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Population Genetic Structure in Hyacinth Macaws (*Anodorhynchus hyacinthinus*) and Identification of the Probable Origin of Confiscated Individuals

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Abstract

Understanding the intraspecific genetic composition of populations in different geographic locations is important for the conservation of species. If genetic variability is structured, conservation strategies should seek to preserve the diversity of units. Also, origin of individuals can be determined, which is important for guiding actions against animal trafficking. The hyacinth macaw (*Anodorhynchus hyacinthinus*) is located in allopatric regions, vulnerable to extinction and suffering animal trafficking pressure. Therefore, we characterized its population genetic structure based on 10 microsatellites from 98 individuals and 2123 bp of mitochondrial sequence (ND5, cytochrome b, and ND2) from 80 individuals. Moderate to high levels of differentiation were observed among 3 geographic regions of Brazil: the north/northeast of the country, the north Pantanal, and the south Pantanal. Differentiation between the 2 regions within the Pantanal was not expected, as they are relatively close and there is no known barrier to macaw movement between these regions. These genetically differentiated groups were estimated to have diverged 16 000 to 42 000 years ago. The low genetic variability observed seems not to be the result of past bottlenecks, although a star-shaped haplotype network and the mismatch distribution suggest that there was recent demographic expansion in the north and northeast. Environmental changes in the Holocene could have caused this expansion. Given the genetic structure observed, the most probable regions of origin of 24 confiscated individuals were identified. Thus, these data helped to trace illegal traffic routes and identify natural populations that are being illegally harvested.

Resumen

El conocimiento de la composición genética intraespecífica, en poblaciones de diferentes ubicaciones geográficas, es importante para la conservación de especies. Si la variabilidad genética es estructurada, las estrategias de conservación ayudaran a preservar la diversidad de unidades; además, es posible conocer el origen de los individuos, lo cual es importante para determinar el modo de acción contra el tráfico de animales. El Guacamayo Jacinto (*Anodorhynchus hyacinthinus*), encontrado en regiones alopátricas, es vulnerable al tráfico animal y por tanto a su extinción. Por

lo anterior, fue caracterizada su estructura genética poblacional usando 10 microsatélites en 98 individuos, y 2.123 bp de secuencia mitocondrial (*ND5, Citocromo b y ND2*) en 80 especímenes. Fueron observados niveles de diferenciación demoderado a alto entre tres regiones geográficas de Brasil. Norte/Nor-oriente del país, Norte del Pantanal y sur del Pantanal. Se encontraron diferencias entre las dos regiones del Pantanal, lo cual no era esperado porque están relativamente cerca y porque no hay barreras para el movimiento del Guacamayo. Fue estimado que la diferencia genética entre estos grupos ocurrió hace 16 – 42 mil años. La baja variabilidad genética observada no parece ser el resultado de los cuellos de botella del pasado, aunque la red de haplotipo en forma de estrella y el desajuste en la distribución sugieren que hubo expansión demográfica reciente en el norte y en el nor-oriente. Los cambios ambientales en el Holoceno podrían haber causado esta expansión. Dada la estructura genética observada, fue identificada la región de origen más probable de los 24 individuos confiscados. Así, estos datos ayudan a trazar las rutas de tráfico ilegal e identificar las poblaciones naturales que se están recogiendo ilegalmente.

Subject areas: Conservation genetics and biodiversity; Population structure and phylogeography
Key words: conservation, genetic diversity, microsatellite, mitochondrial DNA, Psittacidae

Information about population genetic structure can be crucial for combating illegal wildlife trade. It can aid in identifying the geographic origin of confiscated individuals and in planning focused preventive law enforcement actions in illegal harvesting hotspot areas. Moreover, if it is feasible to return seized individuals to the wild, genetic information can guide decisions of where to release the birds. For example, based on the mitochondrial genetic structure of the blue-and-yellow macaw, *Ara ararauna* (Caparroz et al. 2009), 6 of 13 confiscated individuals were identified as candidates for release in northwestern Goiás in Brazil (Fernandes and Caparroz 2013). Also, captive breeding programs composed of individuals confiscated from illegal trade can benefit from the identification of potential pairs from the same genetic lineage, avoiding outbreeding depression (Ballou et al. 2010).

The hyacinth macaw (*Anodorhynchus hyacinthinus*) is considered vulnerable (IUCN 2014) and is primarily threatened by habitat destruction and intense illegal trade. It also has specialized diet and nest site preferences and many pairs reproduce only every other year (Guedes and Harper 1995). It occurs in Brazil, although a few recent records indicate that it is returning to Bolivia (Herrera M, personal communication), where a small population was extirpated by capture for the pet trade in the late 1980s (Herrera M, personal communication). The total number of birds in the wild is estimated to be 6500, mainly distributed in 3 regions in Brazil: the north (east Amazonia), the northeast (a region where the states of Tocantins, Piauí, Maranhão, Bahia, Goiás, Mato Grosso, and Minas Gerais meet), and the pantanal wetlands in Mato Grosso and Mato Grosso do Sul (where the majority of individuals occur; BirdLife International 2013a). These three areas have distinct vegetation and environmental conditions; in the north, hyacinth macaws inhabit the Amazon rainforest, dominated by dense primary forest. In the central and northeastern regions of the country, hyacinth macaws use the Cerrado, a grassland with scattered small trees. The states of Mato Grosso and Mato Grosso do Sul are dominated by the Pantanal, a periodically flooded alluvial plain under direct influence of 4 major ecosystems: rain forest, Cerrado, Atlantic coastal tropical dry forest, and Chaco inland tropical dry forest. We know of no other plant or animal species with the same distribution, nor of biogeographical hypotheses to explain it. Although hyacinth macaws have a large dispersal capacity, as shown by radiotelemetry studies (Seixas and Guedes 2002; Antas et al. 2010), field observations suggest that some pairs use the same breeding site over several years (Guedes 1993). This behavior can generate population structure, such as in Mauritius parakeets, which have high levels of philopatry and genetic structure (Raisin et al. 2012).

Faria et al. (2008), using 2 single-locus minisatellites and 2 microsatellites, observed that individuals from 2 different locations within the Pantanal (Abobral and Miranda) were not differentiated from each other, but were genetically distinct from birds from the northeastern state of Piauí. However, their analysis of 472 bp of mitochondrial control region sequence revealed no differentiation. Analyses of 5 confiscated birds suggested that they did not come from the Pantanal, although their origin could not be determined.

