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POPULATION GENETIC STUDIES OF MITOCHONDRIAL PSEUDO-CONTROL REGION IN THE ENDANGERED ARARIPE MANAKIN (*ANTILOPHIA BOKERMANNI*)

Péricles S. Rêgo,[1,2,5] Juliana Araripe,[1] Weber A. G. Silva,[3] Ciro Albano,[3] Thieres Pinto,[3] Alberto Campos,[3] Marcelo Vallinoto,[1,4] Iracilda Sampaio,[1] and Horacio Schneider[1]

[1]*Instituto de Estudos Costeiros, Universidade Federal do Pará, Bragança, Pará, Brazil;*
[2]*Centro de Ciências Naturais e Exatas, Universidade Estadual do Maranhão, São Luís, Brazil;*
[3]*Associação de Pesquisa e Preservação de Ecossistemas Aquáticos, Fortaleza, Brazil; and*
[4]*Centro de Investigação em Biodiversidade e Recursos Genéticos, Campus Agrário de Vairão, Universidade do Porto, Vairão, Portugal*

Abstract.—The Araripe Manakin (Passeriformes: Pipridae: *Antilophia bokermanni*) is the most threatened passeriform species and is classified as critically endangered. With an estimated population of only 800 individuals, this species is endemic to a small area (~30 km^2) of forest on the slopes of the Araripe Plateau in northeastern Brazil. The urgent need to implement an effective conservation program for the Araripe Manakin has stimulated intensive research into various aspects of its biology. We sequenced a segment of the mtDNA between the genes ND6 and 12S rDNA, which includes a pseudo-control region. This region was analyzed in 30 specimens of *A. bokermanni* with the aim of measuring intraspecific genetic diversity and population structure. Although the segment's position is the same as described in other bird species, *A. bokermanni* differs in some aspects, such as its length of 200 base pairs and the absence of indels or tandem repeats. Our analysis provides no evidence of population substructuring or a history of population expansion. The species' genetic variability is slightly reduced in comparison with its sister species *A. galeata*, but their similarity indicates a relatively recent process of separation. *Received 17 December 2008, accepted 27 August 2009.*

Key words: *Antilophia bokermanni*, Araripe Manakin, conservation, population genetics, pseudo-control region.

Estudios de Genética Poblacional sobre la Falsa Región de Control en la Especie Amenazada *Antilophia bokermanni*

Resumen.—*Antilophia bokermanni* (Passeriformes: Pipridae) es la especie de paseriforme más amenazada y está clasificada como en peligro crítico. Con una población estimada de sólo 800 individuos, esta especie es endémica de un área pequeña (~30 km^2) de bosque en las faldas de la meseta de Araripe en el noreste de Brasil. La necesidad de implementar de forma urgente un programa de conservación efectivo para *A. bokermanni* ha estimulado intensas investigaciones sobre varios aspectos de su biología. Secuenciamos un segmento del ADNmt ubicado entre los genes ND6 y 12S ADNr, el cual incluye una falsa región de control. Esta región fue analizada en 30 especímenes de *A. bokermanni* con el objeto de medir la diversidad genética intraespecífica y la estructura poblacional. Aunque la posición del segmento es la misma que ha sido descrita en otras especies de aves, *A. bokermanni* difiere en algunos aspectos, como su longitud de 200 pares de bases y la ausencia de inserciones o deleciones y de repeticiones en tándem. Nuestros análisis no brindan evidencia de subestructura poblacional ni de una historia de expansión poblacional. La variabilidad genética de la especie es ligeramente reducida en comparación con su especie hermana *A. galeata*, pero su similitud indica un proceso de separación relativamente reciente.

The Araripe Manakin (Passeriformes: Pipridae: *Antilophia bokermanni*) was described in 1998 by Galileu Coelho and Weber Silva in what has been considered one of the most important ornithological finds of the past few decades. Although very similar to its sister species the Helmeted Manakin (*Antilophia galeata* Lichtenstein, 1823), which is found in the gallery forests of the central Brazilian Cerrado biome, the Araripe Manakin differs principally in its vocalizations and the predominantly whitish coloration of the adult males (Coelho and Silva 1998). Although most other piprids have lek mating systems, these two species differ in

[5]Present address: Laboratório de Genética e Biologia Molecular, Campus Universitário de Bragança, Universidade Federal do Pará, Bragança-PA, 68600-000, Brazil.
E-mail: periclessena@yahoo.com.br

