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Genetic structuring among populations of the great egret, Ardea alba egretta, *in major Brazilian wetlands*

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ABSTRACT

1. Waterbirds are increasingly affected by climate change and human disturbances to the wetlands on which they roost, forage and breed. The evolutionary response of populations to such changes is influenced by genetic variability and gene flow patterns, which enable long-term survival. Thus, genetic monitoring of waterbird populations can provide valuable information to support conservation measures and management policies for wetlands.

2. This study assessed past and contemporary levels of genetic diversity, estimated effective population sizes (*Ne*) and investigated gene flow patterns among populations of the great egret, *Ardea alba egretta*, settled in major Brazilian wetlands.

3. Samples ($N = 200$) were collected from the northern, central western, south-eastern and southern regions of Brazil. AMOVA, *F*-statistics, assignment tests, Bayesian clustering analyses and *Ne* were estimated based on mitochondrial DNA (mtDNA) and microsatellite loci.

4. The populations share most mitochondrial haplotypes, suggesting a common recent past. Mismatch analyses, *Fs* and *D* statistics, and *SSD* and *Rg* indices indicated significant signs of expansion in most populations. The time since expansion suggests that egrets colonized southern latitudes more recently, probably accompanying the supposed historical environmental changes in South America, with more stable habitats toward equatorial regions.

5. MtDNA Φ_{ST} revealed significant differentiation between the northern and both the central western and southern populations. Nuclear loci demonstrated significant structuring between the central western and southern populations, which showed similar effective sizes.

6. Despite the considerable dispersal potential of the great egret, there is limited gene flow among populations located in different Brazilian wetlands. Therefore, colonies from different regions should be preserved, with special attention to the northern populations, whose allelic constitution differs from the other. This approach can be used to genetically monitor similar species in other wetlands or to great egret populations in other regions of the Americas.

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KEY WORDS: human disturbance; conservation; gene flow; floodplains; habitat management; molecular markers

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INTRODUCTION

Waterbirds, such as herons, storks, ibises and spoonbills (Ciconiiformes and Pelecaniformes), are found around the world. Some cosmopolitan species occupy broad geographical ranges, exhibit considerable dispersal capacity and forage over extensive regions. Many species establish reproductive colonies and forage in wetlands, which constitute their primary habitat. Hence, waterbirds are extremely dependent on such ephemeral, patchy habitats with specific biotic and abiotic conditions and hydrological requirements (Herring and Gawlik, 2013).

Waterbirds have developed adaptations to the normal cycle of flood and drought for efficient nesting and foraging on floodplains (Gimenes and Anjos, 2007). At present, however, many important wetlands worldwide are in decline or even in a critical state owing to habitat fragmentation and loss, pollution, dam building, drainage, and high levels of precipitation, among other pressures (Rosselli and Stiles, 2012; Junk *et al*., 2013). Human disturbances increasingly change the hydrologic patterns of wetlands (Rosselli and Stiles, 2012; Junk *et al*., 2013), which alters the reproductive and foraging ecology of waterbirds and exerts a negative influence on population dynamics, thereby threatening such species (Gimenes and Anjos, 2007; Scherer *et al*., 2011; Gray *et al*., 2013). Monitoring waterbird populations can therefore assist in evaluating the health of wetlands. Indeed, these birds have been used as bioindicators in such ecosystems (Frederick *et al*., 2009). This approach has helped develop restoration plans, management policies and conservation measures for many important floodplains worldwide (Frederick *et al*., 2009; Gray *et al*., 2013). Among waterbirds, the family Ardeidae, which includes herons, egrets and bitterns, is well represented worldwide and members of this group have been used as biological indicators in wetlands (Connell *et al*., 2002; Kim *et al*., 2010). Thus, monitoring egret populations can provide useful information to support the conservation of freshwater floodplains.

Genetic methods help estimate key biological parameters for birds, which are difficult to assess during field observations, and have contributed much to bird conservation (Haig *et al*., 2011). Molecular markers can also provide information on unknown aspects of population dynamics and history that can assist decision-makers in determining evolutionary units for conservation. The adaptive potential of waterbird populations to novel conditions as well as their ability to cope with threats and respond to environmental changes are influenced by genetic variation levels, gene flow patterns, past evolutionary history and effective population sizes (Friesen *et al*., 2007; Palstra and Fraser, 2012). Understanding the distribution patterns of genetic diversity (i.e. genetic structuring) is essential to predicting the ability of waterbird populations to survive in a particular environment.

Historical processes (Geraci *et al*., 2012) and behavioural traits, such as dispersal patterns, phylopatry and mating systems, influence the genetic structure of natural populations of waterbirds (Friesen *et al*., 2007). Hence, genetic differentiation patterns may reflect ecological and behavioural responses to past history (Lankau *et al*., 2011). Widely distributed populations are expected to harbour more genetic variation (Lankau *et al*., 2011) and adapt better to variable habitat conditions. Widely distributed colonial breeding waterbirds are therefore good models for the investigation of evolutionary processes that may have influenced gene flow patterns at different geographic scales (Reudink *et al*., 2011; Geraci *et al*., 2012). Theoretically, gene flow promoted by the considerable dispersal potential of such birds could counterbalance differentiation imposed by breeding site fidelity (Friesen *et al*., 2007). Determining whether dispersive and widespread colonial waterbirds comprise genetically panmictic populations throughout their distribution range is important for the identification of independent population units for conservation (Friesen *et al*., 2007). Despite their importance to the field of conservation, studies on such issues in colonial waterbirds remain scarce (Reudink *et al*., 2011; Geraci *et al*., 2012). Notably, the few published articles on these birds have reported significant population differentiation despite the lack of apparent barriers for genetic exchange (Wiley

et al., 2012; Miño and Del Lama, 2014) as well as panmixia in the presence of perceived barriers to gene flow (Oomen *et al*., 2011; Reudink *et al*., 2011).

Among waterbirds, egrets have been under-investigated with regard to population genetics. Indeed, to the best of our knowledge, only a handful of studies are available on natural populations (Bates *et al*., 2009; Zhou *et al*., 2010; Hill *et al*., 2012). Therefore, new studies addressing species and geographical ranges that have been unexplored thus far can contribute to understand better the nature and characteristics of processes that model the genetic structure of waterbird populations. Monitoring waterbirds with a genetic approach may also provide valuable information for the establishment of management policies and protective measures for wetlands (Lopes *et al*., 2007). Examples of genetic monitoring for wetland conservation include the assessment of genetic variation among threatened wetland plants (Godt *et al*., 1995; Edwards and Sharitz, 2000), the identification of evolutionary lineages of freshwater isopods (Gouws and Stewart, 2007), the use of molecular markers to examine the stock composition of the Chinook salmon (Teel *et al*., 2009) and, more recently, the investigation of population structure, genetic diversity and demographic history of populations of wetland passerines (Ceresa *et al*., 2015).