In terms of conservation, understanding the demographic history and the responses of a species to past climate pressures may be useful for predicting susceptibility to future changes. If it is possible to hypothesize when divergence, expansion, or population bottlenecks occurred within a species, existing populations can be managed to avoid or minimize decreases in size and genetic diversity (Frankham et al. 2010). Hyacinth macaw populations have ecological and biological characteristics that can influence their genetic composition: a geographical distribution described as allopatric in 3 distinct regions, possible philopatry, and vulnerability to extinction. Knowledge of demographic and evolutionary history can reveal causes of possible population bottlenecks and knowledge of when and if populations separated geographically can also help in deciding the integrated or separate management of these groups. Because the low genetic variability found in hyacinth macaws (Faria et al. 2008; Presti et al. 2011) could be due to a population reduction, it is important to analyze data for signs of instability in demographic parameters. Therefore, in order to better characterize genetic variability and population structure in the hyacinth macaw, as well as to understand its demographic history, we analyzed samples of individuals from the 3 main areas of occurrence using 10 microsatellites and mitochondrial DNA sequences. Samples from confiscated chicks were then genotyped to determine their origin. Thus, in the present study, we addressed the following questions:

1) Are geographically isolated macaws genetically differentiated?
2) If there is differentiation, can we identify the geographic origin of confiscated individuals?
3) What is the demographic history of the species? There was a population bottleneck or expansion?

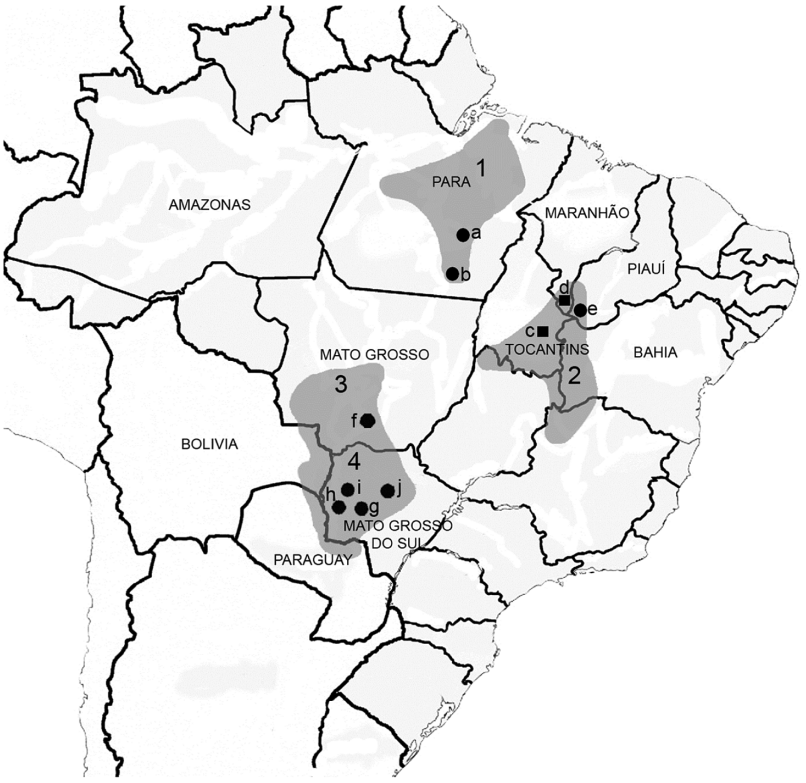

Figure 1. Geographic distribution (gray) of the hyacinth macaw, *Anodorhynchus hyacinthinus* (Birdlife International 2013a), and sampling locations. • Samples with known origin; ■ Samples without accurate origin but from the corresponding Brazilian states. 1: N, north (a, Redenção; b, Carajás); 2: NE, northeast (c, Tocantins; d, Maranhão; e, São Gonçalo de Gurgueia); 3: NP, north Pantanal (f, Barão de Melgaço); SP, south Pantanal (g, Miranda; h, Abobral; i, Nhecolândia; j, Rio Negro). The numbers and letters correspond to the localities in Table 1.

Materials and Methods

Samples

Blood samples (0.1 ml) were collected from a total of 90 wild hyacinth macaws from various locations representing the geographic distribution of the species (Figure 1; Table 1). Sampling in the southern Pantanal (SP) covered 4 subregions with differences in landscape and faunal composition (Silva and Abdon 1998): Miranda (MI), Abobral (AB), Nhecolândia (NH), and Rio Negro (RN). Wild chicks were captured, sampled, and returned to their corresponding nest, using methods described in detail elsewhere (Guedes and Seixas 2002, Presti et al. 2009). Samples from 08 captive birds of known origin and 24 individuals confiscated from the illegal trade were also obtained (Table 1). Samples were stored in absolute ethanol at –20 °C at the Laboratório de Genética e Evolução Molecular de Aves of the Instituto de Biociências, Universidade de São Paulo. DNA was isolated using a standard proteinase K and phenol-chloroform extraction protocol (Bruford et al. 1992).

Microsatellites

We used 10 microsatellite primer pairs that were previously tested in hyacinth macaws (Presti et al. 2011). Six were developed for *Ara ararauna* (UnaCT21, UnaCT32, UnaCT43, UnaCT74, UnaGT55: Caparroz et al. 2003; and UnaCT41: Gebhardt and Waits 2008), 1 for *Amazona guildingii* (AgGT19; Russello et al. 2001), 1 for *Anodorhynchus hyacinthinus* (HYA1172;Davis S, unpublished data), and 2 for *Psittacus erithacus* (Peeμ11 and Peeμ16; Taylor and Parkin 2007). Polymerase chain reaction (PCR) was performed using forward primers with an M13 tail at their 5′end. This sequence was used as a priming site for fluorescent (HEX or TET, Applied Biosystems) M13 primers, following Boutin-Ganache et al. (2001). Each PCR reaction contained 0.1 μL of *Taq*DNA polymerase (5 U/μL, Pharmacia), 0.3 μL of reverse primer (10 μM), 0.2 μL of fluorescent M13 primer (10 μM), 0.1 μL of forward primer (10 μM), 0.4 μL of $MgCl_2$ (25 mM), 1 μL dNTPs (4 mM), 1.2 μL of buffer (10×), 20–50 ng of DNA, and Milli-Q water to complete 12 μL. PCR conditions were as follows: initial denaturation at 95 °C for 10 min; followed by 35 cycles of 95 °C for 1 min, 53–58 °C for 40 s (Different temperatures for the primers were used. See Supplementary Table S1 online), 72 °C for 40 s; and a final extension of 72 °C for 7 min. Approximately 2 μL of the products were electrophoresed through 1.5% agarose gels to evaluate amplification success and to estimate their concentration. An aliquot of 2 μL of this product was mixed with 0.5 μL of GeneScan™ROX™-500 standards (2 fmol, Applied Biosystems) and 7.5 μL of 0.1% Tween 20. About 10 μL of this mixture were analyzed in a Mega BACE 1000 automated sequencer (GE Healthcare). Genotypes were determined using the program Mega BACE™ Genetic Profiler Software Suite v2.2 (GE Healthcare).

