Microsatellite variability of the wood stork *Mycteria americana* (Aves, Ciconiidae) in Cuba: implications for its conservation

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Abstract

Microsatellite variability of the wood stork Mycteria americana *(Aves, Ciconiidae) in Cuba: implications for its conservation. Mycteria americana* (Aves, Ciconiidae) is the only species of stork found in the Caribbean. It is a permanent yet rare resident in Cuba, with only two reproductively active colonies. In this work, we used five microsatellite loci to characterize 37 individuals from these colonies, located in two of the most important wetlands of Cuba, the Zapata Swamp and the Sabana–Camagüey Archipelago. We found low genetic variability, with similar values to those reported for North and South American populations of the species, and little but significant genetic differentiation between colonies. Our results highlight the need to improve the management and conservation planning of the species in Cuba because the combination of low genetic variation, small colonies, anthropogenic influence and climatic factors could threaten its persistence.

Key words: Genetic diversity, Genetic structure, Waterbirds, Wetlands

Resumen

Variabilidad de los microsatélites de la cigüeña americana Mycteria americana *(Aves, Ciconiidae) en Cuba: implicaciones para su conservación. Mycteria americana* (Aves, Ciconiidae) es la única especie de cigüeña distribuida en el Caribe. En Cuba se considera residente permanente, pero rara, y solo se conocen dos colonias reproductivamente activas. En este trabajo se emplearon cinco loci de microsatélites para caracterizar genéticamente a 37 individuos de esas colonias, localizadas en dos de los humedales más importantes de Cuba: la ciénaga de Zapata y el archipiélago Sabana–Camagüey. Se observó una baja variabilidad en los índices de variabilidad genética, cuyos valores fueron similares a los referidos para las poblaciones de la especie en Norteamérica y Suramérica, y poca diferenciación genética entre las colonias que, sin embargo, era significativa. Nuestros resultados destacan la necesidad de mejorar la planificación del manejo y la conservación de la especie en Cuba debido a que la combinación de la baja variación genética, el pequeño tamaño de las colonias, la influencia humana y los factores climáticos podrían amenazar su persistencia.

Palabras claves: Diversidad genética, Estructura genética, Aves acúaticas, Humedales

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Introduction

Wetland birds face increasing threats due to factors such as the destruction and fragmentation of their habitats. Wetlands are among the most threatened ecosystems in the world due to accelerated climate change, the anthropogenic impact caused by expanding coastal development, agricultural land conversion, and urbanization (Junk et al., 2013). Waterbirds occupying islands could be especially susceptible due to their relative isolation, comparatively small population size, and the effects of climate change such as sea level rise.

Mycteria americana Linnaeus, 1758 (Aves, Ciconiidae), the wood stork, is one of the most conspicuous and rare aquatic species found in the Cuban archipelago, being the only stork species present in the Caribbean (Raffaele at al., 2010). It is widely distributed from the southeastern of the United States to the north of Argentina (Coulter et al., 1999). In the Caribbean islands, it resides permanently only in Cuba and the Dominican Republic (Latta et al., 2010; Garrido and Kirckonnell, 2010), while in Jamaica, Bahamas and Dominica it is classified as a casual visitor (Latta et al., 2010). In Cuba, its distribution includes the southern coast of Pinar del Río, the Zapata Swamp, the Sabana–Camagüey Archipelago and the Birama Swamp according to Garrido and Kirkconnell (2010). However, only two nesting sites have been identified and described to date. Historically, these sites have low numbers of breeding pairs, always fewer than 50 pairs, and despite their legal protection, the number of colonies and nests has progressively decreased. In 2015, for example, the largest colony in the Sabana–Camagüey Archipelago contained only 26 nests, while there were only 8 nests at Las Salinas in the Zapata Swamp (Llanes–Quevedo et al., 2015).