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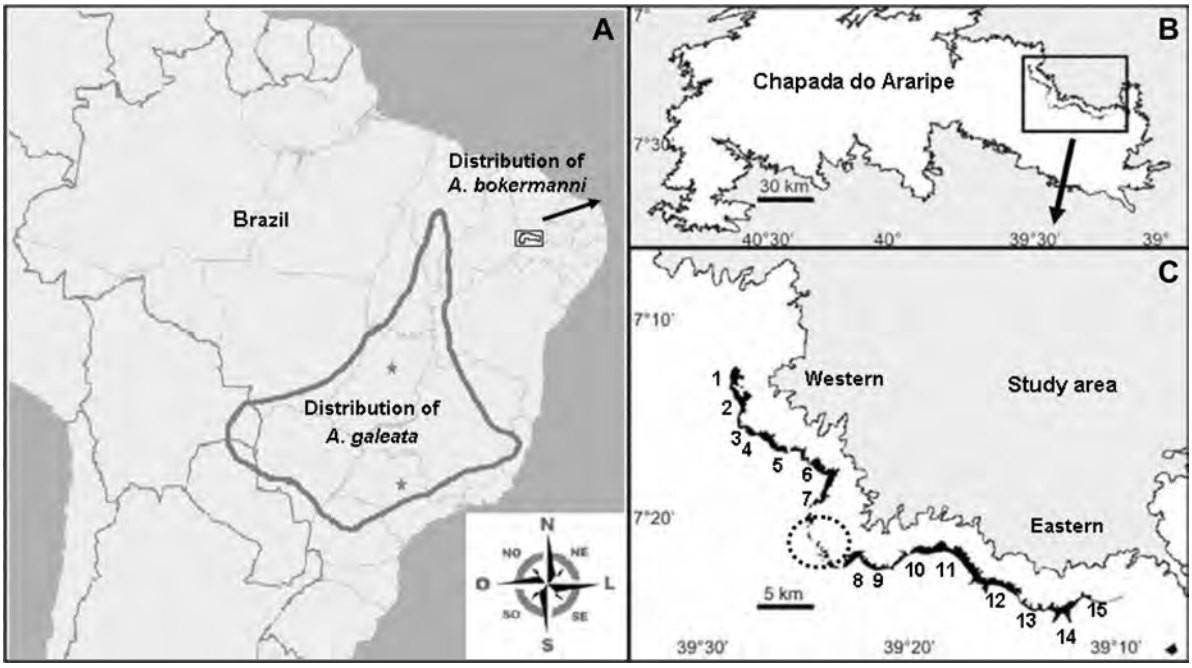

Fig. 1. (A) Collecting localities for *Antilophia bokermanni* (rectangle) and *A. galeata* (stars). (B) Distribution of *A. bokermanni* on the Araripe Plateau (Chapada do Araripe), Brazil. (C) Location of the collecting sites for *A. bokermanni* (numbers) and the lacuna (dotted circle) within its geographic range.

having a socially monogamous mating system that lacks elaborate courtship rituals (Prum 1990, 1992).

The Araripe Manakin, which is endemic to the Brazilian state of Ceará, has been classified as being in critical danger of extinction by both Brazilian and international organizations (Ministério do Meio Ambiente 2003, Hirschfeld 2008) because of its restricted distribution, the reduced size of its population, and habitat loss. The most recent study of the species indicated that its current geographic distribution covers no more than 30 km^2 of the forests on the slopes of the Araripe Plateau (Hirschfeld 2008). This area, which contains the best-conserved tracts of habitat, consists of a narrow strip of forest <500 m wide that stretches <60 km between the extremes of the species' range on the plateau's northeastern slope (Fig. 1A). The Araripe Manakin is found only in the plateau's humid forests, including the less well-preserved areas, which indicates a degree of tolerance of anthropogenic habitat disturbance. The presence of running water from mountain springs and the microclimate of the slope have been identified as the primary factors that influence habitat selection.

Field data from 2001 through 2004 indicate that the species is absent from the center of its distribution (dotted circle in Fig. 1C), which means that the population is divided into a western subpopulation and an eastern subpopulation. Preliminary surveys indicated a population of 50 to 250 individuals (BirdLife International 2000). More recently, population size was assessed more systematically by determining the number of adult males per spring and extrapolating this value according to the number and quality of springs within the known distribution. This resulted in a probably more realistic estimation of total population size (including adults and juveniles) of ~800 individuals, which is still low enough to be of considerable concern for conservation (Hirschfeld 2008).

