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Genetic structure in Brazilian breeding colonies of the Roseate Spoonbill (*Platalea ajaja*, Aves: Threskiornithidae)

C.I. Miño and S.N. Del Lama

Departamento de Genética e Evolução, Universidade Federal de São Carlos, São Carlos, SP, Brasil
Corresponding author: C.I. Miño
E-mail: carolinaianido@yahoo.com.ar

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ABSTRACT. Roseate Spoonbills (*Platalea ajaja*, Linnaeus) are wading birds present in two of the most important Brazilian wetlands: the Pantanal wetlands and Rio Grande do Sul marshes. Natural populations of these species have not been previously studied with variable nuclear molecular markers. In order to support decision making regarding the management and conservation of these populations, we estimated and characterized the distribution of genetic variability among five Brazilian breeding colonies. The average observed heterozygosity in Brazilian Roseate Spoonbill populations (*Ho* = 0.575) did not differ significantly from the value determined in a U.S. wild-caught sample of 15 individuals, using data generated by the same set of microsatellite loci. Considering that the U.S. population underwent a recent reduction in size, we discuss this result supposing that the U.S. population was not genetically affected or that both populations had suffered a bottleneck. Global F_{ST} indicated the lack of genetic differentiation among colonies, indicating the occurrence of past and/or present gene flow among them. Analysis of molecular variance revealed that most of the genetic variation is distributed within

the colonies. Results are explained by a recent origin of colonies or by high levels of gene flow. Management decisions should take into consideration the fact that, even in the presence of high genetic exchange, ecological adaptations to different environments are important for species survival.

Key words: Genetic variability, Microsatellites, Population structure, Nuclear molecular markers, Wading bird

INTRODUCTION

Accurate knowledge of local population structure can provide important insights into species biology, including social structure and the dispersal behavior of individuals (Dobson et al., 1998). Most of our knowledge of how genetic variability is partitioned among avian populations comes from studies on species from temperate latitudes. Genetic structure and dispersal studies of Neotropical birds, however, are scarce (Brown et al., 2004).

The Roseate Spoonbill (*Platalea ajaja*, Linnaeus 1758; Aves: Ciconiiformes) is a widely distributed Neotropical aquatic bird that breeds colonially from the southeastern United States (U.S.) to central Argentina. U.S. Spoonbill populations underwent a profound bottleneck from 1890 to 1920 (Powell and Bjork, 1990), and the species was listed as one of special concern (Florida Fish and Wildlife Conservation Commission, 1997). Brazilian breeding colonies of these birds are located in several wetlands at different latitudes, including the Pantanal region (Yamashita and Valle, 1990) and the lagoon areas along the coast of the State of Rio Grande do Sul (Encarnação and Diniz, 1992). Breeding period and composition are different in the colonies of these regions: Pantanal ones are established between July and October together with other species of Ciconiiformes (e.g., Wood Stork, Great Egret, and Snowy Egret) (Yamashita and Valle, 1990), whereas in Rio Grande do Sul, they occur during the period of September-February with White-necked Herons, Night Herons, Great Egrets, and Cattle Egrets (Silva and Bello Fallavena, 1995). To our knowledge, Brazilian reproductive and wintering areas have not been affected by population bottlenecks due to environmental stress or to anthropogenic disturbances. del Hoyo et al. (1992) classified Pantanal populations as declining, but they did not show data to support this information. On the other hand, Harris et al. (2005) point out that the Pantanal, one of the most continuous wetland areas in South America (approximately 1,000,000 km^2; Neiff, 2001), is still well preserved.

Only one genetic study has been conducted on natural populations of the Roseate Spoonbill by Santos (2005), who performed an analysis of the mitochondrial DNA (mtDNA) control region and found no significant levels of genetic differentiation between individuals from the Brazilian Pantanal and Rio Grande do Sul regions.

In the present study, we estimated and characterized the distribution of genetic variability among Roseate Spoonbill populations from five Brazilian breeding colonies, using five polymorphic microsatellite loci. Our aim was to investigate genetic connectivity among breeding

colonies to provide data that may be of key importance in supporting decision making regarding the management and conservation of these populations.

MATERIAL AND METHODS

Sample collection

A total of 69 samples were collected by either drawing blood or plucking growing feathers. The samples were from nestlings in four Pantanal wetland colonies in the States of Mato Grosso and Mato Grosso do Sul: Fazenda Ipiranga (N: 11; 16°25'S; 56°36'W), Porto da Fazenda (N: 8; 16°27'S; 56°07'W), Baia de Gaíva (N: 20; 16°39'S; 57°10'W), and Fazenda Retirinho (N: 18; 19°50'S; 56°02'W), and in one colony in the State of Rio Grande do Sul: Banhado do Taim (N: 12; 32°29'S; 52°32'W) (Figure 1, Table 1).