The species does not exhibit migratory habits and has a tendency to use the same reproductive sites over the years (Frederick and Ogden, 1997). In general, movements of individuals or colonies seem to be a response to the fluctuation of environmental conditions and successive failed attempts at reproduction at the sites (Coulter et al., 1999). Several studies have reported the dispersal movements of the species, but these are restricted to specific areas of occurrence, such as the United States (Hylton, 2004; Borkhataria, 2009), Argentina and Brazil (Antas, 1994; Del Lama et al., 2015). The entire range of the species has not been studied to date. However, the movements of wood storks are supposedly limited in the Carribean by the absence of thermals that birds require to fly across long distances (Coulter et al., 1999).

The wood stork is considered as Least Concern according to IUCN (BirdLife International, 2016). Nevertheless, throughout its distribution, it has been affected by the loss and alteration of foraging and reproductive sites. Habitat loss and degradation due to anthropogenic activities such as occupation of areas, alteration of drainage for agriculture, and tourism have already led to declines in population numbers of this species in various regions. For example, in the United States, populations of over 100,000 individuals were reduced to about 3,000 between the 1960s and 1970s, (Coulter et al., 1999), and as a result, the species was classified as Endangered. In the Caribbean, wood stork populations have been classified as locally endangered and even as extirpated in the Dominican Republic (Raffaele at al., 2010; Latta et al., 2010), while in Cuba, although it is considered a rare species that occupies vulnerable habitats, it does not present any category of threat. Nevertheless, *M. americana* colonies in Cuba face considerable and increasing threats, such as local hunting and tourism development. These elements, together with the reduced census size of the reproductive colonies (Llanes–Quevedo et al., 2015), the regular occurrence of severe hydro–meteorological phenomena, and the lack of studies enabling development of adequate management measures for the species, may jeopardize the future of *M. americana* in the country. Hence, the goal of the present study was to examine levels of genetic diversity, inbreeding and population differentiation in breeding colonies of *M. americana* in Cuba, using microsatellite markers. Knowing the genetic characteristics of the species in Cuba would provide valuable information to design and improve management plans for its conservation.

Material and methods

Sample collection

Sampling was conducted in *M. americana* breeding colonies of in Cayo Romano, Camagüey province, and Las Salinas in the Zapata Swamp, Matanzas province in the reproductive season 2015–2016 (fig. 1). These breeding sites, included in areas classified as Wildlife Refuges, are located in two of the most important wetlands in Cuba: the Zapata Swamp and Sabana–Camagüey Archipelago (Denis, 2006). In general, the nesting areas are similar in terms of vegetation, soil types, temperature and average rainfall; they are separated by approximately 400 km (Llanes–Quevedo et al., 2015).

Samples consisted of feathers taken from chicks, one or two per nest: from 25 chicks from 17 nests in Cayo Romano colony, and from 12 chicks from 8 nests in Las Salinas colony. Chicks were captured and handled for a short time. Capture and sampling was performed following the Ornithological Council Guidelines for Ethical Animal Experimentation (Fair et al., 2010) and approved by the Cuban authorities from the Center for Environment Inspection and Control (CICA permits of access and collection in protected natural areas n° 2015/21 and 2015/87). The feathers were plucked from the dorsum and the belly. The injuries produced were treated with alcohol to prevent infection once the sample was obtained and the chicks were released at the capture site. Feathers were deposited in a 2–mL Eppendorf tube containing 90 % ethanol.

DNA extraction and amplification of molecular markers

Total genomic DNA was extracted from 10 mg of plucked feathers with a Nucleo Spin Tissue Kit (Ma-

