

# Genetic variation of the endangered Araripe Manakin (*Antilophia bokermanni*) indicates a history of demographic decline

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**ABSTRACT:** The Araripe Manakin (*Antilophia bokermanni*) is a “Critically Endangered” bird species endemic to northeastern Brazil. The habitat of the species has suffered intense fragmentation and degradation in recent years, resulting in a decline in population numbers. The present study evaluated the genetic diversity and structure of this population through the analysis of the Hypervariable Domain I of the mitochondrial Control Region and two nuclear introns (I7BF and G3PDH). Results revealed an absence of population substructuring and population decline beginning during the late Pleistocene, approximately 50,000 years ago. The evidence indicates that the effective population size of the Araripe Manakin has declined gradually over time ever since, a process that may have been intensified as a result of the recent anthropogenic impacts on the habitat of the species.

**KEY-WORDS:** coalescent theory, conservation, control region, Pipridae, population genetics.

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## INTRODUCTION

The Araripe Manakin (*Antilophia bokermanni*) is the world's most threatened species of manakin, according to the International Union for the Conservation of Nature (BirdLife International 2016). This species is endemic to a small tract of humid forest on the slopes of the Araripe Plateau in Ceará state in northeastern Brazil. The principal threats to the survival of the species are the ongoing anthropogenic degradation and loss of habitat, which is thought to contain less than one thousand individuals (Silva *et al.* 2011, BirdLife International 2016).

The risks faced by *A. bokermanni* highlight the need for the collection of reliable data for the effective management and conservation of the remaining populations, including insights into the evolutionary history of the species. Silva *et al.* (2011) estimated that the numbers of individuals have declined by more than a third over the past twenty years. This may have intensified the process of genetic drift, further reducing the genetic variability of its populations, while also increasing the probability of inbreeding, which favors the appearance of genetic anomalies and diseases,

and may lead to a loss of fitness (Frankham 2005). A recent study of the Araripe Manakin revealed slightly lower genetic diversity in comparison with its sister species, *A. galeata* (Rêgo *et al.* 2010).

Historical evolutionary processes are generally underestimated during the development of conservation policies for threatened species (Chaves *et al.* 2011), despite the importance of understanding the demographic history of threatened populations, such as those of *A. bokermanni*, for the development of reliable conservation programs (Cornetti *et al.* 2014). In this context, genetic data can support a better understanding of demographic processes that contributed to the current genetic diversity of the species (Chaves *et al.* 2011).

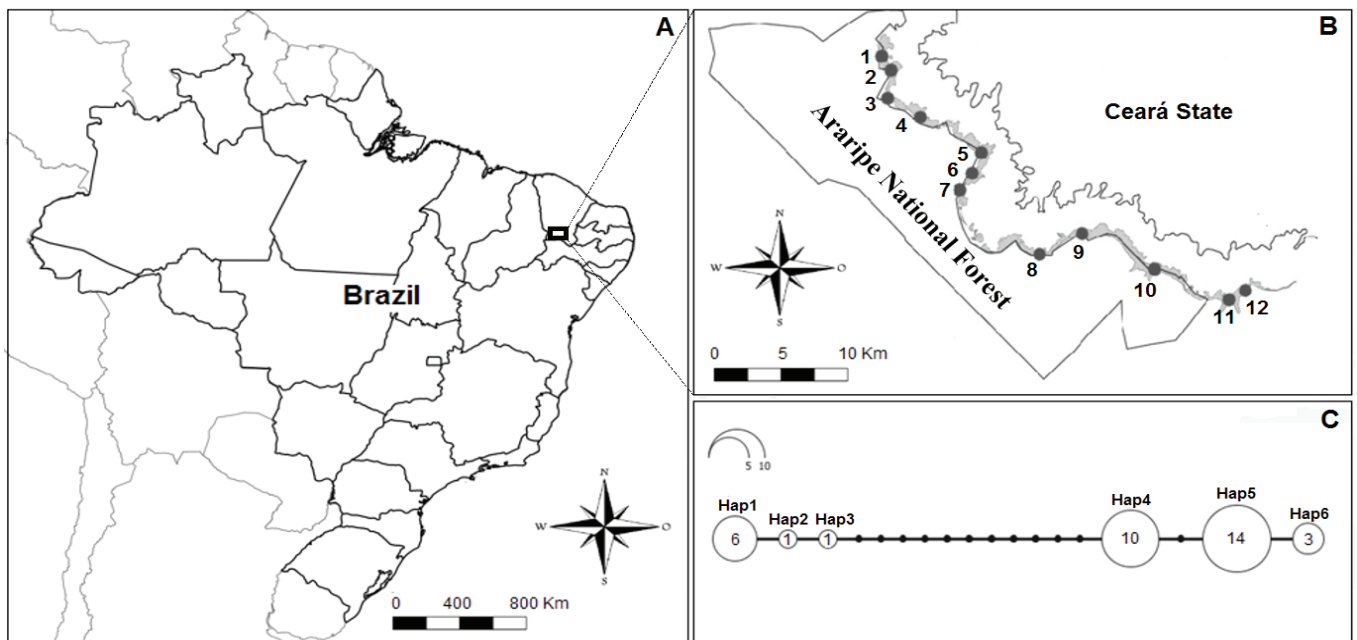
In the present study, the genetic diversity of *A. bokermanni* populations was analyzed based on sequences of the mitochondrial control region and two nuclear introns. Based on analyses of coalescent theory, the data were used to detect possible signs of historical demographic variation, and describe the spatial distribution of the genetic variation of the present-day population of the Araripe Manakin.