To contribute to current conservation needs for major Brazilian wetlands and expand knowledge on the population genetics of ardeids associated with these areas, the present study focused on populations of the great egret, *Ardea alba egretta* Gmelin 1789 (Aves: Ardeidae: Ciconiiformes, but see Pratt, 2011). This subspecies occurs throughout the Americas, from southern Canada to southern Argentina and Chile (Morales, 2000; Kushlan and Hancock, 2005) and is evenly distributed among Brazilian wetlands (GBIF, 2015), where it has been used as an indicator of threatened environments (Gomes *et al*., 2009). Great egrets move locally in response to breeding or wintering needs and are resident or partially migratory birds throughout most of their range in South America (Brazilian Committee on Ornithological Records, 2011; IUCN, 2014). Although this species is globally listed as 'least concern' by the International Union for Conservation of Nature (IUCN, 2014), with trends indicating increasing populations (Morales, 2000), it is increasingly affected by the many threats to the wetlands in which it forages, roosts and breeds. This is alarming, as previous studies on foraging ecology (Gimenes and Anjos, 2007), microhabitat use (Pinto *et al*., 2013) and breeding behaviour (Nunes and Tomas, 2004; Scherer *et al*., 2011) have shown that the great egret in Brazil tends to occupy regions with very specific abiotic characteristics. In southern Brazilian wetlands, for example, the presence of this species is influenced by precise air temperature and relative humidity levels, which affect foraging patterns due to reduced prey availability on hotter days (Pinto *et al*., 2013). Thus, changes in the major environmental variables of the wetlands used by the great egret are likely to exert an influence on population dynamics. Determining the extent of genetic differentiation and similarities among populations is considered one of the research needs for this waterbird (Kushlan and Hancock, 2005).

The general aim of the present study was to contribute novel genetic information on great egret populations to support the conservation of Brazilian wetlands, given their current fragile status (Junk *et al*., 2014). The specific objectives were to: (1) assess historical and contemporary levels of genetic diversity; (2) estimate the number of breeders that effectively contribute to the gene pool; (3) infer historical and contemporary demographic processes; and (4) evaluate genetic structure or gene flow to define independent population units for conservation. Great egrets can travel large distances, which can lead to gene flow between populations from different regions (i.e. homogenization of allele frequencies) but, at the same time, they occupy particular breeding sites annually for many years (Ogden *et al*., 1980; Bancroft *et al*., 1988; Pinto *et al*., 2013). Hence, we expected that populations distributed throughout the country may show some degree of significant genetic differentiation. Regarding historic processes, climatic changes in the Pleiostocene and interglacial periods affected Brazilian wetlands differently, particularly with respect to temperature, humidity and water levels

(Colinvaux and De Oliveira, 2000). We hypothesize that Brazilian great egret populations shifted their ranges in response to such alterations, mirroring past wetland availability for feeding, roosting or breeding. In colder peaks during the Last Glacial Maximum (LGM, *c.* 20 000 yr BP; Aleixo, 2004), populations probably occupied narrow ranges toward equatorial latitudes of the country and expanded to southern ranges after this colder period. We therefore expected to find lower levels of genetic diversity in southern colonies as well as stronger signs of expansion in populations located at southerly latitudes.

METHODS

Samples (blood or feathers, see below) were obtained from 18 sites (Table S1, Supplementary material) located in four major regions spanning a 30° latitudinal range throughout Brazil: north (states of Amapá and Amazonas, N = 20); central west (Pantanal wetland, state of Mato Grosso, N = 71); south east (state of São Paulo, N = 13); and south (state of Rio Grande do Sul, N = 96) (Figure 1). The sampling sites were chosen to include representative wetlands of different geological natures, with variable influences from tides and rainfall as well as diverse vegetation community structures, since these landscape characteristics can shape the evolutionary responses of birds to environmental change (see Table 1 in Junk *et al.*, 2014). The Pantanal wetland is dominated by savannah and stunted-tree woodland/deciduous scrub forest (Diegues, 1994). The Amapá coastal wetlands are situated in a woody/humid tropical forest with the presence of swamps (Diegues, 1994). The marshes of Rio Grande do Sul belong to humid/subtropical forest biomes (Diegues, 1994).

Colonies were visited 13 times during the breeding seasons at each site, which vary in time based on periods of maximum local food availability for adults to raise chicks. For example, in southern Brazil, colonies were sampled in January, whereas colonies in the central western and northern regions were visited in September/October. Nests were randomly selected from different areas within each colony. Blood samples were taken from nestlings aged two weeks, which were manually captured in the nests. To avoid including relatives, which can bias genetic estimates, only one chick per nest was sampled. The vegetation substrate of the nests varied depending on the sampling site. In the states of Amapá and Rio Grande do Sul, the nests were located in shrubs (Scherer *et al.*, 2011), whereas in the Pantanal wetland nests were built in trees at a height of about 10 m (Yamashita and Valle, 1990). Nests built in shrubs were accessed manually, with the aid of a ladder when necessary, while access to nests built in trees required climbing equipment. Blood samples (0.15 mL) were obtained from the brachial vein of nestlings using sterile disposable syringes with 3% EDTA as anticoagulant, stored in sterile microtubes with absolute ethanol and kept at –20°C until processing. The nestlings were safely returned to their nests after sampling.

Intact feathers moulted from adults were collected at two sites (one in Amapá and one in Amazonas, northern region, Figure 1) and these samples were used only for historical analyses. Feathers more than 3 m apart from each other were gathered from the ground and stored separately in paper bags until processing. Only feathers in good condition were collected – i.e. with no signs of ageing or excessive exposure to harsh weather or moisture. The feathers collected were assumed to have been recently moulted by birds from nearby colonies. As different multilocus genotypes were found in these samples, each was assigned to different individuals.