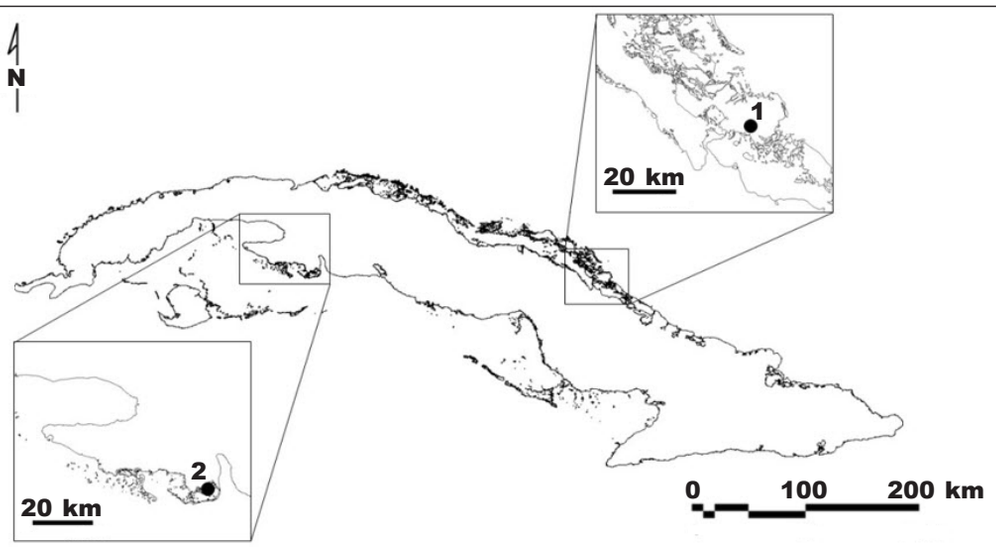

Fig. 1. Map of the Cuban Archipelago showing collection sites of *Mycteria americana* in the reproductive season 2015–2016: 1, Cayo Romano breeding colony, Cayo Romano, Camagüey (n = 25); and 2, Zapata Swamp breeding colony, Zapata Swamp, Matanzas (n = 12).

Fig. 1. Mapa del archipiélago cubano en el que se muestran los sitios de recolección de Mycteria americana *en la temporada reproductiva de 2015–2016: 1, colonia de reproducción Cayo Romano, en cayo Romano, Camagüey (n = 25); y 2, colonia de reproducción Zapata Swamp, en la ciénaga de Zapata, Matanzas (n = 12).*

cherey–Nagel, Dün, Germany). DNA was diluted in 50 µl of sterile water, and the quality and amount of DNA were estimated by electrophoresis (agarose 1 %). The five most polymorphic microsatellite loci described by Tomasulo–Seccomandi et al. (2003) were used to genotype samples: WSµ03, WSµ08, WSµ09, WSµ14 and WSµ20.

PCRs were performed in 15 µl of mix containing 1x buffer (2 mM MgCl2), 10 pM of each primer, 0.5 mM of each dNTP, one unit of Taq polymerase, and 50–150 ng of template DNA. PCR consisted of the following steps: 1) initial denaturing, 5 min at 94 °C; 2) 30 cycles of 35 sec of denaturing at 94 °C, 60 sec of annealing at the appropriate temperature (50–60 °C) and 1 min of extension at 72 °C; and 3) 10 min of extension at 72 °C.

DNA fragments were separated by vertical polyacrylamide gel electrophoresis for 2 hr at 65 W using a Sequi–Gen GT sequencing cell. Genotypes were determined visually and to minimize error in alelle calling, at least two people performed the readings. In addition, to control for genotyping error, we re–amplified all microsatellite loci for 20 % of the samples. Allele sizes were estimated by comparison with the pGem molecular weight markers and GenePrint CTT Multiplex and GenePrint FFV Multiplex Systems (Promega, 2006).

Genetic diversity

We analysed genotypes for null allele with Micro–Checker 2.2.3 (Van Oosterhout et al., 2004). We considered polymorphic all loci with at least two alleles, when the frequency of the most common allele did not exceed 95 % (Graur and Li, 2002). We calculated the standard measures of genetic variation for microsatellite loci: number of alleles per locus, number of private alleles, effective number of alleles, observed heterozygosity, expected heterozygosity, and inbreeding coefficients with Genalex v6.5 (Peakall and Smouse, 2012) and Arlequin v3.5 (Excoffier and Lischer, 2010) for each colony. Allelic richness was valued in FSTAT v2.9.3.2 (Goudet, 2001). The effective population size was estimated based on the sibship assignment method described by Wang (2009) and implemented in Colony v2.0 (Jones and Wang, 2010).