## METHODS

### Population sampling and laboratory procedures

Samples of blood or feathers were obtained from 37 Araripe Manakins obtained from 12 sites distributed throughout the geographic range of the species (Fig. 1A, B). Samples were stored in 95% ethanol at -20°C. Total genomic DNA was isolated using a Wizard® Genomic DNA Purification kit (Promega) according to the manufacturer's instructions. Polymerase Chain Reaction (PCR) was used to amplify Hypervariable Domain I (HDI) of the mitochondrial Control Region, which is widely used for the evaluation of diversity in studies of population genetics (*e.g.* Sammler *et al.* 2012, Jackson *et al.* 2013). We also selected two unlinked nuclear introns for multilocus analyses, intron 7 of the

Beta-Fibrinogen (I7BF) and intron of the glycerol-3-phosphate dehydrogenase 11 gene (G3PDH). Primers used were Dloop H-739 (Sorenson *et al.* 1999) and CytB-End (Bensch & Harlid 2000) for HDI, FIB-BI7H and FIB-BI7L (Prychitko & Moore 1997) for I7BF, and G3PDH13b and G3PDH14b (Fjeldså *et al.* 2003) for G3PDH. Amplification condition consisted of an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturarion at 94°C for 30 s, hybridization at locus specific temperatures and times (HDI, 52°C for 30 s; I7BF, 50°C for 35 s and G3PDH, 55°C for 30 s), extension at 72°C for 1 min and a final extension at 72°C for 10 min. PCR products were purified using Polyethylene Glycol 8000 (PEG, 1 g/mL), sequenced using BigDye® Terminator v. 3.1 Cycle Sequencing kit (Applied Biosystems™) and run in an ABI3500 XL automatic sequencer (Applied Biosystems™).



**Figure 1.** (A) Location of the Araripe Plateau in Ceará state, northeastern Brazil. (B) Distribution of *Antilophia bokermanni* and sites at which samples were collected in upland rainforest. (C) Haplotype network, only for HDI control region, and the number of individuals per haplotype.

### Genetic computational analysis

Sequences were aligned in Clustal-W (Thompson *et al.* 1994) and edited in Bioedit 7.2 (Hall 1999). Best-fit models of nucleotide evolution were determined in Mega 6 (Tamura *et al.* 2013), based on the Bayesian Information Criterion (BIC) with the HKY model being chosen for mtDNA, and the T93 model for the two nuclear introns, which were used in the Bayesian Skyline Plot analyses (see below). The possible recombination of the markers was verified using the *phi* test (Bruen *et al.* 2006), run in Splits Tree 4 4.14.4 (Huson & Bryant 2006). Genetic diversity was estimated based on the indices of haplotype (*h*) and nucleotide ( $\pi$ ) diversity, which were calculated in Dnasp 5.1 (Librado & Rozas 2009). Gametic phases of