Studies of the viability of endangered species have increasingly included the application of genetic tools to aid in identifying and understanding the factors that influence their survival (Frankham et al. 2002). Phenomena such as loss of genetic variability, inbreeding, and subpopulation structuring are problematic for species with highly reduced ranges or population size (Saccheri et al. 1998, Westemeier et al. 1998, Primack and Rodrigues 2001). Mitochondrial DNA (mtDNA) has been widely used as a molecular marker because of its usefulness in analyzing genetic diversity, population structure, and demographic history (Avise 2000). In the present study, we sequenced the segment of mtDNA between the genes ND6 and 12S rRNA in 30 specimens of *A. bokermanni*. In many species of birds, this segment is known as the pseudo-control region (ψCR; Mindell et al. 1998, Haring et al. 1999, Bensch and Härlid 2000). For comparison, we also analyzed samples from the sister species, *A. galeata*, which is distributed in the Cerrado and Pantanal of Brazil, northeastern Paraguay, and northeastern Bolivia (del Hoyo et al. 2004). Although both species occur in the Cerrado biome, there is no evidence of sympatry or contact between the species. As part of a baseline effort to develop effective conservation strategies, we used our genetic data to evaluate the demographic history of *A. bokermanni* and to evaluate the hypothesis that the eastern and western subpopulations differ genetically.

Methods

Sampling.—Samples were collected between May 2003 and February 2004 in the northeastern portion of the Araripe Plateau (Chapada do Araripe) in the Brazilian state of Ceará (39°18′08″S, 7°18′18″W). The locations of collecting sites were determined by confirming the presence of the species through observations or

records of vocalizations (Fig. 1C). Our analysis did not include sexing of samples, but the sex ratio for *A. bokermanni* appears to be 1:1 (P. S. Rêgo unpubl. data). The specimens were captured in mist nets set up adjacent to the springs, and samples of blood or feather bulbs were collected and stored in 70% ethanol. Once each bird was processed, a wing feather was clipped in a unique manner to prevent resampling. Tissue samples of *A. galeata* from São Carlos in the Brazilian state of São Paulo (22°00′51″S, 47°53′28″W) and from Brasília (15°46′36″S, 47°55′41″W) (see stars in Fig. 1A) were provided by researchers from the Federal University of São Carlos and the University of Brasília, respectively.

Extraction, amplification, and sequencing of DNA.—The samples were registered according to the regulations of the Genetics and Molecular Biology Laboratory of the Federal University of Pará and were stored in freezers until analysis. DNA was extracted by ribonuclease digestion for 1 h at 37°C, and then proteinase K was added for 2–4 h (or overnight) at 55°C. The product was washed liberally in phenol-chloroform and precipitated with isopropanol (Sambrook et al. 1989).

Polymerase chain reaction (PCR) was used to amplify the control region (D-loop) of the mitochondrial genome, including the flanking segments of the genes ND6 and 12S rRNA. The primers used in the present study were those described by Sorenson et al. (1999): ND6 L-16525 (5′–ACA AAC ACC ACT AAC ATT CCA CC–3′) and 12S rRNA H-1858 (5′–TCG ATT ACA GAA CAG GCT CCT CTA G–3′). Each reaction was conducted in a final volume of 50 µL containing 8 µL of the mixture of deoxynucleotides (1.25 mM), 5 µL of the 10× *Taq* buffer, 2 µL of $MgCl_2$ (25 mM), 1 µL of each primer (200 ng μL^{-1}), ~150 ng of the total DNA, 0.5 µL of the *Taq* polymerase enzyme (5 U μL^{-1}; Invitrogen, Carlsbad, California), and sterile distilled water to complete the final reaction volume. Amplification conditions consisted of an initial 5-min step for denaturation at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C. Final extension took 5 min at 72°C.

