



Population genetic structure of the blue-fronted Amazon (*Amazona aestiva*, Psittacidae: Aves) based on nuclear microsatellite loci: implications for conservation

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ABSTRACT. The blue-fronted Amazon (*Amazona aestiva*) is a widely distributed Neotropical parrot and one of the most captured parrots in nature to supply the illegal trade of wild animals. The objectives of the present study were to analyze the genetic structure of *A. aestiva* to identify management units and support conservation planning and to verified if *A. aestiva* populations have undergone a recent bottleneck due to habitat loss and capture for the pet trade. The genetic structure was accessed by analyzing six microsatellite loci in 74 individuals of *A. aestiva*, including samples from the two subspecies (*A. a. aestiva* and *A. a. xanthopteryx*), from five populations: four in Brazil and one in Argentina. A significant genetic dif-

ferentiation ($\theta = 0.007$, $P = 0.005$) could be detected only between the most distant populations, Tocantins and Argentina, localized at the northeast and southwest limits of the sample sites, respectively. There was no evidence of inbreeding within or between populations, suggesting random mating among individuals. These results suggest a clinal distribution of genetic variability, as observed for variation in plumage color of the two *A. aestiva* subspecies. Bottleneck analysis did not show a recent reduction in population size. Thus, for the management and conservation of the species, the populations from Argentina and Tocantins should be considered as different management units, and the other populations from the center of the geographical distribution as another management unit.

Key words: *Amazona aestiva*; Microsatellite; Genetic structure; Bottleneck; Conservation

INTRODUCTION

Habitat fragmentation may reduce population size and modify dispersal and population geographic distribution, affecting ecological and genetic processes in remaining populations (Gilpin and Soulé, 1986). Small and isolated populations may become extinct by demographic or environmental stochastic fluctuations or by natural catastrophes, and still may show a genetic decline in response to processes such as inbreeding depression and genetic drift (Franklin, 1980; Gilpin and Soulé, 1986; Frankham, 1995). Additionally, restricted gene flow among fragmented populations may lead to genetic differentiation, reinforcing the effects of genetic drift and inbreeding (Soulé, 1987; Gilpin, 1987; Frankham et al., 2002). Besides, population fragmentation due to habitat loss and landscape modification may reinforce the isolation-by-distance process (Wright, 1943) due to long-distance gene flow constraint.

Throughout the last three decades, the identification of genetically distinct populations has been a key issue in conservation biology (Wayne, 1992; Paetkau, 1999; Frankham et al., 2002). Ryder (1986) and Moritz (1994) distinguished two types of conservation units, namely evolutionary significant unit and management unit. An evolutionary significant unit is defined as a population that is substantially reproductively isolated from other groups and that represents an important component in the evolutionary process of a species, whereas a management unit is a population with significant allelic divergence at nuclear or mitochondrial loci between populations. The relevance of these concepts and the genetic information in species conservation, including data on population structure, has been intensively recognized to develop and apply adequate conservation and management strategies (Paetkau et al., 1999; Eizirik et al., 2001; Coulon et al., 2006; Dethmers et al., 2006).

The blue-fronted Amazon (*Amazona aestiva*) is a widely distributed Neotropical parrot occurring from Northeast Brazil to Argentina (Collar, 1997). Two subspecies are recognized, based mainly on shoulder color and geographic distribution. *A. a. aestiva* has a red shoulder and is distributed in the Northeast and Central-East of Brazil, while *A. a.*

xanthopteryx has a yellow shoulder and is distributed in the Southwest of Brazil, Bolivia, Paraguay, and Argentina. In the Brazilian States of Mato Grosso and Mato Grosso do Sul, an intermediate phenotype with mixed red and yellow shoulder can also be found (Darrieu, 1983). However, phylogenetic studies have shown evidence that this species comprises a paraphyletic group with *A. ochrocephala* complex (Eberhard and Bermingham, 2004; Russello and Amato, 2004; Ribas et al., 2007).