the introns were established using the Phase algorithm (Stephens & Donnelly 2003), with posterior probabilities of at least 0.6 were considered to be resolved (Harrigan *et al.* 2008). A hierarchical analysis of molecular variance (AMOVA) was run in Arlequin 3.5.2.2 (Excoffier & Lischer 2010), for which we inferred the presence of two groups, one located in the northwestern portion of the distribution of the species (sites 1–7), and the other in the southeastern portion (sites 8–12), based on the discontinuity in the distribution of the humid forest (Table 1; Fig. 1) (see Rêgo *et al.* 2010, Silva *et al.* 2011). The number of independent *A. bokermanni* populations, based on the assumption of non-spatially hierarchical genetic mixing at an individual level, was inferred from the mitochondrial database using the Bayesian Analysis

of Population Structure software (Baps 6; Corander *et al.* 2008). A haplotype network was also inferred using the maximum likelihood approach in Haploviewer (Salzburger *et al.* 2011) to provide a visual representation of the relationships among haplotypes.

Two approaches were used to evaluate the occurrence of historic changes in the size of the *A. bokermanni* population. Firstly, deviations from neutral evolution was based on three tests, Fu's  $F_s$  (Fu 1997), Tajima  $D$  (Tajima 1989), and  $R_2$  (Ramos-Onsins & Rozas 2002), followed by a mismatch distribution analysis, in order to evaluate whether the *A. bokermanni* population is in equilibrium, expansion or has suffered a bottleneck (Rogers & Harpending 1992). These tests were computed in Dnasp 5.1. Secondly, Beast 1.8 software (Drummond & Rambaut 2007) was used to estimate a Bayesian Skyline Plot (BSP) for the mitochondrial data, while

an Extended Bayesian Skyline Plot (EBSP) was used for a simultaneous analysis of the three markers (Heled & Drummond 2008) to test historical fluctuations in population size. These analyses were run for  $200 \times 10^8$  generations with sample genealogies being sampled every 10,000 generations, in a strict molecular clock model, in which the first 10% of generations were discarded as burn-in. The calibration of the molecular clock was based on the intraspecific mutation rate estimated by Norman *et al.* (2014) for the HDI (0.0348 substitutions per site per lineage per million years), and 0.0135 substitutions per site per lineage per million years for nuclear introns (Ellegren 2007). One year was assumed as generation time in the calculation of the effective number of females ( $N_{ef}$ ). The BSP/EBSP and the Effective Sample Sizes (ESS) were determined in Tracer 1.6 (Rambaut & Drummond 2007).

**Table 1.** Sample sites and their geographic coordinates, number of samples analyzed, and the haplotypes observed for HDI control region only, on Araripe Manakin (*Antilophia bokermanni*).

	Sample sites	Geographic coordinates	No. of samples	Hap1	Hap2	Hap3	Hap4	Hap5	Hap6
Northwest	1	39°28'28"W; 7°13'48"S	6	-	-	-	2	4	-
	2	39°28'20"W; 7°14'18"S	2	1	-	-	-	1	-
	3	39°28'14"W; 7°15'41"S	2	1	-	-	1	-	-
	4	39°26'21"W; 7°17'01"S	2	-	1	-	-	1	-
	5	39°23'51"W; 7°18'43"S	3	1	-	-	1	1	-
	6	39°24'29"W; 7°19'42"S	1	-	-	-	-	-	1
	7	39°24'45"W; 7°19'58"S	2	-	-	-	2	-	-
Southeast	8	39°21'36"W; 7°22'49"S	1	-	-	-	-	1	-
	9	39°18'48"W; 7°21'57"S	6	2	-	1	2	1	-
	10	39°13'37"W; 7°24'46"S	2	-	-	-	-	-	2
	11	39°10'01"W; 7°24'24"S	3	1	-	-	-	2	-
	12	39°12'23"W; 7°24'34"S	5	-	-	-	2	3	-
	<b>Total</b>		35	6	1	1	10	14	3