The products of the amplification were purified using the ExoSAP-IT kit (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom), for which 2 µL of the enzymatic solution was added to each 5 µL aliquot of the PCR reaction, and then incubated at 37°C for 15 min and then a further 15 min at 80°C. The products of this purification were submitted to a new PCR reaction, this time by the dideoxyterminal method (Sanger et al. 1977), with reagents of the Big Dye kit (ABI Prism Dye Terminator Cycle Sequencing Ready Reaction, Applied Biosystems, Foster City, California). Sequencing reactions were conducted in a final volume of 10 µL, containing 1 µL of the purified samples, 0.5 µL of one of the primers (concentration of 200 ng μL^{-1}), 3 µL of buffer (Tris 0.4 M pH 9.0/$MgCl_2$ 25 mM), 1 µL of Big Dye mix, and 4.5 µL of sterile distilled water to complete the final volume. This volume was submitted to a program of different temperatures: 25 cycles of 50 s at 96°C, 5 s at 50°C, and 4 min at 60°C. Reagents not incorporated during the reaction were eliminated by washing with isopropanol. The nucleotide sequences of the gene fragments produced by this reaction were determined in an ABI 377 (PerkinElmer, Waltham, Massachusetts) automated sequencer.

Sequence alignment and population analyses.—The sequences obtained were subjected to automatic multiple alignment with the CLUSTAL-W application (Thompson et al. 1994), using the penalty parameters suggested by Schneider (2003). The file thus generated was converted into the FASTA format and transferred to the BIOEDIT sequence editor (Hall 1999) for visual inspection of the alignment and possible correction of the coding of any observed insertions or deletions. Nucleotide composition, the number of haplotypes and polymorphic sites, and the rates of divergence between the sequences of the two species were computed using MEGA, version 3.1 (Kumar et al. 2004). Phylogenetic arrangements of the haplotypes of the two species were obtained through the PAUP* program, version 4.10 (Swofford 2002), using the neighbor-joining (NJ), maximum-parsimony (MP), and maximum-likelihood (ML) methods. The significance and robustness of the arrangements obtained were estimated on the basis of bootstrap values with 1,000 pseudoreplications. The possible presence of the hypothesized barrier to gene flow across the center of the distribution was tested using Monmonier's Maximum Differentiation Algorithm, run in ALLELES IN SPACE, version 1.0 (Miller 2005).

The programs DNASP, version 3.51 (Rozas et al. 2003), and ARLEQUIN, version 3.01 (Excoffier et al. 2005), were used to calculate the following parameters of genetic diversity and population structuring in *A. bokermanni*: haplotype (*h*; Nei 1987) and nucleotide (π; Nei 1987) diversity; the fixation index, F_{ST} (Weir and Hill 2002); hierarchical analysis of molecular variance (AMOVA; Excoffier et al. 1992) and the number of migrants per generation (M = Nm). NETWORK, version 4.5 (Bandelt et al. 1999), was used to produce a haplotype network through the method of median vectors. This allows a comparison between the observed distribution of haplotypes and that generated by the analysis of population structuring.

Population growth, stability, or decline was evaluated by analyzing the distribution of the number of observed pairwise haplotype differences, known as the mismatch distribution (Rogers and Harpending 1992), for the population as a whole and for the two hypothesized subpopulations, using DNASP. Values expected according to the sudden-population-expansion model were computed and plotted together with the observed values, and the significance of the sum of the squared deviations (SSD) was used to test the hypothesis of expansion. Three parameters of the sudden-expansion model were also tested using ARLEQUIN: Harpending's raggedness (*r*), the values of θ before and after the expansion process (θ_0 and θ_1), and the estimate of the time of expansion measured in mutational units (Tau – τ). The last is a statistical parameter applied to the mismatch plot to assess robustness and significance, as well as the time course of population expansion (*t*).

Deviations from the model of neutral evolution of infinite sites were tested using Tajima's *D* (Tajima 1989) and Fu's *F*s (Fu 1997), run in ARLEQUIN. These neutrality tests are designed to assess whether the population is evolving under the influence of some selective pressure or experiencing demographic events (expansion or reduction), if the null hypothesis of neutrality is rejected ($P < 0.05$).

Results

A total of 30 samples of *A. bokermanni* were collected from 15 sites and analyzed together with 20 samples of *A. galeata* from Brasília ($n = 5$) and São Carlos ($n = 15$) (Table 1). In contrast to results in most other birds, the segments obtained by amplification and sequencing were much smaller than expected for the control region (D-loop). By comparing sequences deposited in GenBank,

Table 1. Sample information and collecting sites in Ceará (*Antilophia bokermanni*) and in São Paulo and Brasilia (*A. galeata*), Brazil.