DNA isolation, sequencing and genotyping

Genomic DNA was extracted from blood using a standard phenol–chloroform procedure (Sambrook and Russell, 2001). Genomic DNA was extracted from feathers using the methods described by Miño and Del Lama (2009). A fragment of the mitochondrial control region (mtDNA CR) was initially amplified by polymerase chain reaction (PCR) with the *L1652* (Sorenson *et al.*, 1999) and *HCSB-1* (Lopes *et al.*, 2006) primers. A 586-bp fragment of domain I (CRI) was then amplified using more internal specific primers described by

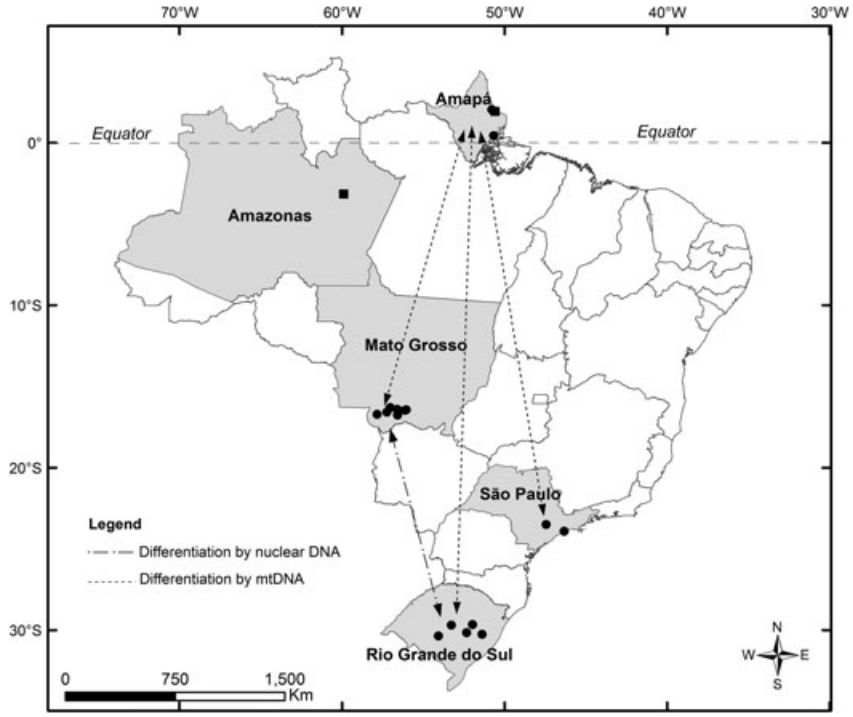

Figure 1. Map of Brazil showing approximate location of great egret sampling sites in northern (states of Amazonas and Amapá), central western (state of Mato Grosso), south eastern (state of São Paulo) and southern (state of Rio Grande do Sul); states are shaded in grey. Black dots represent colonies and black squares indicate roosting sites. Patterns of significant genetic differentiation among populations are summarized in the map, as demonstrated by mitochondrial (dotted arrows) and nuclear data (dashed-dotted arrows).

Table 1. Results of mitochondrial diversity and neutrality tests for great egret populations from four major regions in Brazil. Diversity estimates and results of neutrality tests based on 586-bp sequence of mitochondrial DNA control region domain I; *H*: number of haplotypes; *N*: number of individuals genotyped; *PS*: number of polymorphic sites; haplotype (*h*) and nuclear diversity (π) ± standard deviation (SD); *Fs*: Fu's statistic (Fu, 1997); *D*: Tajima's statistic (Tajima, 1989); R_2: Ramos-Onsins and Rozas' statistic (Ramos-Onsins and Rozas, 2002); *Tau*(τ): mode of unimodal mismatch distribution; population size estimates before (θ_0) and after (θ_1) expansion event; and mean time since population expansion (*yr BP*: years before present); asterisks indicate significant values (*$P \leq 0.05$, ** $P \leq 0.01$)

Region	North	North	South East	Central West	South
Brazilian state	Amazonas	Amapá	São Paulo	Mato Grosso	Rio Grande do Sul
H (N)	6 (6)	11 (14)	8 (13)	37 (71)	36 (96)
PS	9	16	14	38	36
h ± *SD*	1.00 ± 0.09	0.93 ± 0.06	0.88 ± 0.07	0.93 ± 0.02	0.86 ± 0.03
π ± *SD*	0.005 ± 0.004	0.008 ± -0.005	0.005 ±–0.003	0.006 ± –0.003	0.005 ± 0.003
Fs	–2.98**	–4.028*	–2.01	–25.97**	–26.315**
D	–0.82	–0.17	–1.20	–1.59*	–1.691*
R_2	0.09**	0.12	0.09	0.05**	0.04**
Tau (τ)	3.65	6.30	-	2.93	0.66
Θ_0	0	0.00	-	2.94	3.04
Θ_1	99999.0	19.20	-	16.55	44.79
Divergence rate (10%)	62.22	107.49	-	50.10	11.26
Time since expansion (yr BP)	159 003	274 770	-	128 039	28 783

Corrêa (2009) (Ardea L3: CAC CTA ACA CAA AAC ACA AAC and Ardea H1: CGT CTG TAT GCT CAC GTC TTC), following protocols and conditions described in Moralez-Silva and Del Lama (2014). Sequencing was carried out in an ABI Prism 3730 sequencer (Applied Biosystems, Perkin Elmer Corporation, Foster City, CA, USA). Sequences were aligned with Clustal W (Chenna *et al.*, 2003) and visually checked and trimmed using BioEdit v7.0.9.0 (Hall, 1999).

Sixteen microsatellite primers were initially screened in *A. alba egretta*: nine developed for *Ardea herodias* (McGuire and Noor, 2002), four developed for *Mycteria americana* (WS03, WS04, WS18, WS20, Van Den Bussche *et al.*, 1999; Tomasulo-Seccomandi *et al.*, 2003) and three developed for *Platalea ajaja* (Ajμ1, Ajμ2, Ajμ3; Sawyer and Benjamin, 2006). After extensive optimization of the PCR conditions starting from original protocols, seven loci provided consistent polymorphic genotypes: *Ah211*, *Ah217*, *Ah320*, *Ah414*, *Ah522*, Ah630 and *WS03* (primer details are given in (Table S2). Optimized PCRs were carried out in a final volume of 15 μL containing 1× PCR buffer [75 mmol L^{-1} Tris HCL (pH 9.0, 25°C), 50 mmol L^{-1} of KCL, 20 mmol L^{-1} of $(NH_4)_2SO_4$], 1.5 mmol L^{-1} of $MgCl_2$, 1 U of *Taq* DNA polymerase (Biotools), 0.25 of mmol L^{-1} dNTPs (Amersham Biosciences) and 50 to 80 ng of DNA. For dynamically-labelled primers (Table S2), PCR mixes included 0.20 μmol L^{-1} of specific forward primer with the M13 sequence (Alpha DNA, Montreal, Canada), 0.80 μmol L^{-1} of specific reverse primer and 0.80 μmol L^{-1} of M13 primer with a fluorescent tag. For primers with fixed fluorescence, mixes included 0.20 μmol L^{-1} of forward and reverse primers (Alpha DNA). The following were the cycling parameters: 94°C for 5 min; 30 cycles at 94°C for 30 s, annealing for 45 s at a specific temperature for each primer (Table S2) and 72°C for 45 s; eight cycles at 94°C for 30 s, M13 primer annealing at 53°C for 45 s and 72°C for 30 s; and 72°C for 10 min. A modified cycling profile lacking the eight cycles for M13 annealing was used with fixed-fluorescent primers. PCRs were carried out in an Eppendorf Mastercycler® gradient thermal cycler (Eppendorf AG, Hamburg, Germany). Genotype data were collected using a MegaBace™ 1000 sequencer with ET 550-R as the size standard and allele sizes were estimated using the Genetic Profiler Software Suite v2.2 (GE Healthcare Life Sciences, Pittsburgh, PA, USA).