We performed the linkage disequilibrium and Hardy–Weinberg equilibrium analyses with GenePop v4.1.0 (Rousset, 2008), according to the Markov chain method with 1,000 dememorizations, 1,000 batches and 10,000 iterations. Markers were tested for neutrality using the 'detsel' R package v1.0.2 (Vitalis, 2012).

Genetic structure

To assess the level of genetic differentiation between colonies, we computed the F_{ST} index of Weir and Cockerham (1984). The variance components in allele frequencies between colonies and individuals were calculated using a hierarchical AMOVA implemented in Arlequin v3.5 (Excoffier and Lischer, 2010).

Genetic structure was was further tested using Bayesian clustering of the microsatellites data in Structure v.2.3.4 (Pritchard et al., 2000). The running parameters were no *a priori* information on sampling location, an admixture model, and correlated allele frequencies, λ of one. Run length was set to 1,000,000 MCMC (Markov chain Monte Carlo) replicates after a burn–in period of 500,000 and the number of clusters (*K*) from 1 to 4, with 20 replicates for each value of *K*. To select the most probable *K* number, we examined the obtained likelihood values for each run and the variance between these values for each K value. We selected the value with the best posterior probability and the smallest variance between repetitions (Pritchard et al., 2000) as that which best represented the number of population clusters present in the sample.

Results

Genetic diversity

The genetic characterization of breeding colonies of *M. americana* in Cuba showed that the five autosomal microsatellite loci were polymorphic but with a low number of alleles (between 2 and 3). The total number of alleles was 13 with an average of 2.60 alleles per locus. The genotypic proportions of all loci evaluated were in agreement with expectations under Hardy Weinberg equilibrium, except for WS08 in the Cayo Romano colony. No evidence of non–random association was found between segregating alleles at any loci–pair (linkage disequilibrium); there was no evidence of null alleles or deviation from neutrality in any of the studied samples.

The mean number of effective alleles (Ne) was 2.11, with locus WS03 contributing the most (at 2.75) and WS20 the least (1.65). The percentage of Ne per locus was greater than 50 % at all loci. The average value of allelic richness (Rs) was 2.49; WS03 was the most diverse locus with 3 alleles, while WS09 and WS20 presented the lowest value with 2 alleles. Heterozygosity (Ho) was less than 0.5 in all loci, with a minimum value of 0.25 for locus WS20 and a maximum of 0.48 for WS03.The WS03 locus showed the highest expected heterozygosity (He = 0.63) and WS20 showed the lowest (He = 0.38). No rare alleles were found (frequency ≤ 5 %) and only one private allele was detected for the WS14 locus in the Cayo Romano colony. Cayo Romano also showed slightly higher genetic variability indexes, but this difference was not statistically significant (table 1). The average inbreeding coefficient was 0.28, with individual inbreeding coefficients ranging from 0.26 (Cayo Romano colony) to 0.31 (Zapata Swamp colony). Effective population size was 16 (95 % confidence interval, CI: 8 to 34) for Cayo Romano and 9 (95 % CI: 4 to 26) for Zapata Swamp.

Genetic structure

The F_{ST} statistic between the two colonies was 0.06 (p = 0.03). From the analysis of molecular variance (AMOVA) we determined that 93.1 % of the variation was explained by differences between individuals within the localities, while the remaining 6.98 % was explained by differences between localities. On the other hand, the Bayesian clustering analysis showed that, for K = 2, all individuals had equal probability (50 %) of belonging to any group (fig. 2A). A single population cluster seems to best explain the variation in allele frequencies observed in the wood storks in Cuba, as K = 1 had the highest logarithm of the likelihood (close to zero) and also the lower variance (fig. 2B).