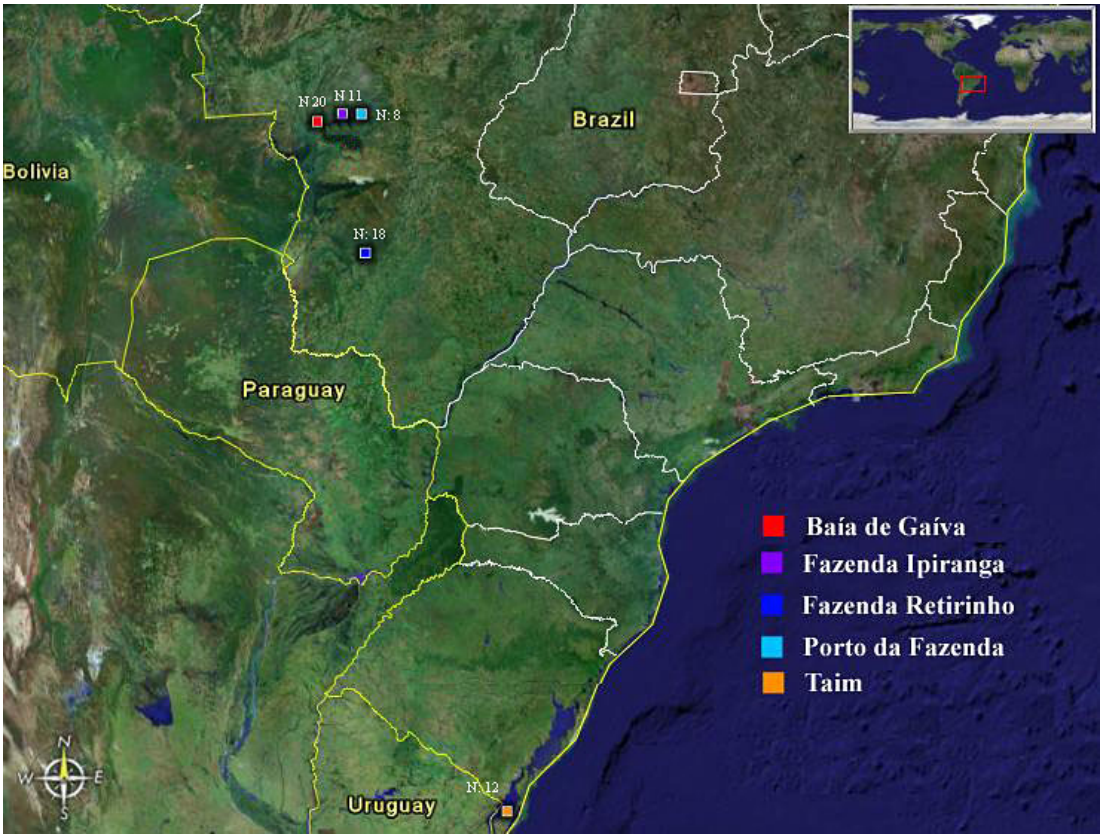

Figure 1. Map showing breeding sites and number of individuals of Roseate Spoonbill sampled in two Brazilian regions.

Genomic DNA isolation

Blood samples were centrifuged, and cell and plasma fractions were frozen separately in liquid nitrogen. Growing feathers were also frozen in liquid nitrogen. DNA was extracted

Table 1. Roseate Spoonbill samples collected from Brazilian breeding colonies.

Collection site	Number of samples	Collection date
Baia de Gaíva (Pantanal, MT)	20	2003
Fazenda Ipiranga (Pantanal, MT)	11	2003
Porto da Fazenda (Pantanal, MT)	8	2000
Fazenda Retirinho (Pantanal, MS)	18	1999
Banhado do Taim (Rio Grande do Sul State)	12	2002
Total	69	

following the phenol-chloroform method (Sambrook et al., 1989). A tube containing no bird tissue was included in each group of DNA extractions as a contamination control.