Genetic diversity, historical demography and effective population size

Haplotype (*h*) and nucleotide diversity (π) (Nei, 1987) of mtDNA were estimated using ARLEQUIN v 3.1 (Excoffier *et al.*, 2005). Deviation from selective neutrality was evaluated by *Fs* (Fu, 1997) and *D* (Tajima, 1989) statistics using ARLEQUIN and by R_2 (Ramos-Onsins and Rozas, 2002) using DNASP v.5 (Librado and Rozas, 2009). Relationships among haplotypes were assessed using a median-joining method (Bandelt *et al.*, 1999) in Network 4.5.1.6 (Fluxus Technology). Demographic expansion was tested through mismatch distribution analyses (Rogers and Harpending, 1992) in ARLEQUIN and DNASP, estimating population sizes before (θ_0) and after (θ_1) the expansion event in units of mutational time. The fit between observed and expected distributions of pairwise differences was evaluated using the sum of square deviations (*SSD*) (Schneider and Excoffier, 1999) and statistical 'raggedness' (*Rg*) (Harpending *et al.*, 1993; Harpending, 1994). Time (t) in years since population expansion was estimated based on mismatch results, using the formula $t = \tau /2u$, in which u is the cumulative probability of substitutions across the sequence and tau (τ) is the mode of the unimodal mismatch distribution. The web-based service 'Mismatch Calculator' (Schenekar and Weiss, 2011) was used to conduct this analysis, with the format 'Divergence rate - %Div/MY' and the following parameters: the τ value obtained for each region, 586 bp of sequence length, a generation time of 2 years (as measured by age at first breeding) (Kushlan and Hancock, 2005) and a 10% divergence rate (Lopes *et al.*, 2006).

Microsatellite data were checked for the presence of null alleles and/or stuttering separately for each sample using MICRO-CHECKER 2.2.3 (van Oosterhout *et al.*, 2004). The number of alleles, allele frequencies, observed heterozygosity (H_O), unbiased expected heterozygosity (H_E) and tests for assigning individuals to potential source populations (Paetkau *et al.*, 2004) were computed using GENALEX v6.5 (Peakall and Smouse, 2012). Heterozygosity levels were compared among colonies using the non-parametric Mann–Whitney test with the aid of BioEstat v 5.0 (Ayres *et al.*, 2003). Deviations from the Hardy–Weinberg equilibrium (*HWE*) and linkage disequilibrium were assessed in GENEPOP v 3.4 (Raymond and Rousset, 1995). Allelic richness and inbreeding coefficients (F_{IS}) (Wright, 1951) were estimated in FSTAT v1.2

(Goudet, 1995). The effective population size (*Ne*) was estimated using the 'sibship' assignment method implemented in COLONY (Jones and Wang, 2010) with the following parameters: monogamy for males and females, full-likelihood option and medium length of the run. Microsatellite analyses were conducted only for regions with larger sample sizes (central west ($N = 56$) and south ($N = 75$)).

Genetic structuring analyses

The hierarchical distribution of mitochondrial variation was inspected using analysis of molecular variance (AMOVA) (Excoffier *et al.*, 1992) with the colonies grouped into regions (north, central west, south east and south, Table S1, Supplementary material) and pairwise Φ_{ST} between regions were computed in ARLEQUIN. For nuclear loci, genetic structuring was assessed by AMOVA in ARLEQUIN and pairwise F_{ST} estimates (θ, Weir and Cockerham, 1984) were determined using FSTAT, correcting for multiple comparisons with the Bonferroni procedure (Rice, 1989). Nuclear structuring was investigated on two levels: (1) individual colonies – three in the central western region and five in the southern region; and (2) with colonies grouped into regions (i.e. contrasting both regions). To infer the most likely number of differentiated genotypic clusters, Bayesian analyses were run in STRUCTURE v2.2 (Pritchard *et al.*, 2000). Twenty independent runs were performed for $K = 1$ to 8 (total number of different colonies analysed for nuclear data), with the admixture model default parameters and burn-ins of 10^5 Markov chain Monte Carlo replicates, followed by 10^6 replicates of data collection. The procedure described by Evanno *et al.* (2005) was applied to STRUCTURE outputs using STRUCTURE HARVESTER v0.6.8 (Earl and von Holdt, 2012). An isolation-by-distance pattern was tested by reduced major axis (RMA) regression of genetic and geographic matrices using IBDWS v3.16 (Jensen *et al.*, 2005), with 1000 randomizations. For this analysis, genetic distance was expressed as $F_{ST}/(1-F_{ST})$ and geographic distance was expressed as a logarithm of pairwise Euclidean distances between colonies (Rousset, 1997). All analyses described above were conducted including loci with signs of null alleles in the dataset, as these were highly polymorphic, but with first correcting allele frequencies using the Brookfield 1 method (van Oosterhout *et al.*, 2004). Removing loci with null alleles from the dataset did not substantially change the results.

RESULTS

Genetic diversity, historical demography and effective population size

Fifty-eight polymorphic sites defined 76 mtDNA haplotypes in the overall sample. The most frequent and widespread haplotype (H9) occurred in 56 individuals outside the state of Amapá (northern region). Haplotype and nucleotide diversity were slightly higher in the northern and central western samples (Table 1). The minimum-spanning network showed a star-shaped pattern with the four regions occupying both interior and terminal positions (Figure S1). Assuming neutrality of the mtDNA CRI, significant *Fs*, *D* and R_2 tests (Table 1) were interpreted as a sign of expansion in the northern, central western and southern regions. The southern population had the most recent time since expansion and the largest variation in estimates of population size ($\theta_1 - \theta_0$) among those with larger sample sizes (Table 1). Population expansion was complementarily supported by unimodal patterns of mismatch analyses, with non-significant *SSD* and *Rg* values (Figure 2).