Habitat destruction due to agricultural and urban landscape expansion has been the main threat to *A. aestiva*, reducing natural cavities for nesting and food availability (Collar and Juniper, 1992). Furthermore, this species is the most frequently captured parrot in the world for the pet trade because of its exuberant color pattern, sociability and ability to reproduce human words. These two processes have contributed to a decrease in population size in the last decades, especially in some more disturbed and fragmented regions (Beissinger and Bucher, 1992). Despite that, only a few reports about the biology and ecology of *A. aestiva* are available and are restricted to studies performed in Argentina (Fernandez-Juricic et al., 1998; Banchs and Moschione, 2006) and in the Pantanal, Brazil (Seixas and Mourão, 2000, 2002, 2003). Genetic studies are almost exclusively about the phylogenetic relationship of *A. aestiva* and other related species (Eberhard and Bermingham, 2004; Russello and Amato, 2004; Ribas et al., 2007).

Considering the high pressure on the blue-fronted Amazon from illegal capture and the scarcity of population genetic studies, we are interested in generating useful information for *A. aestiva* conservation efforts. In the present study, we report on the population genetic structure of *A. aestiva* based on six microsatellite loci and on a bottleneck analysis to test the hypothesis that populations suffered a recent reduction in size. The consequences for conservation are also discussed.

MATERIAL AND METHODS

Samples and genetic analysis

Throughout 2001 to 2006, we collected blood samples from 74 nestlings from four localities in Brazil and one in Argentina (Table 1, Figure 1). Total genomic DNA was extracted using standard proteinase K/SDS digestion followed by phenol:chloroform:isoamyl alcohol purification as described by Bruford et al. (1992).

Table 1. Sampling localities and sample size (N = 74) of *Amazona aestiva*.

Locality/State	Code	Geographic coordinates	N	Year*
Brazil				
Gurupi/Tocantins	TO	11°43'44"S, 49°04'08"W	15	2006
Brasília/Distrito Federal	DF	14°47'03"S, 47°55'25"W	11	2004/2005/2006
Chapada Gaúcha/Minas Gerais	MG	15°17'06"S, 46°03'33"W	10	2001
Pantanal Sul Mato-grossense/Mato Grosso do Sul	MS	20°15'24"S, 56°21'21"W	24	2001/2002/2004/2005
Argentina				
Provincia del Chaco	AR	28°04'28"S, 59°13'48"W	14	2004

N = number of individuals. *Refer to the breeding season when blood samples were collected.