MICROCHECKER (Brookfield 1996) was used to check for the presence of null alleles, genotyping errors, and large allele dropout. For some analyses (Hardy–Weinberg equilibrium, diversity parameters, F_{ST}, and R_{ST}), we assumed the presence of 4 populations according to geographic origin: southern Pantanal (SP; Mato Grosso do Sul state), northern Pantanal (NP; Barão do Melgaço/Mato Grosso state), northeast (NE; São Gonçalo de Gurgueia/PI, Tocantins and Maranhão), and north (N; Redenção and Carajás/PA). Loci were tested for Hardy–Weinberg equilibrium and linkage disequilibrium using GENEPOP 3.3 (Raymond and Rousset 1995). Genetic

Table 1. Sampling locality of hyacinth macaw

Locality	#	Nmic	Nmit	Year	Collector/collection	Basic diversity indices			
						Microsatellite		DNAmit	
						H_O	H_E	H	π
South Pantanal (SP) (4)									
Miranda, MS (SP/MI)	g	17	16	2002	N.M.R. Guedes	0.423	0.402	0.492	0.00060
Abobral, MS (SP/AB)	h	5	5	2000	N.M.R. Guedes	0.461	0.446	0.600	0.00085
Nhecolândia, MS (SP/NH)	i	13	10	2000–2002	N.M.R. Guedes	0.400	0.453	0	0
Rio Negro, MS (SP/RN)	j	6	4	2001–2002	N.M.R. Guedes	0.467	0.427	0.833	0.00079
North Pantanal (NP) (3)						0.365	0.411	0.324	0.00016
Barão de Melgaço, MT	f	20	17	2002–2004	P.T.Z. Antas				
Northest (NE) (2)						0.458	0.472	0.831	0.00058
São Gonçalo de Gurgueia, PI	e	6	5	1999	P. Martuscelli, C. Yamashita				
		8	7	2007	A. R. O. Marques				
Tocantins[a]	c	3	3	2003	Brasília Zoo, DF				
Maranhão[a]	d	2	2	2004	Rio de Janeiro Zoo				
North (N) (1)						0.484	0.520	0.182	0.00009
Redenção, PA			2	1997	C. Baider				
Carajás, PA			9	2007	F. T. Presti, A. R. O. Marques				
Total			80						
Localities of apprehensions									
AP1 – Corumbá and Jaraguari, MS		10	–	2004	N.M.R.Guedes				
AP2 – Porto Alegre, RS		4	–	2005	G. Marciano				
AP3 – Miranda, MS		2	–	2005	N.M.R. Guedes				
AP4 – CastelloBranco highway, SP		8	–	2006	Boituva Zoo, SP				

(corresponding to locality in Figure 1), number of samples of hyacinth macaw used for microsatellite (Nmic) and mitochondrial DNA (Nmit) analyses, year of collection, collector or collection, and basic diversity indices (microsatellite: observed H_O and expected H_E heterozygosities, and mtDNA: values of haplotype (K), and nucleotide (π) diversities).

MS, Mato Grosso do Sul state; MT, Mato Grosso state; PA, Pará state; PI, Piauí state; RS, Rio Grande do Sul state; SP, São Paulo state.

[a]Exact locality unknown but state known.

variation in each geographic region was evaluated using the number of alleles and the observed and expected heterozygosities for each locus using GENEPOP 3.3 (Raymond and Rousset 1995).

Mitochondrial DNA

We obtained partial sequences from strands of the mitochondrial genes ND5, cytochrome b, and ND2. Amplification reactions were performed with 4.9 µL of Milli-Q water, 1 µL of buffer (10×), 1 µL of dNTPs (4 mM), 1 µL of each primer (10 mM; primer sequences provided in Supplementary Table S2 online), 0.1 µL of *Taq*DNA polymerase (5 U/µL, Pharmacia), and 1 µL of DNA (20–50 ng). PCR conditions were as follows: 1) For the single amplicon including segments of both ND5 and cytochrome b, initial denaturation at 95 °C for 5 min was followed by 35 cycles at 95 °C for 30 s, 52 °C for 30 s, and 72 °C for 40 s; and a final extension at 72 °C for 7 min. 2) For ND2, initial denaturation at 95 °C for 5 min was followed by 35 cycles at 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 40 s; and a final extension at 72 °C for 7 min.

Sequences were edited and aligned using CodonCode Aligner v.1.6.3 (CodonCode Corporation). The number of haplotypes (H); haplotype diversity (k); nucleotide diversity (π); and neutral indices D_T (Tajima 1989), Fs (Fu 1997), and R_2 (Ramos-Onsins and Rozas 2002) were obtained using DnaSP 4.10.9 (Rozas et al. 2003). Statistical significance of neutrality tests was based on 10 000 simulations of coalescence (Rozas et al. 2003).

Population Genetic Structure

To estimate the number of populations (k), we simulated 1 to 10 populations based on microsatellite data and using a Bayesian clustering approach implemented in STRUCTURE 2.2 (Prichard et al. 2000). The parameters used in the analysis were 5000 burn-in steps and 1 000 000 Markov Monte Carlo chains (MCMC) iterations. Each simulation was replicated 20 times, as recommended (Evanno et al. 2005). To determine the best value of K, we estimated the delta K (Evanno et al. 2005) and calculated the average of the log of a posteriori probability using Excel. The degree of differentiation between individuals from pairs of locations sampled was estimated with the fixation indices F_{ST} (for microsatellite and mitochondrial DNA data) and R_{ST} (only for microsatellite data). F_{ST} values were estimated as described by Weir and Cockerham (1984), based on the analysis of the observed variance of allele frequencies among different populations assuming the infinite allele mutation model (IAM; Kimura and Crow 1964). These values were calculated using Arlequin 2.0 (Schneider et al. 2000). R_{ST} values were estimated as described by Slatkin (1995) assuming step-by-step change model (Ohta and Kimura 1973) using RSTcalc (Goodman 1997). The significance of these indices (P value) was estimated by permutation tests (Weir and Cockerham 1984), and the indices were adjusted using the Bonferroni correction (Rice 1989).

A haplotype network was obtained based on concatenated mitochondrial data using the median-joining method with subsequent maximum parsimony analysis, using NETWORK (http://www.fluxus-technology.com; Polzin and Daneschmand 2003).

Demographic Inferences

To search for evidence of bottlenecks, we examined microsatellite data using the heterozygosity excess method (Cornuet and Luikart 1996), as implemented in Bottleneck 1.2.02 (Piry et al. 1999). Three mutation models were used: an infinite alleles model (IAM), a single-step mutation model, and a settable intermediate 2-phase model (Estoup et al. 2002).

Three statistical tests to assess the significance of differences between observed and expected heterozygosities were used: a sign test, a standardized differences test, and a Wilcoxon sign-rank test (Luikart et al. 1998).

Mismatch distribution analyses (Rogers and Harpending 1992) were performed in DnaSP 4.10.9 (Rozas et al. 2003) for all individuals, using population divisions indicated by population structure analyses. Concatenated mitochondrial sequence data were used to estimate the date of the expansion event based on tau ($t = \tau/2\mu$, in which t was time in generations, and μ was mutation rate; Rogers and Harpending 1992) using Arlequin 3.5 (Excoffier and Lischer 2010). The 95% confidence interval for τ was estimated from 1000 bootstrap replicates. We assumed a rate of nucleotide substitution of 2.1% per million years (Weir and Schluter 2008) and a generation time of 9 years (as estimated in a similarly sized and phylogenetic closeness macaw, *Ara ararauna*; Caparroz et al. 2009).

