are the number of bands present in individuals A and B, respectively (Wetton *et al.* 1987; Bruford *et al.* 1992). Only bands of the same electrophoretic mobility (migration distance of band centres within 0.5mm) between two individuals were considered to be the same allele. Sex-specific bands were excluded from this analysis. The mean band sharing coefficient for each population was estimated from pairwise comparisons of DNA profiles from only one chick per nest.

Considering that each scored band is an independent marker, we estimated the mean probability that all bands in an individual's profile are present in another unrelated individual chosen at random as $<x^n$, where x corresponds to the mean band sharing coefficient and n the mean number of scored bands (Jeffreys *et al.* 1985a; Bruford *et al.* 1992). The frequency (q) of each scorable allele was

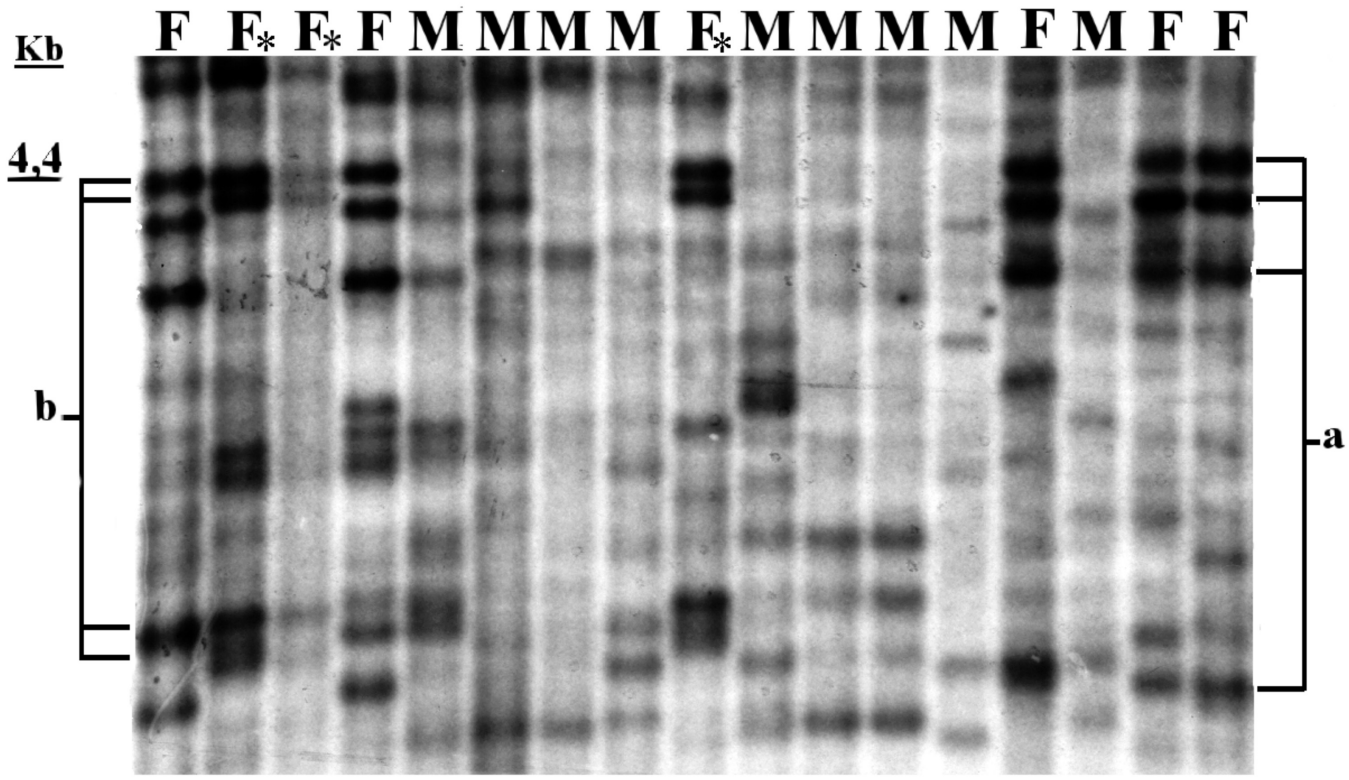

Figure 3. Band profiles observed using the human multilocus minisatellite probe 33.15 for the Green-winged Macaw population. The values of the molecular size marker are showed in the left side of the autoradiograph. The letters show the different sex-specific profiles found in this population. (*) Females with *b* profile sex-specific, (M) male, (F) female.

estimated by as: $q = 1 - (1-x)^{1/2}$ (Jeffreys *et al.* 1985b). Assuming absence of mutation, linkage or allelism, the mean similarity index between full sibs was estimated by as: $x_i = (4+5q-6q^2+q^3)/[4(2-q)]$, (Jeffreys *et al.* 1985c). The mean heterozigosity was estimated by as: $H = 2(1-q)/(2-q)$, (Sundt *et al.* 1994).