Discussion

Genetic diversity

In the present study, we used five autosomal microsatellite loci for genetic characterization of the two breeding colonies of *M. americana* in Cuba. Both colonies showed all polymorphic loci, but the number of alleles was low, coinciding with that reported for two populations, one from Fazenda Ipiranga, Mato Grosso, Brazil and one from Tamiami West, Everglades, Florida, USA (Tomasulo–Seccomandi et al., 2003), and also with later studies comprising several colonies from Pantanal (Lopes et al., 2007) and Amapa region (Miño et al., 2011) in Brazil.

As already proposed by other authors, the low polymorphism found in the present study seems to be intrinsic to the species (Lopes et al., 2006; Miño et al., 2017). Low genetic diversity of *M. americana* has also been detected through mtDNA sequences (Lopes et al., 2006, 2007), other microsatellite loci (Van Den Bussche et al., 1999), and alloenzyme analyses (Strangel et al., 1990). According to Eo (2011), effective population sizes and ecological and environmental features of aquatic birds produce (compared with other types of birds) low rates of molecular evolution.

Although it is not a universal pattern, genetic diversity is commonly reduced in peripheral populations (Volis et al., 2016). These populations differ from central populations mainly because of genetic drift. As a consequence of the random sampling in reproduction, there is usually a loss, by chance, of less frequent alleles and a fixation of the most frequent alleles, thus reducing the genetic diversity of the population. The two Cuban colonies could be considered peripheral because they are located at one of the extremes of the species distribution and their number of breeding pairs is low. However, they present similar and even higher genetic diversity indexes than those of the North American and Brazilian populations; that is, the discrete genetic variation found for *M. americana* in Cuba is not significantly lower than that reported in continental populations. In this sense, our results suggest that Cuban colonies may remain connected, to some extent, with those of North or South America through the exchange of individuals and genes, although this theory should be complemented by future studies using banding and mitochondrial markers.

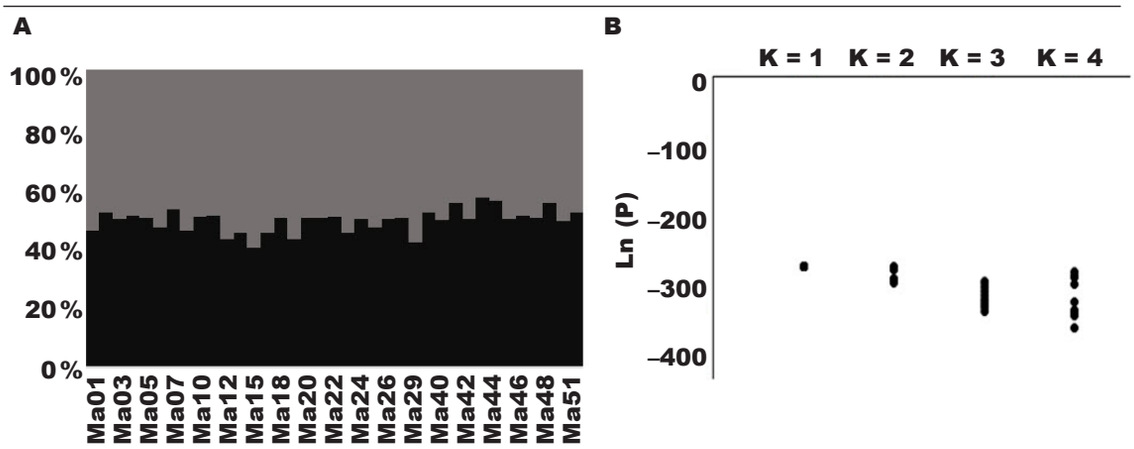

Fig. 2. A, Probability of assignment of *Mycteria americana* individuals to each colony estimated by Structure v.2.3, based on allele frequencies. Vertical bars represent the individuals and are divided into segments corresponding to the probability of assignment to Cayo Romano (gray) or Zapata Swamp (black). B, log of the likelihood value versus most probable number of populations (K) obtained with Structure