The estimated coalescence time of each inferred population was obtained from concatenated mitochondrial data using MDiv (Nielsen and Wakeley 2001). The program uses simulations and MCMC to estimate the maximum likelihood of the following parameters: genetic diversity theta ($\theta = 2\ Ne \times \mu$), migration rate ($M = Ne \times m$), and coalescence time ($T = t/Ne$), in which Ne was the effective population size, μ the substitution rate, m the number of migrants, and t the time of divergence. Four simultaneous analyses were performed with different random seeds, each with 5 000 000 cycles and a burn-in of 1 250 000, assuming different values of M_{max} ranging from 2.5 to 5 and T_{max} 10 to 20 using the Hasegawa–Kishino–Yano substitution model. To calculate divergence times, the mutation rate was assumed to be 1.6–2.1% per million years (Shields and Wilson 1987; Fleischer and Mcintosh 2001; Weir and Schluter 2008). Assuming a generation time of 9 years and 2123 bp of mitochondrial DNA, we used a minimum μ of 15.2856×10^{-5} and a maximum μ of 20.062×10^{-5} substitutions/loci/generation.

Confiscated Chicks

The microsatellite composition of the inferred wild populations was used as a standard data set to assign the multilocus genotypes of 24 confiscated chicks, using the same 10 microsatellites as above, and assignment tests as implemented in Arlequin 2.0 (Schneider et al. 2000).

Data Archiving

In accordance with data archiving standards (Baker 2013), we have deposited the primary data underlying these analyses with Dryad. All generated sequences were submitted to Genbank (NCBI): microsatellite sequences (EU301666-EU301675, KR051398–KR051400, and KM076741-KM076742) and the mitochondrial DNA sequences (KR076865-KR076949 and KR076950-KR077029).

Results

Descriptive Statistics and Underlying Assumptions

Allele frequencies at all microsatellite loci did not deviate from Hardy–Weinberg equilibrium, and we found no evidence of linkage disequilibrium between any pair of loci. There was also no evidence for dropout of large alleles, genotyping errors, or null alleles for any microsatellite (data not shown). The mean number of alleles for each geographic region varied from 2 to 9, and the mean expected and observed heterozygosities were 0.447 and 0.438, respectively (Table 1; Supplementary Table S1 online).

We obtained partial sequences from ND5 (117bp), cytochrome b (1,048 bp), and ND2 (958 bp) for 80 individuals. The alignment matrix of concatenated sequences had 2123 characters. Sequences had no unexpected stop codons. Only 8 polymorphic sites were observed, with nucleotide (π) and haplotype diversity (h) values for the species as a whole and for each geographic region were 0.82, 0.324 for NP, 0.481 for SP, 0.182 for N, and 0.831 for NE (Supplementary Table S4 online). The neutrality tests DT, FS, and R2 based on concatenated mitochondrial sequence revealed no evidence for selection (Supplementary Table S5 online). In general, the number of polymorphic sites found is lower than in other species of birds; however, unlike the haplotype diversity (h) is considered high. According to Grant and Bowen (1998), h values above 0.5 are considered high, but interpretation of high h values can vary with nucleotide diversity (π). High h values with low π values indicate an accumulation of mutations after a population bottleneck event that was followed by a rapid population increase (Grant and Bowen 1998).

Population Genetic Structure

Within the Pantanal, differences of landscape characterize different subregions (Silva and Abdon 1998). These divisions are based on differences in source material and soil type, drainage, altitude, and vegetation associated with watersheds. These differences can affect the populations of hyacinth macaws as they may use different ecological resources (Silva and Abdon 1998). Thus, to test if birds from 4 subregions within SP (Miranda, Nhecolândia, Abobral, and Rio Negro) presented any detectable differentiation, we performed a Bayesian analysis of the microsatellite data.

Bayesian analysis of the microsatellite data revealed that a k of 4 had the highest likelihood (Figure 2; Supplementary Figure S1 online). These 4 populations were NP, SP, N, and NE. However, individuals from N and NE were not well differentiated (Figure 2). Fixation indices based on microsatellite data indicated a moderate degree of genetic differentiation among all pairs of regions (NP, SP, N, and NE), except F_{ST} between N and NE (Table 2). F_{ST} indices based on concatenated mitochondrial DNA sequences also indicated strong differentiation between NP, SP, and N+NE (Table 3).

Each of the regions (NP, SP, N, and NE) contained exclusive haplotypes (Figure 3; Supplementary Table S3 online), while haplotype 2 was present in all regions and at high frequency in some. Four haplotypes were observed in individuals from SP; the 2 most frequent were shared with individuals from different subregions (Figure 3). The general structure of the haplotype network showed many low-frequency haplotypes, especially in NE, possibly indicating recent population expansion. However, as sample sizes from most regions were not large, it is possible that addition of more samples could result in higher frequencies for these haplotypes.

Among subregions within the South Pantanal, no additional genetic structure was observed (Supplementary Figure S2 online). Fixation indices based on microsatellite data revealed no differentiation among subregions of SP, with negative values that were not significantly different from zero ($P > 0.05$), except R_{ST} between Miranda and Nhecolândia ($P < 0.05$; Table 2). Therefore, all further analyses considered SP to be one population.

Some microsatellite alleles were unique to particular regions. Two alleles from both UnaCT21 and UnaCT43 were exclusive to the north Pantanal (NP), and one allele each from UnaCT21, UnaCT43, and UnaCT74 only occurred in the north (N) and the northeast (NE). UnaCT32 was monomorphic in NP and polymorphic in the

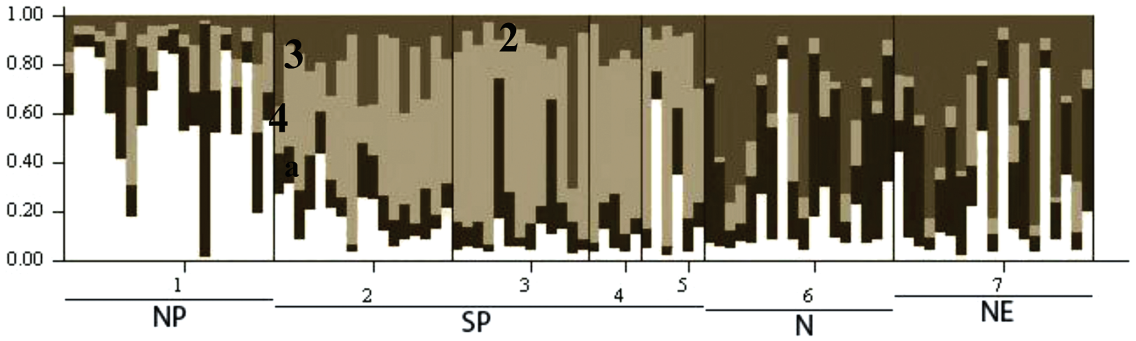

Figure 2. Result of the Bayesian clustering analysis of 10 microsatellites (k = 4) of 98 hyacinth macaws. NP, north Pantanal; SP, south Pantanal; N, north; NE, northeast.