Samples from central western and southern Brazil exhibited 39 alleles at microsatellites (2 to 18 alleles per locus) (Table S3). There was no evidence of linkage disequilibrium between any pair of loci. Only 10 out of 69 HWE tests departed significantly from expectations, four of which included the Barra do Ribeiro sample (southern region). The presence of null alleles was suggested by the excess of homozygotes in loci Ah414 (mean frequency: 0.11) and Ah522 (mean frequency: 0.12). The central western population exhibited a mean of 4.82 alleles and allelic richness of 3.47 and the southern population exhibited a mean of 5.38 alleles and allelic richness of 4.81 (Table 2). These regions harboured similar heterozygosity levels: central

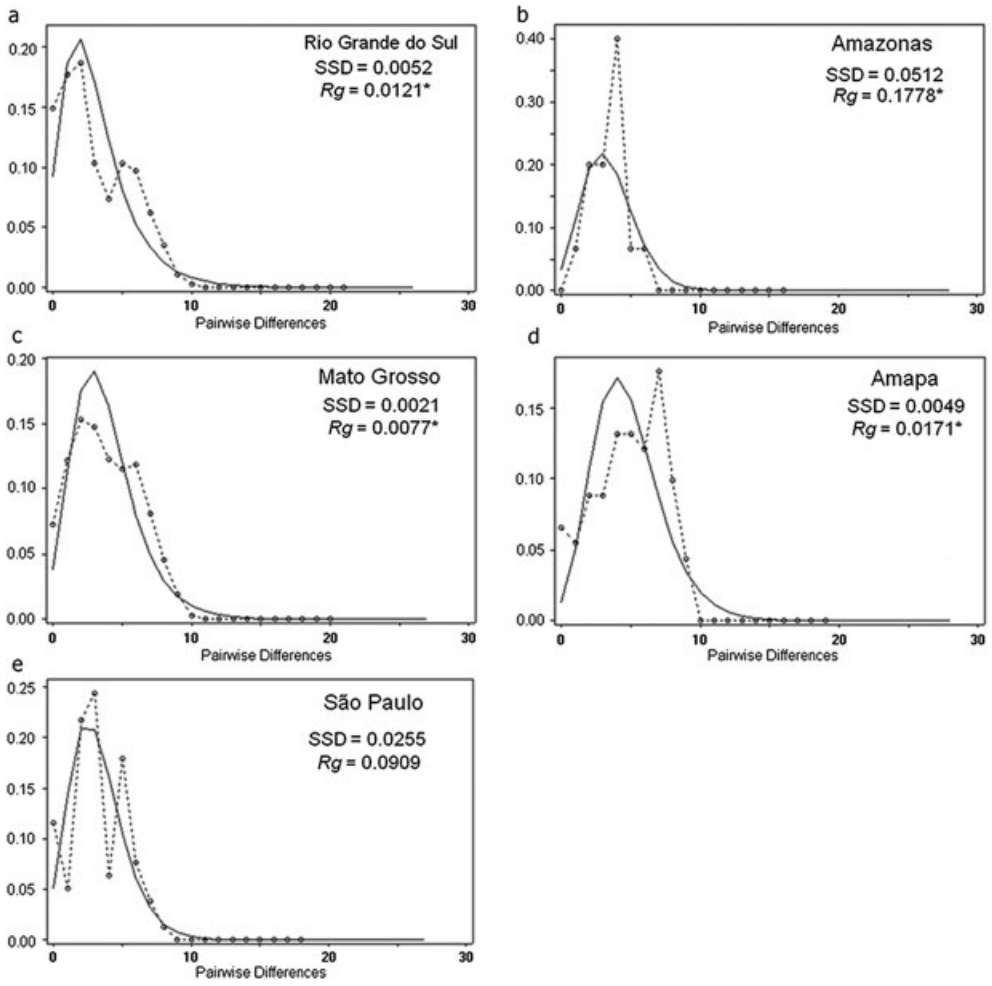

Figure 2. Mismatch analyses of great egret population from major geographic regions in Brazil; observed (dotted line) and expected (continuous line) distribution curves of frequencies of pairwise differences between mitochondrial DNA control region haplotypes; sum of square deviations (*SSD*) and raggedness (*Rg*) values are shown in each sub-figure. Asterisks indicate significant *Rg* values (*P*-values ≤ 0.05, not shown), suggesting population expansion in all samples except the population in the state of São Paulo.

Table 2. Nuclear diversity and effective population sizes observed in great egret populations from two major regions in Brazil. Summary estimates of genetic diversity over seven microsatellite loci: *A*: number of alleles; A_R: allelic richness; H_O: observed heterozygosity; H_E: expected heterozygosity; F_{IS}: inbreeding coefficient (no significant deviation from zero); and effective population size (*Ne*)

Region	Colonies	*A*	A_R	H_O	H_E	F_{IS}	*Ne*
Central West	Tucum	3.11	3.76	0.56	0.62	0.05	23
	Porto da Fazenda	6.14	3.75	0.62	0.63	-0.02	15
	Praialzinho	5.28	3.36	0.59	0.60	0.01	17
South	Barra do Ribeiro	6.71	4.56	0.56	0.62	0.11	12
	Santa Maria	4.57	3.74	0.40	0.48	0.02	37
	Serrinha	4.85	3.36	0.39	0.50	0.11	55
	Mariante	4.14	2.54	0.48	0.53	0.03	36
	Pântano Grande	3.85	3.14	0.55	0.59	0.01	14

west: mean $H_E = 0.54$ and mean $H_O = 0.48$; south: mean $H_E = 0.60$ and mean $H_O = 0.54$ (Mann–Whitney test, $P > 0.05$). Effective population size estimates for individual colonies ranged from 12 to 55 (Table 2). Pooling colonies within regions, the *Ne* was 29 (95% CI: 17 to 53) in the central western population and 31 (95% CI: 19 to 50) in the southern population.

Genetic structuring patterns

MtDNA Φ_{ST} demonstrated significant structuring between the state of Amapá (northern region) and all other samples (Table 3). Accordingly, 2.7% of mtDNA diversity was explained by significant differences among regions (P = 0.01). At the colony level, microsatellites demonstrated a lack of structuring among most colonies in the southern region (Table 4). However, the Barra do Ribeiro colony differed significantly from three other colonies in this region. In the central western region, the Praialzinho colony differed significantly from both the Tucum and Porto da Fazenda colonies (Table 4).