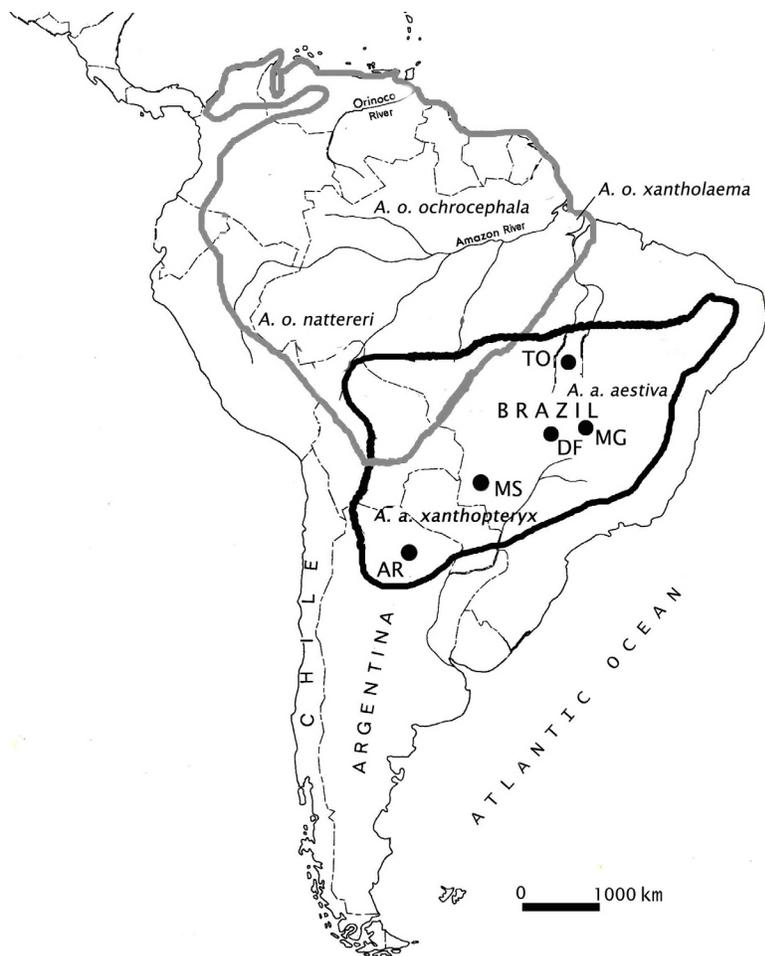


Figure 1. Geographic distribution of *Amazona aestiva* subspecies (black line) and of some *A. ochrocephala* subspecies (grey line). Black circles indicate sampling localities (see Table 1 for locality codes).

Six microsatellite loci (AgGT07, AgGT12, AgGT21, AgGT29, AgGT72, and AgGT83) described by Russello et al. (2001, 2005), and previously optimized for *A. aestiva* (Caparroz et al., 2007) were used to genotype all individuals. Although nine loci amplified for *A. aestiva*, the loci UnaCT43 and AgGT22 were excluded from the analysis because of deviation from Hardy-Weinberg equilibrium. Additionally, comparative analyses of population genetic structure including these loci indicated significant changes in the results that may not be explained by geographic distribution of populations. The locus AgGT90 was in linkage disequilibrium with AgGT07, and hence the locus AgGT90 was excluded from analysis (see Caparroz et al., 2007).

Forward primers were labeled with a fluorescent dye, 6-FAM, HEX or NED. The reactions were performed separately for each locus in a 10- μ L volume, containing 0.5 μ M of each

primer, 1 unit *Taq* DNA Polymerase (Phoneutria, Brazil), 200 μ M of each dNTP, 1X reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂) and 9.0 ng DNA template. Amplifications were performed using a PE 9700 thermal controller (Applied Biosystems, USA) with the following conditions: one cycle of 95°C for 7 min, 35 cycles of 95°C for 1 min, 48-59°C for 1 min (according to each locus), and 72°C for 1 min, and one cycle of 72°C for 20 min of final extension. Reaction conditions for some primers were optimized using the touchdown cycling program as described by Russello et al. (2001). The amplified products were diluted in a 1:2 multiplex as following: AgGT72 (6-FAM) and AgGT21 (HEX); AgGT83 (HEX) and AgGT12 (NED), and AgGT29 (HEX) and AgGT07 (NED). One microliter of the diluted reaction was added to 0.25 μ L of DNA ladder standard (GeneScan 500 internal lane standard, ROX, Applied Biosystems), 0.45 μ L loading buffer (25 mM EDTA and 50 mg/mL Blue-Dextran) and 2.3 μ L deionized formamide. Afterwards, the products were separated on 5% denaturing polyacrylamide gels in a DNA automated sequencer (ABI Prism 377, Applied Biosystems). Fragments were automatically sized using the Genescan and Genotyper 2.1 softwares (Applied Biosystems).

Genetic structure

Microsatellite loci were characterized for the number of alleles per locus and population, the allelic richness for population, and for observed and expected heterozygosities (H_E) under Hardy-Weinberg equilibrium (Nei, 1978). Genetic structure was accessed by Wright's (1951) statistics: f , θ and F , obtained from an analysis of variance of allele frequencies (Cockerham, 1969). As most mutations in microsatellites involve the addition or subtraction of a small number of repeat units, according to a stepwise mutation model (Ota and Kimura, 1973; Valdes et al., 1993; Slatkin, 1995), population genetic differentiation was also estimated by R_{ST} (Slatkin, 1995). R_{ST} is obtained by analysis of variance of allele size and may be interpreted as the correlation between allele sizes of different individuals in the same population (Goodman, 1997). This is analogous to θ , with the exception that for θ the correlation between allele frequencies of different individuals in the same population considers an infinite allele model (Cockerham, 1969; Weir and Cockerham, 1984). Analyses were performed using the FSTATS 2.9.3.2 software (Goudet, 2002). Statistical significance tests were based on 10,000 randomizations followed by sequential Bonferroni's correction (Goudet et al., 1996).