Table 2. F_{ST} (below diagonal) and R_{ST} values (above diagonal) between individuals from different locations based on 10 microsatellites of hyacinth macaw

Locality	NP (20)	SP/MI (17)	SP/NH (13)	SP/AB (5)	SP/RN (6)	N (18)	NE (19)
NP (20)	–	**0.0444**	**0.0782**	0.0746	0.0716	**0.0941**	**0.0723**
SP/MI (17)	**0.07108**	–	0.0268	–0.0115	0.0104	**0.0829**	**0.0469**
SP/NH (13)	**0.12415**	0.03310	–	–0.0311	–0.0102	**0.0667**	**0.0896**
SP/AB (5)	**0.15031**	0.00968	–0.01030	–	–0.0284	0.0599	0.0685
SP/RN (6)	**0.09408**	0.00270	–0.02468	–0.00976	–	**0.0981**	**0.1005**
N (18)	**0.08172**	**0.08954**	**0.09599**	**0.09809**	**0.09542**	–	**0.0250**
NE (19)	**0.06893**	**0.05713**	**0.10019**	**0.10476**	**0.09374**	0.01402	–

Significant values are in bold ($P < 0.01$) or underlined ($P < 0.05$).
N, north; NE, northeast; NP, north Pantanal; SP, south Pantanal; SP/AB, Abobral; SP/MI, Miranda; SP/NH, Nhecolândia; SP/RN, Rio Negro.

Table 3. F_{ST} values based on 2123 bp of concatenated mitochondrial DNA sequence (ND5, ND2, and Cyt-b) of hyacinth macaw

Locality	NP (17)	SP/MI (16)	SP/NH (10)	SP/AB (5)	SP/RN (4)	N (11)
NP (17)	–					
SP/MI (16)	**0.61496**	–				
SP/NH (10)	**0.62636**	–0.04523	–			
SP/AB (5)	**0.85758**	0.08499	0.22078	–		
SP/RN (4)	**0.73034**	–0.11880	–0.16412	0.23077	–	
N (11)	**0.73454**	**0.39589**	**0.41550**	**0.88397**	**0.60217**	–
NE (17)	**0.52679**	**0.31351**	**0.29303**	**0.50723**	**0.35768**	0.08704

Significant values are in bold ($P < 0.01$). In parentheses are the numbers of samples.
N, north; NE, northeast; NP, north Pantanal; SP, south Pantanal; SP/AB, Abobral; SP/MI, Miranda; SP/NH, Nhecolândia; SP/RN, Rio Negro.

other regions. UnaCT43 was monomorphic in the south Pantanal (SP; Supplementary Table S1 online).

Demographic Inferences

Microsatellite data revealed no significant excess of heterozygotes, suggesting that populations were in mutation-drift equilibrium, such that no sign of a population bottleneck was detected. Neutrality tests did not indicate any signs of population expansion. On the other hand, the mismatch distributions obtained for each of the four populations had contrasting patterns (Figure 4): N and NE had unimodal distributions, indicating population expansion, while NP and SP had bimodal distributions, indicating no population expansion.

Estimated dates of demographic expansion based on mean τ and their 95% confidence intervals were 8842 years for NP (0–23 332); 92 767.5 years for SP (0–1 210 028); and 24 390 years for N+NE (11 250–44 730; Table 4). The estimated divergence times (t), assuming a minimum μ of 15.286×10^{-5} and a maximum of 20.062×10^{-5} were 2700–3400 years between NP and SP, 3600–4600 between NP and N+NE, and 1900–2300 years between SP and N+NE.

Confiscated Chicks

Assignment tests indicated that 4 of the individuals from confiscation group 1 (CG1) most likely came from NE, 1 from NP and the remaining 5 from N. All the individuals from CG2 were assigned to NE. The 2 chicks from CG3 most likely came from SP. Finally, for CG4, 5 of the individuals most likely came from N, 2 from NE, and 1 from NP.

As macaws from N and NE were not highly differentiated, an additional analysis was performed, assuming that birds from these regions all belonged to the same population. This analysis assigned 9 birds from CG1 to this N+NE group and 1 to NP; 3 of the individuals from CG2 to N+NE and 1 to NP; all individuals from CG3 to SP; and 5 of the macaws from CB4 to N+NE and 3 to NP (Table 5).

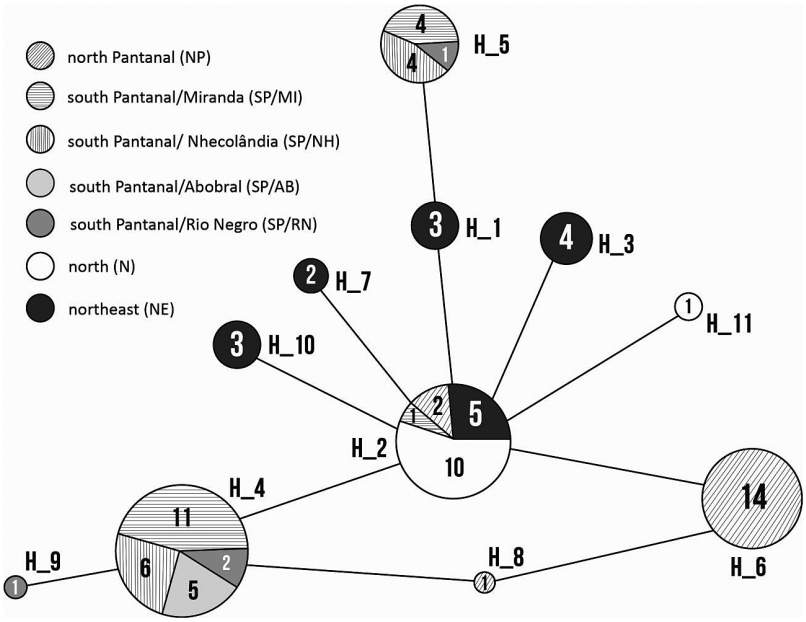

Figure 3. Haplotype network based on 2123 bp of concatenated mitochondrial data (ND5, ND2, and Cyt-b) from 80 hyacinth macaws. The number of individuals in each region or subregion is indicated. The sizes of the circles are proportional to the frequency of the haplotype.