## RESULTS

### Population genetic diversity and structure

Total dataset length and individual count was 348 bp for mitochondrial HDI from 35 specimens, 961 bp for I7BF from 37 specimens and 393 bp for G3PDH intron from 31 specimens, with no evidence of indels. GenBank accession numbers for the sequences of the different molecular markers analyzed: HDI (KY788006 – KY788011), G3PDH (KY788012, Hap1  $n = 24$ ; KY788013, Hap2  $n = 3$ ; KY788014 Hap3,  $n = 4$ ) and I7BF (KY788015, Hap1  $n = 34$ ; KY788016, Hap2  $n = 1$ ; KY788017 Hap3,  $n = 2$ ). The *phi* test found no evidence of any significant recombination in this marker ( $P > 0.9$ ). A total of 15 polymorphic sites was identified for the control region, with six haplotypes (Table 1), and haplotype diversity of 0.741 and nucleotide diversity

of 0.0161. By contrast, introns presented lower levels of genetic diversity, with only three haplotypes for each marker (Table 2), and lower levels of haplotype and nucleotide diversity for both I7BF (0.080 and 0.00008, respectively) and G3PDH (0.210 and 0.00055, respectively). Non-significant  $F_{st}$  values (Table 2) were obtained between the northwestern and southeastern segments of the population, indicating a lack of genetic sub-structuring in both mitochondrial (-0.007) and nuclear (-0.008) markers. The AMOVA indicated that all (100%) the molecular variability was contained within the population as a whole, rather than in the different subpopulations. The structural analysis in Baps indicated the existence of two groups ( $k = 2$ , marginal probability = -108.6688), but without independent lineages when the northwestern and southeastern groupings were included in the analysis (Appendix I). The haplotype network, represented only for HDI (Fig. 1C) shows that the most

common haplotypes (Hap1, Hap4, Hap5) are found throughout the population (Table 1).

### Historical demography

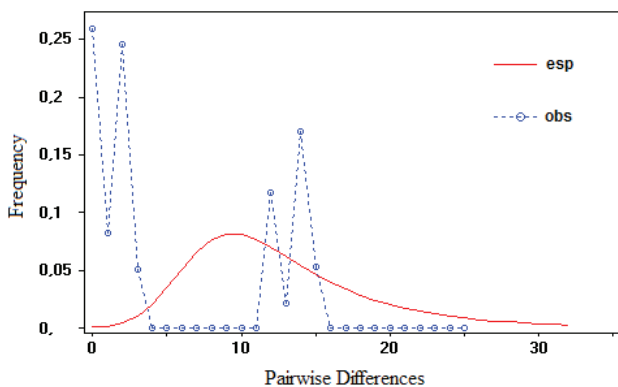
Significant positive results were obtained from the data for the HDI for  $D$  (1.7591;  $P < 0.05$ ),  $F_s$  (5.368;  $P < 0.01$ ) and  $R2$  (0.1868;  $P < 0.01$ ), rejecting the equilibrium population hypothesis. For the nuclear markers, the neutrality and population change tests did not return significant values (Table 2), and did not allow any reliable interpretation based on coalescence inferences. The mismatch distribution, performed only for the

mitochondrial marker, presented a bimodal pattern (Fig. 2), which was consistent with the haplotype network, but distinct from that of a population in equilibrium.

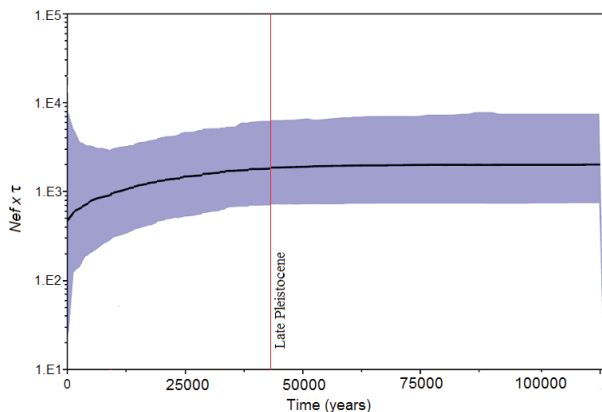
The evaluation of historic changes in population size based on the inferences derived from the EBSP found low ESS values ( $< 200$ ) in the different simulations. On the other hand, the demographic pattern outlined by the BSP (Fig. 3), focusing exclusively on the mitochondrial locus, indicates that mean values of effective size of the *A. bokermanni* population (females only) has been declining steadily over the past 50,000 years. However, the 95% confidence intervals indicated a scenario of relative stability or possibly, a recent expansion.