Bottleneck

To verify if populations were affected by size reduction leading to a bottleneck, we determined the heterozygosity excess by the Wilcoxon sign rank test using the Bottleneck software (Cornuet and Luikart, 1996). The bottleneck process may cause a faster loss of heterozygosity under mutation-drift equilibrium than loss of heterozygosity under Hardy-Weinberg equilibrium. Hence, populations that have experienced recent reduction in effective population size may present higher allele diversity (H_E under Hardy-Weinberg equilibrium) than H_E under mutation-drift equilibrium H_E for a given number of alleles in the population (Cornuet and Luikart, 1996; Luikart et al., 1998a,b). The distribution of H_E under mutation-drift equilibrium for each locus and population was obtained by a simulation of a coalescent process. Because microsatellite loci may evolve following the stepwise mutation model (SMM; Ota and Kimura, 1973) or the two-phase mutation model (TPM; Di Rienzo et al., 1994), we per-

formed the bottleneck test using 100% SMM and combining the two models of mutation, with 70% SMM and 30% TPM (Luikart et al., 1998b).

RESULTS

All microsatellite loci showed no deviation from Hardy-Weinberg equilibrium (Table 2) resulting in nonsignificant f values ($P > 0.00833$, adjusted nominal level 5% with Bonferroni's correction, $p_{BC} = 0.00833$) and all pairs of loci were in linkage equilibrium ($P > 0.00333$, $p_{BC} = 0.00333$). They displayed high levels of polymorphism, with the least variable (AgGT72) and the most variable (AgGT21 and AgGT83) loci with 15 and 23 alleles, respectively. They also presented high level of observed heterozygosity, from 0.808 up to 0.972 (Table 2).

Table 2. Characterization of six microsatellite loci based on 74 *Amazona aestiva*.

Locus	N	A	H _E	H _O	f
AgGT07 ¹	73	18	0.817	0.808	0.012
AgGT12 ¹	70	20	0.932	0.900	0.035
AgGT21 ¹	72	23	0.922	0.819	0.113
AgGT29 ²	74	17	0.857	0.810	0.055
AgGT72 ²	73	15	0.919	0.876	0.046
AgGT83 ¹	72	23	0.936	0.972	-0.038
Overall loci		19.3	0.897	0.864	0.037

All loci are in Hardy-Weinberg equilibrium ($P > 0.00833$) and all pairs of loci are in linkage equilibrium ($P > 0.00333$). ¹Russello et al. (2001); ²Russello et al. (2005). N = number of individuals; A = total number of alleles; H_E = expected heterozygosity; H_O = observed heterozygosity; f = inbreeding coefficient.

All populations showed no deviation from Hardy-Weinberg equilibrium (Table 3) resulting in nonsignificant f values ($P > 0.00167$, $p_{BC} = 0.0133$) and suggesting random mating within populations. They also showed a high level of H_E, from 0.874 up to 0.895 (Table 3). The Minas Gerais (MG) population had the lower mean number of alleles per population while Mato Grosso do Sul (MS) showed the higher mean number (Table 3). The sampling effect could be discarded, since MG also had the lower allelic richness (Table 3).

Table 3. Characterization of five *Amazona aestiva* populations based on six microsatellite loci.

Locality	N	A	Rs	H _E	H _O	f
TO	15	12.16	9.750	0.895	0.905	-0.012
DF	11	10.83	10.030	0.888	0.826	0.052
MG	10	9.50	9.143	0.879	0.900	-0.024
MS	24	14.33	9.592	0.874	0.811	0.091
AR	14	11.66	9.620	0.892	0.903	-0.013
Overall	74	11.69	9.627	0.886	0.869	0.018

f values were not significant for all populations analyzed ($P > 0.00167$). N = sample size; A = mean number of alleles; Rs = allelic richness; H_E = expected heterozygosity; H_O = observed heterozygosity; f = inbreeding coefficient. See Table 1 for locality abbreviations.