Sex ratio of the studied birds was identified by analysis of sex-specific band patterns in DNA fingerprinting profiles obtained using minisatellite probe 33.15, as described by Miyaki *et al.* (1997a) and results were confirmed by PCR using the same primers as described by Griffiths *et al.* (1998) and Miyaki *et al.* (1998).

RESULTS

The DNA fingerprinting profiles of the studied populations obtained by using human multilocus minisatellite probe 33.6 are shown in figure 2. Mean number of fragments detected, mean band sharing coefficients and other estimated data for both wild populations with both minisatellite probes are shown in table 1. Two bands below the analysed range were present in all studied Green-winged Macaws (figure 2). Mean band sharing coefficients estimated from the analysis with each of the probes were higher for Blue and Yellow Macaw population than those observed for the Green-winged Macaw population ($\alpha < 0.05$, non-parametric Mann-Whitney test). In both populations, probe 33.6 detected lower levels of variability than probe 33.15. The mean band sharing coefficients combining the results obtained by both probes were 0.315±0.090 for Blue and Yellow Macaw and 0.231±0.084 for Green-winged Macaw. However, in Blue and Yellow Macaw, 21% of the fragments could be detected by both probes and in Green-winged Macaw this percentage was of 23.

In three Blue and Yellow Macaw nests and five Green-winged Macaw nests we found two nestling. The estimated band sharing coefficients among chicks from the same nest were close to those expected for full siblings (table 2). However, in a Green-winged Macaw nest, the band sharing coefficient between the chicks (x = 0.361 and 0.333 for probes 33.6 and 33.15, respectively) was within the range found between chicks sampled in different nests (table 1).

The hybridization with probe 33.15 revealed intense female-specific bands in both Macaw species. In the Green-winged Macaw population a pattern of four female-specific bands was identified (figure 3). In five out of the eight studied females (62.5%), the bands presented similar sizes compared to those found in captive females (band pattern of 2.9; 3.9; 4.2; 4.4 Kb, Miyaki *et al.* 1997a). However, in three of the females studied (37.5%), there was a different female-linked band pattern of 3.0, 3.1, 4.3, 4.4 Kb. The sex-specific band pattern observed in all Blue and Yellow Macaw females was identical to the one described by Miyaki *et al.* (1997a).

Table 1. Estimates of the genetic similarity obtained using the human multilocus minisatellite probes 33.6 and 33.15 among birds of wild Macaw populations.

Species	Probe	N	n ± sd	b	x ± se (x)	H	x^n	q
A. ararauna	33.6	15	28.17 ± 5.56	0	0.376 ± 0.070	0.883	1.1 x 10-12	0.210
	33.15	15	29.17 ± 4.71	0	0.253 ± 0.062	0.927	3.9 x 10-18	0.136
A. chloroptera	33.6	66	28.00 ± 3.58	2	0.268 ± 0.068	0.922	8.8 x 10-17	0.144
	33.15	66	23.67 ± 3.11	0	0.195 ± 0.092	0.946	1.6 x 10-17	0.103

(N) Number of comparisons, (n ± sd) mean number of scored bands ± 1 standard deviation, (b) number of bands present in all studied birds, (x ± se (x)) mean band sharing coefficient ± 1 standard error, (H) mean heterozigosity, (x^n) probability of unrelated birds sharing the same band profile by chance, (q) mean allelic frequency.

Table 2. Estimated mean band sharing coefficients between chicks of the same nest of two wild Macaw populations obtained by using multilocus minisatellite probes 33.6 and 33.15.

Species	N	Probe	x_s ± se (x)	x_i
A. ararauna	3	33.6	0.704 ± 0.199	0.670
		33.15	0.656 ± 0.028	0.613
A. chloroptera	4	33.6	0.546 ± 0.138	0.619
		33.15	0.579 ± 0.072	0.587

(N) Number of analysed nests with two chicks, (x_s ± se (x)) mean band sharing coefficient between chicks of the same nest ± 1 standard error, (x_i) mean similarity index expected between full siblings.

Five out of nine Blue and Yellow Macaw chicks were male and nine out of 17 Green-winged Macaw were sexed as males. The observed sex ratio within each studied populations was not significantly different from 1:1 ($p > 0.05$, chi-square test).

DISCUSSION

The decay of genetic variability in small populations can be detected by the loss of heterozygozis and/or fixation of alleles (Wright 1931). According Sundt *et al.* (1994) there is a direct relationship between the heterozygozis present in a population and the similarity indexes obtained by DNA fingerprinting analysis.

Estimates of mean genetic similarity obtained by the same probes we used, revealed that, for non-endangered bird species, the indexes are usually below 0,30 (Burke and Bruford 1987; Hanotte *et al.* 1992; Fleischer *et al.* 1994; Tegelström and Sjöberg 1995). The same was true for such estimates in parrots of unknown origin kept in captivity in aviaries, Zoos and official anti-traffic institutions (Miyaki *et al.* 1993, 1995a, 1997b).