Contrasting colonies from different regions, most comparisons (73.3%) yielded significant F_{ST} values (Table 4). When pooling data into regions, a low but significant genetic difference was detected between the southern and central western populations (global F_{ST} = 0.06, P = 0.05). Likewise, 2.37% of nuclear variation was explained by significant differences among regions (P = 0.07), and 5.1% was explained by differences among colonies within regions (P = 0.05). In addition, assignment tests showed that, when colonies were set as potential sources, a mean of 32% of the egrets were correctly assigned to their sites of origin (Table S4). However, when regions were set as sources, the mean proportion of correctly assigned egrets increased to 83%. Bayesian clustering showed that the proportion of individuals assigned to clusters was maximized at K = 2 [ln $P(K)$ = –2519.68] (raw data given in Table S5) and the highest Delta K (1.60) was obtained in two population clusters (Figure S2). There was no evidence of isolation-by-distance (r^2 = 0.04, P = 0.30).

DISCUSSION

The present study describes nuclear and mitochondrial variation in populations of the great egret sampled over a 30° latitudinal range in Brazil and provides insights to past and contemporary demographic processes as well as

Table 3. Mitochondrial estimates of genetic structure among great egret populations from four major regions in Brazil. Pairwise θ_{ST} values based on mitochondrial DNA (below diagonal) and corresponding P-values (above diagonal) depicting patterns of genetic structuring; asterisks indicate significant θ_{ST} values (*$P \leq 0.05$, ** $P \leq 0.01$)

	South	Central West	South East	North	
	Rio Grande do Sul	Mato Grosso	São Paulo	Amazonas	Amapá
Rio Grande do Sul	-	0.26	0.19	0.17	0.01
Mato Grosso	0.001	-	0.17	0.20	0.04
São Paulo	0.020	0.020	-	0.17	0.02
Amazonas	0.020	0.020	0.040	-	0.11
Amapá	0.109**	0.062*	0.125**	0.110	-

Table 4. Microsatellite estimates of genetic structure among great egret populations from two major regions in Brazil. Pairwise F_{ST} values based on microsatellites (below diagonal) and corresponding P-values (above diagonal) obtained for colonies within regions and between regions (shaded cells); Asterisks indicate significant F_{ST} values after Bonferroni correction. Sample sizes are shown in parentheses along diagonal. Shaded cells indicate comparisons between colonies from different regions. Colony codes: TC = Tucum, PF = Porto da Fazenda, PR = Praialzinho; BR = Barra do Ribeiro, SM = Santa Maria, SE = Serrinha, MA = Mariante, PG = Pântano Grande

Region		Central West			South				
	Colony	TC	PF	PR	BR	SM	SE	MA	PG
Central West	TC	(10)	0.08	0.02	0.00	0.01	0.01	0.08	0.01
	PF	0.02	(22)	0.02	0.00	0.00	0.00	0.00	0.00
	PR	0.09*	0.03**	(21)	0.00	0.00	0.00	0.00	0.00
South	BR	0.03**	0.03**	0.06**	(37)	0.03	0.03	0.01	0.12
	SM	0.05	0.09**	0.15**	0.03*	(11)	0.59	0.47	0.15
	SE	0.04	0.10**	0.16**	0.03*	-0.01	(11)	0.37	0.17
	MA	0.03	0.10**	0.15**	0.03**	-0.01	-0.01	(9)	0.08
	PG	0.04	0.08**	0.13**	0.02	0.05	0.03	0.03	(7)

genetic differentiation patterns, contributing new information to a cosmopolitan, yet understudied species with high dispersal potential and expanding knowledge on distribution patterns of genetic variation across populations of ardeids. Moreover, this study offers baseline genetic methods for researchers investigating the great egret around the world. The Brazilian populations demonstrate a recent historical past, as suggested by the pattern of shared mitochondrial haplotypes. Both mtDNA and nuclear markers revealed variable patterns of genetic differentiation at different spatial scales.

Genetic diversity, demographic expansion and effective population size

The greater mtDNA diversity among great egret populations in northern Brazil (Table 1) suggests that lower latitudes could have harboured more stable populations in the past. The combination of a high degree of hapotype diversity and low nuclear diversity seen in the populations studied can be interpreted as a sign of population expansion from a small effective population size. Nuclear diversity was similar in both regions submitted to this analysis (central west and south). Levels of nuclear and mitochondrial diversity in the great egret populations are comparable with those reported in the few previous studies on ardeids. For example, mean nuclear diversity in the present study (π = 0.006, N = 200) was similar to that of the near threatened reddish egret, *Egretta rufescens* (π = 0.005, N = 149) (Bates *et al*., 2009), but lower than that found for the vulnerable Chinese egret, *Egretta eulophotes* (N= 90) (Zhou *et al*., 2010). *Ardea alba egretta* exhibited similar H_E at nuclear loci (Table 2) to that found in the species from which these loci were isolated: the great blue heron, *Ardea herodias* (N = 30, mean H_E = 0.61; McGuire and Noor, 2002). Microsatellite allelic diversity and mean H_E in the great egret populations studied here were similar to those found in the reddish egret (seven specific loci, N = 8-37, H_E = 0.41 – 0.59; Hill *et al*., 2012), whereas mean H_E in the great egret populations was higher than that found in the vulnerable Chinese egret (18 specific loci, N = 20, H_E = 0.18–0.82; Huang *et al*., 2010). Heterozygosity levels at microsatellite loci observed among great egrets in Brazil and levels found in other ardeids conform to the general trend reported in a survey of Pelecaniformes and Ciconiiformes (Eo *et al*., 2011).

The populations studied shared mtDNA haplotypes (Figure S1), suggesting a recent common historical past. The absence of the ancestral haplotype in the Amapá population (northern Brazil) could have simply been due to the small sample size in the region. The populations studied probably underwent a process of demographic expansion, as suggested by the star-shaped minimum-spanning network (Figure S1), large negative Fu's *Fs* and Tajima's *D* values and mismatch analyses (Figure 2). The levels of mtDNA and microsatellite diversity (Table 2), together with the trend observed in estimates of time since population expansion (Table 1), suggest that the great egret established and increased in number earlier at lower and medium latitudes in Brazil (northern, central western and south-eastern regions). This pattern is consistent with supposed Pleistocene climate changes, which affected the regions studied differently, particularly with regard to temperature, humidity and water levels (Colinvaux and De Oliveira, 2000; Bush *et al*., 2004). Equatorial environments of South America maintained a more stable climate throughout the LGM (Brown and Ab' Saber, 1979; Ledru *et al*., 1996; Bush *et al*., 2004). Moreover, lakes and wetlands remained available in the Amazon region at the time (Bush *et al*., 2004). As the great egret requires shallow water for foraging and vegetation for nest-building, favourable climate conditions at lower Brazilian latitudes during the LGM are likely to have allowed the establishment of populations. The palaeontological evidence available for South America indicates that several species of waterbirds did not become extinct during the LGM (Cenizo *et al*., 2015). A description of bird fauna during the interstadial MIS3 (37 800 ± 2300 ybp) in the Pampean region of Argentina reports the occurrence of *Plegadis chihi*, a species in the same order as the great egret (Cenizo *et al*., 2015). Evidence also suggests that extinction events were less dramatic in Brazil and Argentina than on the Pacific coast of South America (Cenizo *et al*., 2015). This probably occurred owing to the fact that the

flying skills of waterbirds allow them to shift their range in response to variable climate conditions and wetland availability.