A low but significant genetic differentiation among populations ($P > 0.01040$, $p_{BC} = 0.00100$; Table 4) was found. Pairwise θ was significant only for the most geographically distant populations, Argentina (AR) and Tocantins (TO) ($P = 0.005$, $p_{BC} = 0.005$; Table 5), suggesting that the significance of θ over all populations may be a bias caused by the differentiation between these two populations. In fact, R_{ST} was not significantly different from zero ($R_{ST} = 0.007$; Table 4), also suggesting that significant differentiation for θ may be a bias. Additionally, F and f ($P > 0.0010$, $p_{BC} = 0.0043$; $P > 0.0019$, $p_{BC} = 0.0317$, respectively; Table 4) were also not significant, suggesting that mating pattern may not play an important role in population differentiation.

Table 4. Population genetic structure of *Amazona aestiva* based on an analysis of variance of allele frequencies and of allele size.

Locus	f	θ	F	R_{ST}
AgGT07 ¹	0.012	0.033	0.019	0.047
AgGT12 ¹	0.035	0.002	0.035	-0.009
AgGT21 ¹	0.113	-0.003	0.112	-0.007
AgGT29 ²	0.055	0.034	0.061	-0.011
AgGT72 ²	0.046	0.007	0.048	0.022
AgGT83 ¹	-0.038	0.005	-0.037	0.000
Overall loci	0.037	0.012*	0.040	0.007

¹Russello et al. (2001); ²Russello et al. (2005). f = inbreeding coefficient; θ = fixation index (significant for value followed by asterisk); F = total inbreeding coefficient; R_{ST} = population genetic differentiation based on allele size.

Table 5. Values of θ for pairwise localities of *Amazona aestiva* based on six microsatellite loci.

Locality	AR	MS	DF	MG
MS	0.007			
DF	0.020	0.010		
MG	0.023	0.021	0.010	
TO	0.007*	0.001	0.011	0.029

* $P = 0.005$. See Table 1 for locality abbreviations.

No evidence of recent reduction in population size could be detected for both, strictly SMM ($P > 0.422$) and 70% SMM and 30% TPM ($P > 0.039$, assuming a significant level of 0.01). Allele frequency distribution showed the L-shape expected by the mutation-drift equilibrium, with most alleles with low frequency and few alleles with high frequency (results not shown).

DISCUSSION

For the six microsatellite loci used in this study, the levels of polymorphism and of H_E for *A. aestiva* (19.3 alleles per locus and $H_E = 0.897$; Table 2) were higher than those observed for other parrots. For some threatened and endemic species, such as the red-spectacled parrot (*Amazona pretrei*; Caparroz et al., 2007), the red-tailed parrot (*Amazona brasiliensis*; Caparroz et al., 2007) and the St. Vincent parrot (*Amazona guildingii*; Russello et al., 2001, 2005), the mean number of alleles per locus (9.5, 7.5 and 4.2, respectively) was lower than the value found for *A. aestiva* as well as the

values of H_E (0.800, 0.676 and 0.548, respectively). Even though *A. aestiva* is non-threatened and widely distributed, other species with the same characteristics show lower levels of polymorphism, such as the African grey parrot (*Psittacus erithacus*; Taylor and Parkin, 2007), which has shown 7.3 alleles per locus and $H_E = 0.745$ for 11 microsatellite markers. These results indicate that *A. aestiva* still maintains high levels of genetic diversity, despite the intense anthropic pressures on its natural populations.

Our results show a low genetic differentiation among populations (Table 4). Although θ may underestimate genetic differentiation, R_{ST} was lower than θ , showing that there is no divergence between identity by descent and identity by state in the loci analyzed. Additionally, pairwise comparison showed significant differentiation only between the most distant populations AR and TO (Table 5), localized in the limits (southwest and northeast) of the sample sites, and f was not significant for overall population or for each population (Tables 3 and 4), suggesting random mating within populations. These results suggest a clinal distribution of genetic variability, as observed for variation in plumage color of the two subspecies of *A. aestiva*. The primary difference between them is the coloration of the shoulder, which changes from red in eastern populations (*A. a. aestiva*) to yellow in western populations (*A. a. xanthopteryx*), with an intermediate phenotype with mixed red and yellow shoulder in the center of the distribution (Darrieu, 1983). We hypothesize that long distance dispersal may be responsible for this result, since the blue-fronted Amazon can fly long distances (Seixas and Mourão, 2000). The low differentiation for bird populations has already been described in other bird species that can potentially fly long distances, such as other Amazons (e.g., *A. auro-palliata*; Wright et al., 2005), macaws (e.g., *A. ararauna*; Caparroz R, Miyaki CY and Baker AJ, unpublished data) and passerines (Dougall-Shackleton and Dougall-Shackleton, 2001).