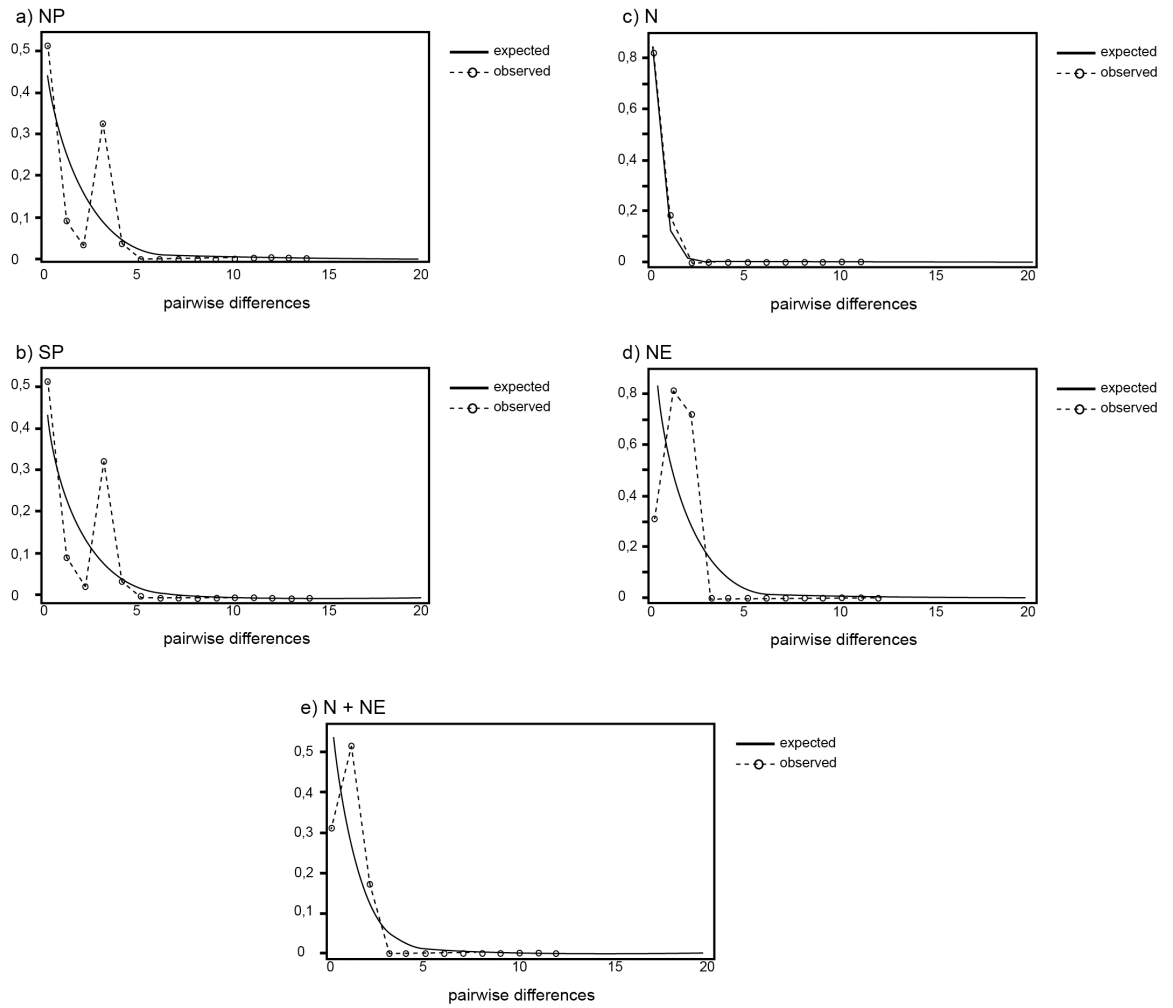

Figure 4. Mismatch distribution based on 2123 bp of concatenated mitochondrial data (ND5, ND2, and Cyt-b) from 80 hyacinth macaws.

Table 4. Parameters of coalescence analysis based on 2123 bp of concatenated mitochondrial DNA sequence (ND5, ND2, and Cyt-b) of 80 hyacinth macaws

Pair of populations	θ	*M*	*T*	Ne	*t* (years)
NP × SP	0.763475	0.12	1.36	2.497	30 567.77
				1.902	23 290.13
NP × N+NE	0.970104	0.15	1.44	3.173	41 125.46
				2.417	31 334.23
SP × N+NE	0.873442	0.215	0.82	2.857	21 085.21
				2.176	16 065.20

Values of Ne and *t* are based on the mutation rates of 15.286×10^{-5} (top) and 20.062×10^{-5} (bottom) substitutions/loci/ generation.

θ, theta; M, migration rate; *T*, coalescence time; Ne, effective population size; *t*, estimated date of divergence (thousands years before present).

Table 5. Numbers of hyacinth macaw individuals that were attributed to each geographic region and percentage of individuals that were correctly attributed to their region of origin (%) based on 10 microsatellites

Region or group of origin	Attributed region			
	NP	SP	N/NE	%
NP (20)	19	1	–	95
SP (41)	2	37	2	90.2
N/NE (37)	3	2	32	86
AP1 (10)	1	–	9	–
AP2 (4)	–	1	3	–
AP3 (2)	–	2	–	–
AP4 (8)	3	–	5	–

In parentheses are the numbers of individuals analyzed.

N, north; NE, northeast; NP, north Pantanal; SP, south Pantanal.

Discussion

Genetic Diversity

Analysis of samples from 98 hyacinth macaws (*Anodorhynchus hyacinthinus*) revealed 2 to 9 alleles per microsatellite locus and a mean observed heterozygosity of 0.438 (Supplementary Table S1 online). Mitochondrial sequences from 80 of those macaws revealed 11 haplotypes, a nucleotide diversity of 0.00071, and only 8 polymorphic sites in a total of 2123 bp (Supplementary Tables S3 and S4 online). As a rough comparison, in 50 blue-and-yellow macaws (a species for which 5 of the 10 microsatellite primers used in the present study were developed), 6 microsatellite loci had 3 to 11 alleles and mean observed heterozygosity of 0.604 (Caparroz et al. 2009). Additionally, 1290 bp of control region sequence from these birds revealed 39 variable sites, 28 haplotypes, and a nucleotide diversity of 0.0074 (Caparroz et al. 2009). Therefore, the general level of genetic diversity found in blue-and-yellow macaws appears to be greater than that observed in hyacinth macaws. This comparison must be made with caution, however, because Caparroz et al. (2009) used different mitochondrial genes than the present study; also, we used heterologous microsatellite loci, while those used for blue-and-yellow macaws were specific. However, the difference in the genetic variability between these 2 species was previously observed in single-locus minisatellite probes, microsatellites, and 472 bp of the control region (Faria and Miyaki 2006; Faria et al. 2008). These results are also consistent with DNA fingerprinting data that had slightly lower mean genetic variability in hyacinth macaws (65.60%; Miyaki CY, unpublished data) than in blue-and-yellow macaws (68.50%; Caparroz et al. 2001). Finally, Leite et al. (2008) observed a high diversity in blue-fronted parrots (*Amazona aestiva*) using 6 microsatellite primers developed for St. Vincent amazons (*Amazon guildinguii*); they found an observed heterozygosity of 0.808–0.972, with 15–23 alleles, values greater than the 0.438 and 2–9 alleles observed in the present study.

The lower level of microsatellite variability in hyacinth macaws compared to that observed in blue-and-yellow macaws could be due to the use of heterologous primers. The genera *Ara*, *Amazona*, and *Psittacus* (species for which the microsatellite primers were originally developed) are not sister groups of the genus *Anodorhynchus* and are not closely related to each other (Tavares et al. 2006; Tokita et al. 2007). In general, polymorphism decreases as phylogenetic distance increases between the target species and the one for which the original primers were developed (Galbusera et al. 2000; Zane et al. 2002; Primmer et al. 2005). However, other studies with birds have observed high genetic variability when heterologous microsatellite primers were used (Primmer et al. 2005; Caparroz et al. 2007; Chan et al. 2009).