**Table 2.** Numbers of individuals and haplotypes sampled and summary statistics of indices of genetic diversity and population neutrality estimated for *Antilophia bokermanni*.  $n$  – number of samples; NH – number of haplotypes; S – variables sites;  $h$  – haplotype diversity;  $\pi$  – nucleotidic diversity, SD – standard deviation;  $D$  and  $F_s$  – Tajima and Fu tests, respectively;  $R2$  – Ramos & Rozas test. \*  $P < 0.05$  (significant); \*\*  $P < 0.02$  (significant).

Marker	Size of sequences	$n$	NH	S	$h \pm SD$	$\pi \pm SD$	$D$	$F_s$	$R2$	$F_{st}$
Control Region-DHI	348	35	6	15	0.741 $\pm$ 0.044	0.0161 $\pm$ 0.0027	1.7591*	5.368**	0.1868*	-0.007
I7BF	961	37	3	2	0.080 $\pm$ 0.043	0.0008 $\pm$ 0.00005	-1.3130	-2.853	0.0592	-0.008
G3PDH	393	31	3	2	0.210 $\pm$ 0.067	0.00055 $\pm$ 0.00018	-0.8350	-1.129	0.0541	



**Figure 2.** Mismatch distribution of the sequences of the HDI Control region of *Antilophia bokermanni*, based on the equilibrium population model. Expected (Exp) and observed (Obs) values are marked, respectively.



**Figure 3.** Bayesian Skyline Plot (for mitochondrial only) representing the effective size of the *A. bokermanni* population over time. The curve represents a gradual and constant decline beginning 50,000 years before present. The solid dark blue line represents the mean and the blue shaded area represents the 95% confidence interval of the historic effective size of the female population ( $N_{ef}$ ).

## DISCUSSION

### Genetic diversity and structure

Despite being among the world's most threatened bird species (BirdLife International 2016), the genetic diversity reported here for the mitochondrial marker of the Araripe Manakin (HDI  $h = 0.741$ ) is much higher than that found typically in the other threatened species of this group, such as *Aquila adalberti* ( $h = 0.321$ , Martínez-Cruz et al. 2004), *Pomarea dimidiata* ( $h = 0.000$ , Chan et al. 2011), and *Ardeotis nigriceps* ( $h = 0.261$ , Ishtiaq et al. 2011). The significantly lower levels of diversity in these species appear to have been produced by severe population bottlenecks, which do not appear to have occurred in *A. bokermanni*, suggesting that population size has been maintained above 500 individuals (Jackson et al. 2013). The unexpectedly high levels of genetic diversity found in the Araripe Manakin may be related to the retention of an ancestral polymorphism, associated with its relatively recent, and as yet incomplete separation from its sister species, *A. galeata* (Rêgo et al. 2010). Overall, then, the genetic diversity found in *A. bokermanni* is not consistent with any drastic reduction in population size (Jackson et al. 2013), but that there has been a slow and recent decline over the course of the evolutionary history of the species.

Despite the substantial fragmentation of the habitat found in the central portion of the range of this species, the results of  $F_{st}$  and the homogeneous distribution of haplotypes within the population (Table 1) indicate a lack of substructuring. A similar pattern was also

observed by Rêgo *et al.* (2010), in their analysis of the pseudo-control region. This may reflect either the relatively recent process of fragmentation, which has yet to affect the genetic structure of this type of marker, or the migration of individuals among fragments of forest. The latter hypothesis would be related to the behavior of the adult males of this species, which normally expel the juvenile offspring from their territories, obliging them to occupy new areas (Silva *et al.* 2011). This would result in high levels of gene flow, which would contribute to a reduction in the potential for inbreeding, and tends to increase in smaller, fragmented populations. This type of behavior also enhances the probability of adaptation to fragmented habitats (Canales-Delgado *et al.* 2012), although the exact genetic consequences of this dispersal pattern in *A. bokermanni* are still unclear.

### Historical demography

Despite the reduced resolution of the EBSP analysis, the demographic history of this species derived from the HDI sequence of the Control Region (derived from the BSP analysis) indicates a possible reduction in the *A. bokermanni* population approximately 50,000 years ago. The BSP derived from this analysis revealed a general trend of population decline, although the confidence intervals are also consistent with a stable population, or even a recent expansion. However, the lack of information derived from the low diversity of these loci limits phylogenetic estimates of the genealogy (Heled & Drummond 2008), and precludes reliable interpretation of the demographic events that have occurred in the Araripe Manikin population using only this approach.