DNA methods

Each sample was genotyped at five microsatellite loci using primers developed for *Mycteria americana* by Tomasulo-Seccomandi et al. (2003) and for *Platalea ajaja* by Sawyer and Benjamin (2006) (Table 2). To account for genotyping errors, 5% of the samples, randomly chosen, were amplified and genotyped three times. Extractions and amplifications were performed in separate rooms. Amplification reactions were prepared in a UV-sterilized cabinet, pipetting was performed using filtered tips, and one control PCR blank was included for each set of amplifications. PCR reactions were performed in a 25-µL final volume with 1 mM Tris-HCL, pH 8.4, 50 mM KCl, 50 µg bovine serum albumin, 2.5 mM $MgCl_2$, 0.25 mM dNTPs, 0.5 U of *Taq* DNA polymerase, and 50 ng template DNA in a GeneAmp® PCR System 2400

Table 2. Microsatellite loci used in the present study.

Locus	Primer sequence (5'-3') and fluorescent marker	Annealing temperature	Na	Fragment sizes	Ref.
WS03	*HEX* - AGAAGCCAAATTGATTAGA ACAAAGTTGCGGAGAA	60°C	3	170-176	1
Aajµ1	*HEX* - GATCACCACCATCTTAAATGATAA CTTCTGTTTGCCTCACATGG	55°C	10	157-184	2
Aajµ2	*6'-FAM* - CTTGATGCAAAGGAAACATCC GAGGTGCTTCCAGTTTCCTG	55°C	9	165-245	2
Aajµ3	*6'-FAM* - CCCATGGCCACATTATAAACTT GCTCTGGAGTAACTTGCTGGA	55°C	7	137-203	2
Aajµ5	*HEX* - GGCTGAACACTGTTGTGCTCT GAACCAAGCCTCCCTGAATA	58°C	5	164-215	2

Na: number of alleles per locus.
[1]Tomasulo-Seccomandi et al., 2003.
[2]Sawyer, 2002.

thermal cycler (Applied Biosystems). Briefly, the PCR reaction profile was: 96°C for 5 min, 10 cycles for 45 s at 96°C, corresponding annealing temperatures for 45 s and 1 min at 72°C; 10 cycles for 30 s at 96°C, corresponding annealing temperatures -0.5°C per cycle (touchdown) for 30 s and 1 min at 72°C; 10 cycles for 30 s at 96°, 50° or 53°C (depending on primer) for 30 s and 1 min at 72°C. All reactions had a final extension for 10 min at 72°C. PCR products were sized in a MegaBACE™1000 automatic sequencer using the Gensize Rox 500 ladder as the internal standard (Amersham Biosciences). Results were analyzed using the GeneticProfiler® software (Amersham Biosciences).

Data analysis

Microsatellite genotyping error rates (allelic dropout and false alleles) were calculated using GIMLET, v1.3.4 (Valière, 2002). A set of standard population genetic analyses was conducted to quantify genetic diversity within and among Roseate Spoonbill colonies. Gene frequencies were calculated using GENEPOP version 3.2 (Raymond and Rousset, 1995) and used to estimate other genetic variability parameters. Allelic richness (El Mousadik and Petit, 1996) was calculated using FSTAT, version 2.9.3.2 (Goudet, 2001). Gametic phase disequilibria and departures from Hardy-Weinberg equilibrium at each locus were determined with exact tests using a Markov chain algorithm (Guo and Thompson, 1992) in GENEPOP version 3.2 (Raymond and Rousset, 1995). Sequential Bonferroni corrections were used to adjust the P values obtained for possible type I errors (Rice, 1989). Levels of genotypic heterozygosity for each breeding colony at each microsatellite locus were assessed by calculating observed (*Ho*) and expected (H_E) heterozygosity (also using GENEPOP, version 3.2). Heterozygosities were compared to the values obtained by Sawyer (2002) using the G test for contingency analysis (Zar, 1996) performed in BIOSTAT 3.0 (Ayres et al., 2003). Wright's (1951) F_{ST} values were calculated for each locus independently, as well as for all loci. Pairwise comparisons between breeding colonies of the Roseate Spoonbill for estimators of F_{ST} were calculated using a variance-weighted determination of allele frequency in ARLEQUIN, version 2.0 (Schneider et al., 2000). Analysis of molecular variance (AMOVA, Excoffier et al., 1992) was also performed in ARLEQUIN using all colonies to estimate the total percentage of variance attributable to differences between colonies and differences among individuals within colonies.