Additionally, the hyacinth macaw is considered threatened, while the blue-and-yellow macaw is not (BirdLife International 2013b; IUCN 2014), and in general, threat categories can be related to population size. The hyacinth macaw population size is estimated at 6500 individuals (BirdLife International 2013a) and the blue-and-yellow macaw population size is unknown, but may be more than 50,000 individuals (Antas, unpublished data). Thus, the lower genetic variability in hyacinth macaws is consistent with threat status, although it is difficult to compare the genetic diversity of organisms that probably have different evolutionary histories.

Scant published literature exists on the genetic variability of South American parrots (Presti and Wasko 2014). Researchers using DNA fingerprinting found high diversity in nonthreatened species, such as the red-and-green macaw, *Ara chloropterus* (Faria and Miayki 2006). On the other hand, for the near-threatened blue-winged macaw, *Primolius maracana* (IUCN 2014) genetic diversity is similar to fully threatened species (Craveiro and Miyaki 2000). In another study, Presti et al (2011) found lower levels of genetic variability in three threatened macaw species (Spix's macaw, *Cyanopsitta spixii*; Lear's macaw, *Anodorhynchus leari*; and hyacinth macaw, *A. hyacinthinus*) compared to nonthreatened species, using heterologous microsatellite loci (developed in the red-and-green macaw, *Ara chloropterus*; and the scarlet macaw, *Ara macao*).

Population Genetic Structure

The Bayesian clustering implemented in STRUCTURE software and R_{ST} indices based on microsatellites (Table 2) revealed the existence of moderate genetic differentiation among 4 major groups: north Pantanal (NP), south Pantanal (SP), north (N), and northeast (NE). F_{ST} values confirmed the moderate (microsatellites) and high (mtDNA) differentiation between the same major regions, except for N and NE (Tables 2 and 3). The differentiation between the Pantanal (north and south) and both N and NE was not surprising, given their geographic distances (~700 km; Tables 2 and 3). These results are in agreement with Faria et al. (2008), who found significant differentiation among populations of hyacinth macaws from Piauí and Abobral/Miranda within SP (F_{ST} = 0.33; $P < 0.001$ and F_{ST} = 0.25; $P < 0.001$, respectively).

As expected, no genetic differentiation was detected among the subregions within the south Pantanal (SP; Miranda, Nhecolândia, Abobral, and Rio Negro), probably due to the short distances (120–200 km) separating them (Table 2). This result suggests that the

different landscapes and delimiting rivers of these subregions (Silva and Abdon 1998) are not barriers to macaw movement.

The low differentiation between N and NE was more surprising and could be explained by 2 hypotheses. First, there may be limited gene flow between them at low undetectable levels. Even though the Bayesian analysis indicated the presence of 4 groups, N and NE were less differentiated (Figure 2). Additionally, as R_{ST} is more sensitive to variation in sample size (Baloux and Lugon-Moulin 2002), the observed R_{ST} values may be overestimates. Third, the isolation between N and NE may have occurred recently so that there has not been enough time for differentiation to occur. In that case, more sensitive tests, such as Bayesian analysis, would be able to detect differentiation, while others, such as F_{ST}, would not. Because mutation rate of microsatellites is greater than that of mitochondrial DNA, it is not surprising that the former marker detected greater genetic structure between N and NE. Thus, the low differentiation between N and NE based on microsatellite data could either be due to ongoing gene flow or recent geographic isolation. A larger number of polymorphic markers and higher sample sizes from these 2 regions may help to better address this issue.

F_{ST} and R_{ST} values (Tables 2 and 3), as well as haplotype networks (Figure 3), all indicated genetic differentiation between NP and SP. These sampling locations were less than 500 km apart, with no known physical barrier separating them. However, although the hyacinth macaw is capable of flying long distances (Forshaw 1989; Seixas and Guedes 2002; Antas et al. 2010; Antas, unpublished data), radio tracking data of adults from NP demonstrated a total average movement distance of only 20 km and an average homerange of 10481 hectares (ha; average 40 days of transmission data, maximum 151 days; Antas et al. 2010). Juveniles monitored with backpack radios had an average homerange of 1011 ha, with a maximum of 4025 ha, and total average movement distance of 19.8 km, with a maximum of 50 km (average 122 days of transmission data, maximum 467 days; Antas et al. 2010). In SP, radiotracking showed that juveniles born locally generally stay close to their nest after fledging, although 1 moved 36 km away (Seixas and Guedes 2002). Because juveniles accompany their parents for almost a full year after fledging (Antas et al. 2010), most do not move long distances from their birth place. Unfortunately, there is no radio tracking data monitoring the later stages of juvenile development. Even with this limited data, indicating that hyacinth macaws do not seem to disperse over long distances, the genetic differentiation between NP and SP was surprising because the Pantanal has no obvious barriers to movement. However, no published studies or museum records indicate the presence of hyacinth macaws in the gap between these 2 regions (Antas, personal communication). It is possible that the area between NP and SP lacks critical nesting and/or feeding habitat for hyacinth macaws; in the present study, we did not have the opportunity to survey this important area.

The difference in F_{ST} values based on the 2 markers (microsatellites and mtDNA sequences, Tables 2 and 3) could be the result of sex-biased dispersal. Maternally inherited mtDNA was more differentiated than biparentally inherited microsatellite loci, which is consistent with greater dispersal in male birds (Greenwood 1980; Clarke et al. 1997). Another parrot species, the blue-and-yellow macaw, also had 2 distinct mitochondrial genetic lineages and yet an absence of genetic structure based on microsatellite data (Caparroz et al. 2009). These patterns in the genetic data are what would be predicted if female hyacinth macaws are philopatric while males disperse farther. For better understanding of these processes, it would be interesting to examine other sex-linked molecular markers, such as the control region of mitochondrial DNA (which is also more variable than the regions studied in this work), or more and species-specific microsatellite markers. Another possible explanation for the difference in F_{ST} values is that the mtDNA has matrilineal inheritance and is haploid, making its effective population size approximately one-fourth that of the nuclear DNA. This would result in an increased rate of genetic drift and rapid approach to the balance between drift and migration in populations without a biased sex ratio or polygamy in males (Birky et al. 1983). In this case, the lower level of genetic differentiation found in nuclear DNA could be due to the short time of population isolation (Keeney et al. 2005), even though the microsatellite mutation rate is greater than that of mtDNA (Brown et al. 1982; Dallas 1992). Furthermore, microsatellite alleles may suffer from homoplasy and thus be prone to underestimation of the differences between populations (Hefti-Gautschi et al. 2009).

We found no bird species in the literature with the same pattern of population structuring as hyacinth macaws. Other parrot species have a variety of patterns. In South America, structuring in the blue-fronted parrot, *Amazona aestiva*, is present only between distant populations, with genetic diversity varying across a gradual cline (Leite et al. 2008). Other parrot species have high genetic structuring, such as the Cuban amazon, *Amazona leucocephala*, which has 5 currently recognized subspecies (Russello et al. 2010). The southern mealy amazon, *Amazona farinosa*, has 2 distinct clades in Central America and South America with estimated divergence from 1.75 to 2.7 million years ago (Wenner et al. 2012).