Complementing these results, and supporting the assumption of a constant decline in the population size of the species, the demographic model presented in the mismatch distribution shows a pattern which may reflect the mixing of lineages that have separated recently or that have suffered a recent decline in numbers, with only the most common haplotypes surviving. The significant neutrality found in the HDI of the Control Region is also compatible with a historical reduction in population size. Assuming that the Araripe Manakin has had a relatively stable demographic history or has undergone a recent expansion, we would conclude that the considerable variation observed in the *N<sub>ef</sub>* values of the BSP resulted from the retention of ancestral polymorphisms. This feature is typical of the species of the genus *Antilophia*, as indicated by the recent separation of its lineages (Rêgo *et al.* 2010). This effect may generate false evidence of changes in population size, which emphasizes the need for caution in the interpretation of results (Grant *et al.* 2012, Heller *et al.* 2013). This restricts the potential for the inference of reliable estimates of effective population

size using this current method. It is also important to note that the mutation rate of the genetic marker analyzed in the present study, while adequate for the evaluation of recent demographic events on an evolutionary time scale (Zink & Barrowclough 2008), would not be sensitive enough to assess the effects of more recent anthropogenic impacts.

The possible recent reduction in the size of population of the Araripe Manakin, within the last 50,000 years, corresponds to the late Pleistocene. This epoch is characterized by successive periods of climate change (wet and dry cycles), which had a profound effect on the dynamics of the Neotropical biotas (Vuilleumier 1971), especially in the more rainforest and open biomes, such as the Cerrado and Caatinga (Werneck 2011), in which the species of the genus *Antilophia* are found.

The subsequent periods of glaciations and interglacials characterized by significant cooling, interspersed with shorter periods of intensely humid climate, resulted in the expansion and retraction of the majority of the gallery and scarp forests in northeastern Brazil (Behling *et al.* 2000), the type of habitat which the Araripe Manakin is associated. These climatic fluctuations may have provoked adverse conditions for the *A. bokermanni* population, which may have suffered a reduction in its genetic diversity during the adaptation process (Frankham 2005). In this context, the reduction and fragmentation of forest habitats may have led to a decrease in effective population size (Croteau *et al.* 2007), as observed in *A. bokermanni*. This indicates that the present-day genetic diversity of this population may have been determined primarily by past environmental and climatic events, during the evolutionary history of the species, rather than ongoing anthropogenic pressures, and the resulting reduction in population numbers (Silva *et al.* 2011).

As in *A. bokermanni*, studies of other passeriform populations in the forests of northeastern Brazil have also found evidence of a historical decline in population size during the same period. The ranges of species such as *Sclerurus scansor cearensis* (d'Horta *et al.* 2011), *Conopophaga lineata cearae* (Batalha-Filho *et al.* 2014), and *Pipra fasciicauda scarlatina* (Ferraz 2016), which are currently restricted to enclaves of humid cloud forest within the Caatinga, may have contracted progressively through the successive fluctuations in climate occurring during this period.

### Conservation implications

Based on the most recent census data, the population of this species may have suffered a loss of up to 36% over the past two decades, resulting from the deforestation of riparian zones, and the illegal catchment of springs, which typically results in the desiccation of the prime riparian

breeding habitat of the species (Silva *et al.* 2011). This type of impact, together with the historic decline in the *A. bokermanni* population, may have severe consequences in genetic terms, such as mating between closely-related individuals and increasing effects of inbreeding depression (Keller & Waller 2002).

The results of the present study emphasize the need for the preservation of the remaining genetic variability and the prevention of further losses, given the importance of this diversity for the adaptation of these organisms to random changes in the environment (Frankham 2005). These findings also reinforce need for the understanding of the genetic diversity of the *A. bokermanni* population. The main factor determining the loss of this diversity has yet to be identified. Further genetic analyses, based on more detailed methods and analyses (*e.g.* microsatellites and SNPs) may provide more conclusive answers for this problem and other important questions. One key objective is to integrate these data in the National Conservation Plan for the Araripe Manakin. These measures and other actions will determine the viability of this population over the medium- to long term, through the implementation of conservation measures and appropriate management actions.

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## APPENDIX I

Hierarchical clustering from Bayesian Analysis of Population Structure. The individual level mixture analysis resulted in two groups in the optimal partition, but not suitable when placed geographically (northwest and southeast of the *Antilophia bokermanni* distribution).