Our data show that in the studied samples, similarity indexes between chicks from different nests were higher for Blue and Yellow Macaw than for Green-winged Macaw and that the estimated mean index for this Blue and Yellow Macaw population was above the observed values for non-endangered populations. The similarity indexes for Green-winged Macaw were within values observed in non-endangered populations. However, the presence of two specific fragments detected with probe 33.6 in all the Green-winged Macaws studied suggests that at least one locus is homozygous in all individuals from this population. The presence of this fragment can be useful as a population marker if it can be proved that it is absent from other populations of the same species. This "marker" was not found in a captive sample of Green-winged Macaw, which was probably originated from various wild localities (Miyaki *et al.* 1993), showing that it is not a species marker. In the Blue and Yellow Macaw population, the same probe detected another fragment in eight out of nine chicks analysed. In the remaining chick, there was a weak hybridization signal in this region. Again, such fragment was not observed in samples of captive kept birds of the same species (Miyaki *et al.* 1993). Such "marker" may have spread in these populations either because it was already present in the founder group or because it became fixed by genetic drift more recently. This last possibility should be considered. These "marker" fragments were not included in the estimates of the similarity indexes as they were below the 4.0 Kb limit considered for analysis.

The Brazilian middle-west region is highly affected by the increasing of agricultural and cattle breeding activities. Aerial survey performed by Silva *et al.* (1992) show an exponential tendency of deforestation of the Pantanal region, severely affecting the area from where our Green-winged Macaw samples were drawn. The Parque Nacional das Emas, where the studied Blue and Yellow Macaw population inhabits, is one of the largest cerrado (Brazilian Savannah) areas that is legally protected (around 132.000ha). Due to its special characteristics that favour mechanical agriculture, this fitogeographic domain has been highly exploited (Espinoza *et al.* 1982; Azevedo and Adámoli 1988) and the surroundings of the protected

area presently show only 30% of the original cerrado vegetation (Mantovani and Pereira 1998).

Macaws achieve reproductive maturity approximately at the age of five. As there has been a continuous and exponential habitat loss in the last 30 to 50 years, especially in the areas where the studied populations inhabit, around 6 to 10 generations have elapsed since high habitat fragmentation started. Depending on the number of birds isolated in the remnant fragments, the probability of loosing the least frequent alleles can be reasonably high.

The threat imposed by human occupation of natural areas can be further increased for species that present site fidelity for reproduction areas. Macaws are considered as presenting such behaviour: there are strong evidences that the Hyacinth Macaw returns to the same reproductive site (Guedes and Harper 1995).

Data on Blue and Yellow Macaws from Parque Nacional das Emas (Bianchi 1998) suggest that the effective number of birds is small, in spite of the disposability of nest cavities in dead palm trees (Mauritia flexuosa). However, for the Green-winged Macaw, habitat destruction, the loss of appropriate nesting holes and a strong nest competition between different species as well as within the same species was documented by Guedes and Harper (1995).

Nest intraspecific competition is the most plausible hypothesis for our findings in one of the nests of Green-winged Macaw where the similarity index between two chicks was within the range obtained for chicks from different nests. Even though such competition was not observed in this nest during the breeding season when the samples were collected, it was documented (NMRG) during the preceding season, when two couples of this species were fighting for this same tree cavity and blood stains were observed on the bird's naked face. The similarity indexes between chicks from the other nests with more than one chick, were within expected values for full sibs, reinforcing the field observations that both species are monogamous as long as the pair always remains together, even when flying in large flocks.

A sex specific polymorphism was detected in the Green-winged Macaw population by means of probe 33.15. Two sibs and an unrelated chick from a different nest presented a peculiar pattern of fragments. Sex specific polymorphism was identified by Miyaki *et al.* (1997a) in the Scarlet Macao (*Ara macao*) and might be useful in studies of population structure as described for *Milvus milvus* (May *et al.* 1993).

In spite of the small number of birds sampled in the present work, no significant deviation from a 1:1 sex ratio was found. The same result was obtained by Miyaki *et al.* (1995b, 1998) in natural population of Hyacinth Macaw from the Pantanal, Mato Grosso do Sul state.

In the present work it was possible to detect, in the Blue and Yellow Macaw population from Parque Nacional das Emas, levels of heterozygozis similar to those that were found among vulnerable bird populations and the presence of a "marker" fragment. Such "marker" fragments were also detected in the Green-winged Macaw population from the Pantanal. Thus, our data suggest that habitat disturbance and fragmentation can be responsible for decay of genetic variability in both Macaw populations here studied. The bird populations characterised as vulnerable could serve as stimuli to create conservation programs next to local human communities, leading to preservation of vital ecosystems.

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