As the species is long-lived and travels long distances (McCrimmon *et al*., 2011), great egret populations seem to be regulated over large temporal and spatial scales. The present widespread distribution in Brazil demonstrates a strong ability to occupy a variety of natural habitats, such as floodplains, savannahs, grasslands and constructed wetlands (McCrimmon *et al*., 2011). However, great egrets require a specific water depth for foraging and suitable vegetation for nest-building. Migratory movements of this waterbird may, therefore, be imposed by local or regional factors (e.g. droughts, floods, cooler temperatures, etc.) expressed over hundreds or even thousands of miles and for several years (McCrimmon *et al*., 2011). Thus, in colder peaks during the LGM, great egrets probably withdrew to a narrow range towards equatorial latitudes in Brazil, where suitable habitats were available. Once the ice melted, birds migrated to the central western, south-eastern and southern regions and established colonies. According to this scenario and as observed in the present study, the intrinsic genetic diversity of equatorial great egret populations would have been maintained, while populations at more southerly latitudes would bear only part of the original variation. The pattern of past expansion found in the great egret populations is similar to that seen in breeding colonies of the wood stork, *Mycteria americana*, settled in the Pantanal wetland (Lopes *et al*., 2007).

Great egret populations from central western and southern Brazil had similar effective population sizes (*Ne c*. 30 birds, Table 2). Considering the mean *Ne*/*Nc* (census size) ratio of 0.23 reported in a review by Palstra and Fraser (2012), the inferred *Nc* would be *c*. 130 great egrets breeding in each region of Brazil. The only available data on census size of great egrets reported 12 715 birds in *c*. 5000 km of the Pantanal wetland (central western Brazil) (Morrison *et al*., 2008). However, this figure does not reflect effective population size, as the survey was performed through direct counts of birds sighted from an aircraft flying over colonies and included non-breeders and juveniles. Therefore, the results from the present genetic study represent the only estimate available on the effective number of breeders that contribute to the gene pool of the species in Brazil. The *Ne* estimated for the great egret based on genetic data is in agreement with the general trend seen in most studies on natural populations of animals: *Ne* is considerably smaller than *Nc* (Palstra and Fraser, 2012). The rather low *Ne* observed in the great egret may reflect the non-monogamous genetic mating system with extra-pair paternity exhibited by this species (Miño *et al*., 2011), which would reduce *Ne* as a consequence of increasing the variance in reproductive success. In the present investigation, *Ne* was estimated separately for each region after the evaluation of genetic structure. This is important, because, despite the high potential for dispersal, great egrets breeding in Brazil form a subdivided population (see below). If the sample had been analysed as if it were representative of a panmictic population, *Ne* could have been underestimated (Ryman *et al*., 2014). Thus, as in many other studies, the present findings underscore the need to assess structuring before conducting the genetic monitoring of population size in other widespread vagile species. However, it should be stressed that *Ne* is a complex parameter that is concurrently influenced by behavioural and demographic traits, such as the genetic mating system, sex ratio, dispersal patterns, social structure and generation time. For a better investigation of effective population sizes of the great egret in Brazil, future studies should apply other genetic methods with different assumptions and based on different sampling schemes. Novel radio tracking techniques may also help clarify the movements of individuals of this species in the country.

Genetic structuring among populations in two major regions

The mitochondrial DNA data demonstrated that the great egret population in northern Brazil differs significantly from populations in the southern, central western and south-eastern regions of the country (Table 3). Microsatellite-based hierarchical AMOVA, F_{ST} (Table 4) and Bayesian clustering analyses (Table S5) also revealed significant differentiation between the central western and southern populations. Likewise, microsatellite-

based assignment tests revealed a 51% increase in correctly assigned individuals when regions rather than colonies were used as the source populations (Table S4). Collectively, these findings indicate that breeding colonies in different regions harbour dissimilar haplotypic and allelic constitutions and the overall population is not completely panmictic. Notably, nuclear loci revealed contrasting patterns of genetic structuring on different geographical scales. Great egret colonies harbour similar allele compositions within regions. However, the colonies exhibited low, but significant differentiation between regions. A study on the roseate spoonbill, *Platalea ajaja* (Miño and Del Lama, 2014), which breeds in sympatry with the great egret, also reported significant differentiation between breeding colonies in northern Brazil compared with those in the central western and southern regions. Mixed structuring patterns on different spatial scales have also been reported for other waterbirds (Bicknell *et al*., 2012; Hill *et al*., 2012), including ardeids. In the reddish egret, for example, microsatellites revealed dramatic genetic differentiation among colonies from three different regions, but a lack of structuring among colonies within regions (Hill *et al*., 2012). To better depict structuring patterns among great egret colonies at different spatial scales, future studies should sample other breeding populations throughout the entire distribution range of this subspecies in the Americas.

While mtDNA demonstrated no significant differentiation between the central western and southern populations, microsatellites showed structuring between these regions. This would appear somewhat surprising, given that nuclear loci normally take longer to reveal differentiation than mitochondrial DNA. Theory predicts that, at equilibrium, microsatellites generally constitute a less sensitive indicator of population structure than mtDNA (Zink and Barrowclough, 2008). However, although mtDNA Φ_{ST} values are higher than microsatellite F_{ST} values under most conditions, this is not always true (Karl *et al*., 2012). For example, a differentiation pattern similar to the one demonstrated by the great egret has been reported for bats, as populations with shared mtDNA haplotypes fell into different microsatellite clusters (Flanders *et al*., 2009). This was interpreted as a reflection of a common ancestry among bat populations pre-dating the LGM, followed by retreat of bats with the same haplotype to separate refugia before subsequent postglacial expansion (Flanders *et al*., 2009). Thus, microsatellite differentiation may reveal what occurred during the time that pre-dated the secondary expansion (Flanders *et al*., 2009). A study on the hairy woodpecker (*Picoides villosus*) also found a single mtDNA group divided into different microsatellite clusters, which was explained by assuming reduced gene flow within the last few generations after colonization (Graham and Burg, 2012). Likewise, the shared mtDNA haplotypes found in great egret populations may be indicative of more ancient events occurring before the LGM, whereas the significant microsatellite differentiation may reflect subsequent expansion to newly colonized areas. An alternative explanation for the pattern observed in the present study would be inappropriate sampling (Zink and Barrowclough, 2008). However, this does not appear to be the case, as the sample sizes for the regions that demonstrated structuring can be considered adequate (central west: N = 56; south: N = 75).