Demographic Inferences

The estimated date for the divergence between genetically differentiated hyacinth macaw groups was between 16000 and 42000 years ago, which corresponds to the end of the Pleistocene (Table 4). This also corresponds to the last glacial maximum period (18000 to 48000 years ago; Behling and Lichte 1997; Behling 2002). During this phase, continental glaciers may have advanced (Sant'Anna Neto and Nery 2005), and areas of open vegetation may have expanded while humid forest area was reduced (Haffer 1967). Around 30000 years ago, the temperature in Brazil could have been about 5 °C colder than the current temperature, which could have affected the distribution of forest and savannah habitats (Stute et al. 1995). However, there is no consensus regarding this scenario in the literature. For example, Colinvaux and De Oliveira (2000) and Melo e Souza et al. (2013) argue that the Amazon basin retained its forests during the last glacial cycle, as there is no direct evidence suggesting forest fragmentation in the Pleistocene and thus, there would have been no major canopy opening and no replacement of the forest by savanna. Despite these controversies, our data do not rule out a Pleistocene-related environmental change that could have caused fragmentation in the hyacinth macaw distribution. Crick (2004) argues that climate changes of this period changed some bird species distributions primarily through changes in the annual cycle and abundance of food resources. Thus, habitat gain or loss could lead to expansion or contraction of a species distribution in response to climate changes, depending on the degree of dependence on the corresponding type of environment. Hyacinth macaws nest in tree hollows or cliffs and feed on palm tree nuts. Assuming that the environment changed, groups of individuals could have been isolated, and this may have resulted in the genetic differentiation observed in this study. Moreover, the species seems to be philopatric (Guedes 1993), and this behavior may favor isolation and reduced gene flow.

Significant neutrality index values may be associated with selective effects and/or demographic changes such as expansion or bottleneck. Neutrality indices were not significant (Supplementary Table S5 online); however, this may also indicate population stability. No

signal of past bottlenecks was observed in microsatellite data, but the haplotype network (a star shape with 3 highly frequent haplotypes and 8 low-frequency haplotypes; Figure 3) was consistent with recent population expansion. Among the low-frequency haplotypes, 5 were from N and NE. The unimodal mismatch distributions (Figure 4) also suggested the presence of expansion in N and NE. It is important to note that the generation time of the hyacinth macaw is unusually long: They only reach reproductive maturity after 7 years and, in general, during the initial breeding seasons, they do not seem to be able to reproduce every year (Guedes 1993). These features could also have affected our results, as it would take a long time to accumulate any demographic signal. Also, the low mtDNA variability (8 polymorphic sites in 2123 bp) may have limited statistical power (Ramos-Onsins and Rozas 2002). Our data suggest that NP and SP have been more constant, while N and NE may have experienced a recent expansion. Pollen data from Lago Calado in Central Amazon indicate an area influenced by long-term water level changes, caused by Quaternary sea-level fluctuations and also by seasonal water level variations in the Amazonian drainage system, including a period of profusion of the palm-swamp genus *Mauritia* in the Holocene (Behling et al. 2001). This kind of environmental change could have favored the population expansion of hyacinth macaws in the north and northeast.

Confiscated Chicks

The suspect caught with the chicks from CG1 and CG2 stated that they were obtained in the Pantanal. However, our microsatellite analysis revealed that they probably came from N and NE. Confiscated chicks were also significantly younger than chicks monitored at the time in SP by the Hyacinth Macaw Project (NMR Guedes, personal communication). Thus, it is probable that these chicks were really caught outside the Pantanal and the suspect was crossing the Pantanal to reach the border of a neighboring country. Lear's macaws (*Anodorhynchus leari*), an endemic and endangered macaw species that occurs in northeast Brazil, have been observed for illegal sale at the Bolivian border (Herrera M, personal communication) that might have been the intended destination for these hyacinth macaw chicks. This information supports a possible animal trafficking route that begins in Northeastern Brazil and crosses the border with Bolivia (Lopes 2003). This example illustrates how genetic analysis can help trace routes of the illegal trade of wild animals and thus aid plans for more effective actions against this crime.

The 2 CG3 chicks were seized in Miranda (one of the 4 SP subregions sampled here) and were genetically assigned to SP. This result was in accordance with the strong suspicion that they had been captured in this region, because they were seized with a suspect native to the region, who later confessed to having captured the 2 chicks in SP.

The assignment test showed that 62.5% of the individuals from CG4 were assigned to N and NE and 37.5% to NP. This arrest occurred on a highway (Castello Branco) that is one of the main terrestrial routes connecting the Pantanal with the city of São Paulo. It is possible that the majority of these chicks were caught outside the Pantanal and some in the Pantanal.

It is important to note that the inferred origin of confiscated chicks could change if data from other hyacinth macaw populations that were not analyzed here were added to the present data set. We do not know how many populations of hyacinth macaw exist in Brazil: there is a need to study the area between NP and SP to verify the possible presence of hyacinth macaws and better infer the genetic differences found and sampled a small resident population north of Pará (Altamira city region) with a totally unknown number of individuals.

Recommendations for Conservation

In this study, we found at least 3 Management Units (*sensu* Moritz 1994) and conclude that these populations should be managed separately whenever possible. This is our main recommendation for this species at this time in its history and considering its still relatively large population size in Pantanal, and we have little information on the biology of hyacinth macaw for other areas of study. We found significant genetic structure among populations, and this may reflect local adaptations that would be lost in case of a joint management of populations (Haig 1998), possibly resulting in outbreeding depression, the loss of fitness in hybridized populations (Frankham 1995). If any demographic restoration or translocation is needed, such as the release of individuals from another population to increase the genetic variability of a genetically depauperate population (genetic reinforcement) or to maintain local adaptations of given populations, genetic information should be taken into account in order to achieve the objective of long-term survival of the species and probably the chances increase with increasing genetic variability (Frankham et al. 2010). Furthermore, because populations of these hyacinth macaws are structured, we were able to use this structuring to identify the origin of seized birds, which would not be possible if management actions mixed birds of different origins. Identifications such as these in turn can be used to plan law enforcement actions against this illegal practice. Many hyacinth macaws have been seized in Brazil (Marini and Garcia 2005, Machado et al. 2008), and genetic analysis can help in describing the trafficking routes of these birds indicating the possible location of occurrence of a confiscated individuals. In addition, this information can be used to know in which region can be given a possible release of the individual, as he will have greater chances of survival in the habitat which he was captured (Wanjtal and Silveira 2000).

In terms of conservation, knowing generated about the demographic history of hyacinth macaw and its responses to past climate processes can be useful for predicting susceptibility to future changes. For example, if we are able to hypothesize the period of divergence between genetic groups, time of expansion, or population bottleneck of a species, and connect such biological events to geological and ecological ones, it is possible to manage future populations to prevent or minimize decreases in population size and genetic diversity. In the case of hyacinth macaws, we hypothesize that climatic changes occurring during the Quaternary could have influenced their geographical distribution and that the species has not experienced population bottleneck. Therefore, its low genetic variability could be associated with a demographic decrease, but also may be due to behavioral characteristics, such as philopatry leading to a degree of inbreeding, or to action of genetic drift in isolated populations.

Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

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