Alternatively, the clinal distribution with differentiation between AR and TO populations, at the extreme of the geographic distribution, may also be explained by population-level phenomena such as introgression. Some subspecies of the *A. ochrocephala* complex and *A. aestiva* comprise a paraphyletic group (Eberhard and Bermingham, 2004; Russello and Amato, 2004; Ribas et al., 2007). Ribas and collaborators (2007) showed that there are two different mitochondrial lineages: one composed of *A. a. aestiva* individuals (middle-north region of the geographic distribution), including some individuals from MG (CG in Ribas et al., 2007) and *A. o. xantholaema* from Marajó Island, northeastern Brazil; the other composed of *A. a. aestiva*, including some individuals from MS (MI in Ribas et al., 2007), *A. a. xanthopteryx* (both from west-southwest region of the geographic distribution), *A. o. nattereri* and *A. o. ochrocephala* from Northwestern Brazil. We hypothesize that these two mitochondrial lineages hybridize in an area that coincides with the region where no significant genetic differentiation was detected (MS, Distrito Federal (DF) and MG populations) with microsatellite markers, which may be a contact region (hybridization zone) among these lineages (Figure 1). Moreover, in Pantanal Sul Mato-Grossense (MS), the blue-fronted Amazon has a mixed shoulder (yellow and red) (Darrieu 1983), morphological evidence that supports the hybridization of these two lineages.

Based on our results, it is not possible to decide which of these hypotheses is more likely, because we did not have enough *A. ochrocephala* samples to perform a population study with microsatellite markers. Thus, a detailed study, including more populations from the overlapping between *A. aestiva* and *A. ochrocephala* subspecies and populations from all *A. ochrocephala* subspecies, should be performed to better understand the evolution and taxonomic relationship of this group.

Implications for conservation

The rapid and intense destruction and fragmentation of natural habitats due to the fast increase in human activities have progressively destroyed the natural habitat of *A. aestiva*, contributing effectively to the reduction of wild populations (Bucher and Martella, 1988; Collar and Juniper, 1992). Additionally, the capture of wild individuals for the pet trade has seriously affected the wild population of these species. These threats may interfere directly in the spatial distribution and in population size of the blue-fronted Amazon in the medium and long term. Nevertheless, the results obtained in this study suggest that these threats still have not had a strong impact on the genetic structure and diversity of the blue-fronted Amazon populations. However, *A. aestiva* has a long life expectancy (almost 70 years - data from captive individuals; Sick, 1997), and fragmentation in the core area of the geographic distribution of this species is a recent event (less than 60 years), considering the life cycle of this species. Possibly, some individuals that are still breeding may be older than the first anthropic disturbances. Thus, the effects of these impacts may have not yet affected the genetic structure and diversity of this species. Furthermore, with a long life expectancy, it is possible that the current population of *A. aestiva* is composed mainly of old individuals, and when these old individuals die, the populations will suffer a sudden, drastic size reduction, which may cause a reduction in genetic variability. In fact, the mortality of nestlings due to predation and cavity loss as a result of habitat loss is very high and may lead to a very low recruitment rate, reinforced by the illegal capture of nestlings for the pet trade (Seixas and Mourão, 2002; Resende FO and Caparroz R, unpublished data). Other studies showed that eggs and offspring of *A. aestiva* are intensely captured in the natural populations (Pinho and Nogueira, 2000; Seixas and Mourão, 2000; Longatto and Seixas, 2004; Banchs and Moschione, 2006), which may interfere directly in the recruitment rate.

We suggest that conservation strategies for *A. aestiva* should consider the evidence of clinal distribution of allele frequency and morphological differentiation in shoulder color. Thus, for the management and conservation of the species, the populations from Argentina and Tocantins should be considered as different management units, and the other populations from the center of the geographical distribution as another management unit. Independent of the taxonomic relationship between *A. aestiva* and *A. ochrocephala* complex (see discussion above), it is important to maintain the different genetic and morphological populations described in this paper.

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