RESULTS

Genetic diversity

Five of the six microsatellite loci tested were polymorphic in all breeding colonies. Locus WS09 was monomorphic in all colonies and it was excluded from further analysis. Individual multi-locus genotyping was reliable in separate amplifications for the repeated samples (across loci: allele dropout: 0.097; false alleles: 0.144). Gametic phase disequilibria between all pairwise microsatellite loci comparisons did not prove significant after performing a sequential Bonferroni correction (Rice, 1989). Genetic independence among loci was thus assumed for all subsequent analyses.

Average allelic richness ranged from 2.69 to 3.04 (Table 3). A total number of 34 alleles were detected across all loci, with allele numbers per locus ranging from 3 (WS03) to 10 (Aajμ1)

(Table 2). A minimum of 17 alleles were detected in the Fazenda Ipiranga and Porto da Fazenda colonies and a maximum of 26 alleles at Baia de Gaíva. Seven alleles were unique to a single-breeding colony (Baia de Gaíva, four alleles; Fazenda Retirinho, three alleles). *Ho* across all loci was 0.575. Average *Ho* for each colony ranged from 0.499 (Fazenda Ipiranga) to 0.712 (Porto da Fazenda) (Table 2). Mean *Ho* per locus was: 0.768 (Aajµ1), 0.622 (Aajµ2), 0.637 (Aajµ3), 0.491 (Aajµ5), and 0.360 (WS03). No population showed significant departure from Hardy-Weinberg equilibrium, and estimates of F_{IS} (Table 3) for each population did not differ significantly from zero ($P > 0.05$), showing that genotypic frequencies are in accordance with that expected for a random-mating population (Hartl and Clark, 1997).

Table 3. Levels of genetic diversity (*A*: allelic richness, *HE*: expected heterozygosity; *Ho*: observed heterozygosity, F_{IS}: Wright, 1951) estimated for five microsatellite loci among five Brazilian Roseate Spoonbill breeding colonies (present study) and *Ho* observed for one wild-caught U.S. population (Sawyer, 2002).

Colony	Estimate	Locus WS03	Locus Aajµ1	Locus Aajµ2	Locus Aajµ3	Locus Aajµ5	Average per colony
Baia de Gaíva							
	A	1.908	4.328	3.359	3.273	2.355	3.044
	HE	0.434	0.869	0.712	0.685	0.545	0.649
	Ho	0.333	0.933	0.714	0.500	0.643	0.624
	F_{IS}	0.239	-0.077	-0.004	0.278	-0.1878	0.038
Fazenda Ipiranga							
	A	1.992	3.811	2.773	2.909	1.992	2.695
	HE	0.621	0.879	0.667	0.636	0.318	0.624
	Ho	0.166	0.666	0.667	0.833	0.167	0.499
	F_{IS}	0.705	0.184	-0.212	-0.351	0.000	0.080
Porto da Fazenda							
	A	1.967	3.762	4.393	2.929	2.000	3.010
	HE	0.467	0.844	0.893	0.821	0.533	0.711
	Ho	0.667	0.800	1.000	0.500	0.667	0.712
	F_{IS}	-0.333	0.000	-0.143	0.333	-0.333	-0.052
Fazenda Retirinho							
	A	2.142	3.695	3.567	2.632	1.978	2.800
	HE	0.521	0.797	0.772	0.579	0.349	0.603
	Ho	0.214	0.692	0.800	0.600	0.400	0.541
	F_{IS}	-0.333	0.000	-0.143	0.333	-0.333	0.087
Banhado do Taim							
	A	1.984	3.553	3.257	3.235	2.309	2.867
	HE	0.554	0.750	0.721	0.735	0.533	0.658
	Ho	0.417	0.750	0.833	0.750	0.583	0.666
	F_{IS}	0.203	0.000	-0.164	-0.021	-0.242	-0.043
Average per locus							
	Ho	0.360	0.768	0.622	0.637	0.491	0.575
U.S. wild-caught							
	Ho	*	0.733	0.667	0.600	0.667	0.667

In the first analysis, including all colonies, global F_{ST} value was not significant (0.002, P > 0.05). In order to determine if this pattern would also be observed among more geographically distant colonies, two groups were defined: one joining only the Pantanal colonies (Baia de Gaíva, Fazenda Ipiranga, Fazenda Retirinho, and Porto da Fazenda) and the other including the sample of the Rio Grande Sul colony (Banhado do Taim). Non-significant levels of genetic differentiation were found in this test. Pairwise estimates of F_{ST} did not differ significantly from zero for any of the breeding colony comparisons, with values ranging from -0.020 (Fazenda Ipiranga *vs* Porto da Fazenda) to 0.015 (Baia de Gaíva *vs* Rio Grande do Sul) (P > 0.05). In agreement with the above results, the AMOVA analysis showed that most of the genetic variation present in the colonies can be explained by differences among individuals within breeding colonies (99%). In summary, Brazilian Roseate Spoobill populations did not show a significant population genetic structure.