Fig. 2. A, Probabilidad de asignación de individuos de Mycteria americana *a cada colonia, estimada por Structure v.2.3 a partir de frecuencias alélicas. Las barras representan a los individuos y se dividen en dos segmentos que corresponden a la probabilidad de asignación a Cayo Romano (gris) y a Zapata Swamp (negro). B, logaritmo del valor de la probabilidad con respecto de el número más probable de poblaciones (K) obtenido con Structure.*

The colonies analyzed in the present work showed low estimates of effective population size and positive and high values of the inbreeding coefficient. These elements constitute risks for their survival in the future because they indicate that the number of reproductive individuals contributing demographically and genetically to the next generation is small (Hedrick, 2000). The reduced effective polpulation size of *M. americana* colonies was similar to that obtained by Miño et al. (2017) for the Pantanal and Amapá regions in Brazil; these authors found lower and, in some cases, negative *Fis* values.

In the present work, we found that the differences between colonies in the genetic diversity indexes were minimal. Nevertheless, all indices were slightly higher in the Cayo Romano colony (table 1). Regarding the higher number of alleles, this could be due to the larger sample size taken in this colony (N = 25). For the other indexes, the higher indices could be associated with the genetic health status of this larger nesting colony in Cuba (Cayo Romano) being better than that of the colony in the Zapata Swamp, which, according to nest counts from 1987 to the present, is declining (Llanes et al., 2015). Our findings could support the urgent management and protection of the species in Cuba where, the Zapata Swamp colony is likely to be impacted in the near future by habitat loss, saline inclusion and coastal erosion (Moya et al., 2005). In contrast, the nesting area of *M. americana* in Cayo Romano is currently classified as medium–low risk (Menéndez Carrera et al., 2015).

Genetic structure

The value of F_{ST} obtained in the present study (F_{ST} = 0.06; p = 0.03) suggests genetic differentiation between the two colonies studied is low, a finding that does not agree with the results obtained by Structure. The significant genetic structuring between colonies of *M. americana* was only recently documented, by Miño et al. (2017), in their comparison of colonies of Pantanal and Amapá. In the remaining studies where this statistic has been used, no evidence of genetic structuring has been found between wood stork colonies in the Pantanal region (Del Lama et al., 2002; Lopes et al., 2004, 2006), in the Everglades (Stangel et al., 1990; Van Den Bussche et al., 1999), or in North and South America. However, the F_{ST} values reported in these studies were calculated for allozymes and microsatellites (described by Van Den Bussche, 1999) that are less variable than those used in the present study.

The Cuban breeding colonies we studied are separated by approximately 400 km, but this does not seem to be an important limitation for individual interchange due to the species' great flight capacity. Studies in Florida have reported that wood storks can travel more than 100 km per day in their dispersal movements between winter and summer residence sites (Hylton, 2004). This incipient genetic differentiation thus suggests some degree of site fidelity, a behavior reported in the literature based on ecological (Frederick and Odgen, 1997) and genetic

Table 1. Estimates of genetic diversity of *Mycteria americana* breeding colonies in Cuba based on five microsatellites loci: Na, number of alleles; Ne, number of effective alleles; Ho, observed heterozygosities; He, expected heterozygosities; and Rs, allelic richness.

Tabla 1. Estimaciones de la diversidad genética de las colonias de reproducción de Mycteria americana *en Cuba a partir de cinco loci de microsatélites: Na, número de alelos; Ne, número de alelos efectivos, Ho, heterocigosis observada; He, heterocigosis esperada; Rs, riqueza alélica.*