The significant genetic structuring found in the great egret populations could reflect the limited dispersal of individuals between regions. This species breeds in patchily distributed ephemeral habitats, which are irregularly occupied over the years, depending on favourable environmental conditions that ensure enough food availability (McCrimmon *et al*., 2011). The settlement of colonies in the Pantanal wetland (central western Brazil) is influenced by a number of concurrent environmental changes and/or anthropogenic factors (Cardoso, 2011), which vary annually. Likewise, the formation of breeding colonies in the southern region (state of Rio Grande do Sul) is affected by human disturbance derived from considerable habitat conversion for agricultural use, mainly rice production, which significantly alters the hydrological dynamics and vegetation structure of natural wetlands (Overbeck *et al*., 2007; Scherer *et al*., 2011). Presumably, the great egret developed its foraging strategies over many years of evolution in natural wetlands (Herring and Gawlik, 2013). When human disturbance affects wetlands, waterbirds face greater difficulties encountering new

foraging resources (Herring and Gawlik, 2013) which, in turn, may affect their breeding possibilities. The differential settlement of breeding colonies in Brazilian wetlands can lead to extinction or relocation depending on the availability of suitable sites, thereby limiting the dispersal of individuals and exerting an influence on the genetic structure of populations on different spatial and temporal scales (Pierson *et al*., 2013). The present results indicate that the allelic constitution of the northernmost population of the great egret (state of Amapá) differs significantly from that of the other populations. It is likely that the northern population occasionally receives immigrants from populations located in North or Central America that do not reach more southerly latitudes. Band recovery data suggest that North American herons winter in Central and South America (Mikuska *et al*., 1998), specifically in Colombia (Hilty and Brown, 1986). It remains to be investigated whether great egrets from North America also reach the Amapá wetlands in Brazil.

Implications for conservation

Brazil has 2 million km^2 of wetlands (Guadagnin *et al*., 2005; Junk *et al*., 2014) that provide breeding, foraging or stopover grounds for many migratory and resident birds (Antas, 1994; Lunardi *et al*., 2012). Despite their importance, only 12 sites in Brazil (72 256 km^2) are currently protected under the Ramsar convention (international treaty on wetland conservation; http://www.ramsar.org/wetland/brazil), while most are neither preserved nor protected (Junk *et al*., 2014). The present study assessed levels and distribution patterns of genetic variation among great egret populations – previously unexplored aspects of this species (Junk *et al*., 2014) – inhabiting floodplains that undergo the many threats that affect most wetlands in Brazil. As the hydrologic regimens in such wetlands are likely to be severely affected in the short term, waterbird populations would have to respond to such changes. The genetic differentiation pattern (mtDNA and microsatellites) found between great egret populations in the state of Amapá (northern region) and those established in both the Pantanal wetland (central western region) and the state of Rio Grande do Sul (southern region) suggests limited connectivity between these wetlands. Therefore, breeding colonies settled on different floodplains should be preserved to maintain the entire gene pool of this cosmopolitan top predator in Brazil. The colonies established in the Amapá coastal wetlands harbour allelic constitutions that differ from all the other populations studied and therefore merit particular attention. The data provided herein serve as a basis for detecting future changes in gene flow patterns or reductions in population size that can compromise the survival of this species because of the increasing threats to their breeding and foraging habitats. Thus, the present findings can assist in establishing conservation measures for the Pantanal wetland and coastal regions in the state of Amapá. These findings may also guide the monitoring of southern great egret populations, some of which occupy man-made reservoirs and rice fields and are therefore subjected to stronger pressure from agricultural activities.

Environmental law in Brazil only protects breeding colonies of birds (Law no. 9605, art. 29, 12 February 1998). However, conservation measures focused only on breeding sites may be unsuccessful. For the effective protection of great egret populations it is also necessary to preserve feeding grounds and non-breeding areas (Kushlan and Hancock, 2005). Thus, long-term genetic monitoring of great egret populations should be carried out in parallel with the management of hydrology, minimization of human disturbance, pollution control and the maintenance of vegetation diversity in the areas surrounding colonies to preserve suitable foraging and breeding grounds. Genetic studies could offer information on the effectiveness of such actions in maintaining the continuing, albeit limited, genetic exchange among populations that inhabit Brazilian wetlands.

Waterbird species use various techniques for foraging and the selection of feeding patches, which make these birds susceptible to anthropogenic changes to their habitats (Gimenes and Anjos, 2007; Herring and Gawlik, 2013). Thus, gathering genetic information on different species belonging to the same group, but with particular trophic requirements can help in the creation of coherent wetland conservation policies. The concordant

genetic pattern observed in populations of two waterbird species that breed together in Brazil – the present investigation involving the great egret and a study on the roseate spoonbill (Miño and Del Lama, 2014) – indicates that northern Brazil is a key area and its conservation should be a high priority, yet this region is not listed as an important area for herons in South America (Morales, 2000). Nevertheless, Brazil has recently declared its 12th Ramsar site (Cabo Orange National Park), illustrating the importance of coastal wetlands in the state of Amapá for the conservation of biodiversity (http://www.ramsar.org/wetland/brazil). Findings from the present study and previous investigations provide a solid argument for supporting the establishment of public policies aimed at preserving Brazilian wetlands, with special attention given to the northern region.

This study provides the first genetic estimate of effective size of great egret populations in major Brazilian wetlands. According to the Ramsar Convention, wetlands should be declared significant conservation areas if they harbour at least 1% of individuals of waterbird populations (Ramsar, 2015). The methodology applied here can help guide future genetic monitoring of great egret populations to detect changes in *Ne* over time. Moreover, these methods can be used to assess *Ne* in other populations and estimate how much genetic variation in species is found in wetlands. Such an approach could assist in the definition of new conservation sites in Brazil, which would allow the country to comply with the commitments stipulated by the Ramsar Convention. Policy makers and resource managers could benefit from the present results when designing management strategies for wetlands. In addition, this study can be included in future comparative research aimed at evaluating how different but sympatric waterbird species respond to environmental changes.

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