DISCUSSION

From a total of six microsatellite loci studied, only the non-specific WS09 was monomorphic in the sample examined. This result is expected because locus WS09 was isolated from *Mycteria americana*, another species from the Ciconiiformes order, while the other five loci are species-specific (Sawyer and Benjamin, 2006). Overall observed heterozygosity was greater than that reported for other Ciconiiformes species. For example, Pantanal Wood Stork colonies showed lower heterozygosity levels (0.389, Van Den Bussche et al., 1999; 0.303, Tomasulo-Seccomandi, 2004) and Pantanal Jabiru Stork populations showed a value of 0.410 (Lopes et al., 2005). According to the G test, levels of genetic variability detected in Brazilian Roseate Spoonbill populations did not differ significantly from that determined by Sawyer (2002), in a U.S. wild-caught sample of 15 individuals, using data generated by the same set of microsatellite loci (Table 3). To explain these similar levels of genetic diversity in the U.S. and Brazilian populations, we can suppose, firstly, that these levels are inherent to the species, and that the northern population was not seriously affected by its documented reduction in size in the early 1900s. Another possible hypothesis for the observed similarity could be that the Brazilian population may have undergone undocumented population declines, as reported for the Pantanal region by del Hoyo et al. (1992). Studies including samples from other areas along the distribution range in the American continent, where there is no evidence of demographic decline, can help in clarifying if the U.S. population was not genetically affected or if both populations suffered a decline and loss of genetic variability.

Analyses based on multi-locus microsatellite genotyping indicate that genetic homogeneity characterizes Brazilian Roseate Spoonbill colonies. Observed F_{ST} values between Pantanal and Rio Grande do Sul populations were low and none differed significantly from zero. We found no evidence to support the possibility that coastal colonies (Banhado do Taim, RS) are genetically different from inland (Pantanal) colonies. Low levels of genetic structuring have been previously detected, using mtDNA and microsatellite data, in Pantanal populations of other Ciconiiformes species such as Jabiru and Wood Storks (Del Lama et al., 2002; Rocha et al., 2004; Lopes, 2006). The lack of evidence for strong genetic structure reported in the present study can be interpreted as follows: 1) colonies are of recent origin and have not had sufficient time to differentiate genetically, or 2) levels of gene flow have been maintained high between breeding colonies of this species. However, when interpreting our results we have to bear in

mind that the performance of the commonly used estimators of genetic structure depends on the applicability of the underlying analytical models and on a number of other factors that are difficult to sort out clearly (Balloux and Lugon-Moulin, 2002). On the other hand, the results presented here, from an analysis using a highly variable nuclear molecular markers, are in accordance with the results previously reported using mtDNA (Santos, 2005), indicating that the population sampled merits recognition as a single-conservation unit (Allendorf and Luikart, 2007).

Genetic information has been widely used in management decision making. However, using only this approach is sometimes inadequate, because levels of genetic divergence and gene flow do not always correlate with ecologically relevant habitat adaptations (Hendry and Taylor, 2004). Moreover, according to Allendorf and Luikart (2007), many kinds of information, including environmental characteristics, may be integrated for identifying units of conservation. Roseate Spoonbills live in quite different habitats in Brazil, one that is strictly a freshwater environment in the Pantanal region, and the habitat where marine and fresh water mix in Banhado do Taim. Populations from these different places can show ecological adaptations that have not yet been investigated. Thus, we propose, at this time, not to consider the Pantanal and southern Brazilian colonies as a unique management unit. This proposal will conserve the evolutionary processes responsible for the actual patterns of genetic diversity, and the preservation of populations from different environments. This fact, as proposed by Allendorf and Luikart (2007), will allow selection pressures to be diverse and let multi-locus genotype diversity to remain high. This strategy should be applied to species lacking genetic differentiation and high gene flow but located in distinct environments so as to consider the local adaptations important to species survival.

In order to confirm if populations from these regions deserve recognition as independent conservation units, future studies in Roseate Spoonbills should focus on other markers (adaptive ones) to better explore the relationship between adaptive and neutral variation. A study with a more representative sample of the species along its distribution range, would also help unravel how genetic diversity is partitioned in these wading birds.

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