	Pop	WSμ03	WSμ08	WSμ09	WSμ14	WSμ20	Mean
Na	Cayo Romano	3.00	3.00	2.00	3.00	2.00	2.60
	Zapata Swamp	3.00	3.00	2.00	2.00	2.00	2.40
Ne	Cayo Romano	2.98	2.21	1.70	2.85	1.75	2.30
	Zapata Swamp	2.53	1.72	2.00	1.86	1.55	1.93
Ho	Cayo Romano	0.46	0.40	0.34	0.49	0.30	0.40
	Zapata Swamp	0.50	0.36	0.20	0.36	0.20	0.33
He	Cayo Romano	0.66	0.55	0.41	0.64	0.42	0.54
	Zapata Swamp	0.61	0.42	0.50	0.46	0.33	0.46
RS	Cayo Romano	3.00	2.96	2.00	2.99	2.00	2.59
	Zapata Swamp	3.00	2.90	2.00	2.00	2.00	2.38

evidence (Miño et al., 2017), although this was not recognized in previous works with molecular markers (Del Lama et al., 2002, Lopes et al., 2004, 2006).

Implications for conservation

The markers used in the present work are neutral and do not therefore allow direct evaluation of the diversity of genes that confer adaptation to current environmental conditions. Nevertheless, they provide data to make inferences regarding important variations that should be maintained as a source for natural selection to act upon when responding to future environmental changes. Although there is wide debate on the subject (Reed and Frankham, 2001; Volis et al., 2016), numerous studies have reported a correlation between the diversity of neutral markers and that of the loci subjected to selection (e.g. Vandewoestijne et al., 2008; Da Silva, 2006). Low genetic diversity associated with a loss of adaptive potential has been identified as one of the most important risks for species persistence and its importance goes beyond its effects on population dynamics, to affect the structure of communities and ecosystem processes (Hughes et al., 2008). The low genetic variability found for *M. americana* in Cuba coincides with that reported in the literature for the North and South American populations of the species. However, Cuban colonies are smaller, creating an extinction risk (Gilpin and Soule, 1986) because of the increased potential for inbreeding (Blomqvist et al., 2010). This situation is further aggravated by the negative influence of anthropogenic activities on reproductive colonies and the use of chicks as food by local people. All these factors could cause the Cuban colonies to enter a vortex of extinction, that is, create negative dynamics that would increase the probability of extinction due to stochastic or catastrophic demographic events (Gilpin and Soulé, 1986).

At this point, we emphazise the need to study fitness variables, given that the variation in quantitative traits is a better indicator of their evolutionary potential than neutral genetic variation (Navarro et al., 2005, Volis et al., 2016). These studies would be useful to assess the real adaptive potential of these colonies and allow more accurate decisions regarding conservation priorities.

Besides the conservation importance of the Cuban colonies of the wood stork in view of their current risky demographic conditions, they could theoretically be of great importance from an evolutionary standpoint. Peripheral and central ones populations tend to differ because of the combined effect of genetic drift and natural selection, and the less predictable effects of the latter on genetic variation. Marginal populations, i.e., those distributed at the extremes of the range of occurrence of species, often occupy lower quality habitats and undergo different selection pressures, which may lead to the emergence of important new allele variants or may constitute a source potential of speciation episodes (Lesica and Allendorf, 1995).

Our findings highlight the importance of planning and implementing measures to ensure effective and lasting protection of the species in Cuba. An essential part of these plans should be the conservation of the habitats that the species occupies, particularly the

wetlands of northern Camagüey, which harbor the most current genetic diversity of the species in Cuba. To effectively conserve this area it is necessary to implement measures that allow the restoration of its ecosystems, mainly affected by the increased building development due to the expansion of human settlement and tourism. Mangroves and seagrasses in this area are already affected by increased turbidity, sediment suspension and hypersalination caused by the obstruction of channels with structures built in or between the cays (Iturralde–Vinent and Serrano, 2015). Special emphasis should also be given to the need for environmental education in order to prevent wood stork consumption by local people. Excessive hunting has already decimated the colonies and presumably led to the extinction of the species in Hispaniola (Latta et al., 2010). Although potentially more complex, ex–situ management of wood storks could also be considered in an attempt to alleviate the effect of human impact, at least while chicks are in the nest.

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