Species	Collecting site[a]	Number of samples	Sample codes
A. bokermanni	(1) Sítio Vale Verde	2	CLU_01, CLU_02
	(2) Fazenda Bebida Nova	2	CVV_01, CVV_02
	(3) Nascente da AABEC	2	CAA_01, CAA_02
	(4) Nascente do Grangeiro	2	CPI_01, CPI_02
	(5) Sítio Melo	2	BME_01, BME_02
	(6) Nascente do Céu	2	BCE_01, BCE_02
	(7) Nascente do Farias	2	BFA_01, BFA_02
	(8) Nascente dos Guaribas	1	BGA_01
	(9) Nascente Riacho do Meio	2	BRM_01, BRM_02
	(10) Nascente Santa Rita	2	BSR_01, BSR_02
	(11) Nascente do Silvério	2	BSI_01, BSI_02
	(12) Fazenda Serra do Mato	2	MHE_01, MHE_02
	(13) Nascente do Furtado	3	MFU_01, MFU_02, MFU_03
	(14) Nascente do Cafundó	1	MCA_01
	(15) Sítio Valentim	3	MVA_01, MVA_02, MVA_03
A. galeata	São Carlos–SP	5	AGA_39, AGA_40, AGA_41, AGA_42, AGA_43
	Brasília–DF	15	AGA_75, AGA_78, AGA_131, AGA_134, AGA_410 AGA_454, AGA_519, AGA_545, AGA_612, AGA_639 AGA_761, AGA_795, AGA_822, AGA_906, AGA_1010

[a] Numbers refer to the sites marked in Figure 1C.

we were able to confirm that the fragment obtained here did not correspond to the control region but rather to a pseudo-control region (ΨCR; Haring et al. 1999). The genus *Antilophia* is therefore consistent with the arrangement of the mitochondrial genome already described in the Passeriformes and other bird orders (Mindell et al. 1998, Bensch and Härlid 2000, Haring et al. 2001), in which a ΨCR, rather than the D-loop, is found between the genes ND6 and 12S rRNA.

Once the sequences were edited, the segment comprised 788 base pairs (bp), of which 200 correspond to the pseudo-control region. The rest of the amplified segment corresponded to parts of the 12S rDNA (~290 bp) and ND6 genes (~150 bp), in addition to $tRNA^{GLU}$ and $tRNA^{PHE}$. No indels (insertions or deletions) were observed, with the exception of a sequence found in *A. galeata* (specimen Aga_75), in which three bases had been deleted. No evidence was found (e.g., no stop codons in the 12S rDNA and ND6 genes, no double bands in the PCR, and no double peaks in the chromatogram) to suggest that the segment is a nuclear copy of a segment of the avian mitochondrial genome (NUMT; Quinn 1997). The sequences that we obtained were deposited in GenBank (accession numbers GQ849229–GQ849258 for *A. bokermanni* and GQ849259–GQ849278 for *A. galeata*).

Seven variable sites (~1% of the total) were identified in the *A. bokermanni* sequences, resulting in 6 distinct haplotypes distributed among the collecting sites (Table 2). Of these 7 polymorphic sites, one was located in the ND6 segment, 3 in the region of the ΨCR, and the remaining 3 in the 12S rDNA, with no variation found in the tRNAs. Seven haplotypes were identified in the 20 specimens of the sister species (*A. galeata*), resulting from 12 variable sites and the deletion in Aga_75. The nucleotide composition of the sequences of both species was highly similar, with a predominance of adenine and cytosine (37.4% and 29.2% of bases, respectively) and a deficiency of guanine (11.7%), which corresponds to the known pattern of mitochondrial sequences in birds (Baker and Marshall 1997, Haring et al. 2001, Ruokonen and Kvist 2002). In all samples from both species, all changes at the variable sites were transitions.

The observed rates of nucleotide divergence in the haplotypes of the two species were very similar (<1.5%, mean = 0.5 ± 0.2 [SD]). The consensus phylogenetic arrangement produced by the three methods (NJ, MP, and ML) revealed a star-like tree with no node resolution (data not shown). This similarity between the two sister species is even more evident in the haplotype network (Fig. 2), which reveals two principal and divergent mitochondrial lineages (H_1 and H_3), which represent 50% of the specimens analyzed. The remaining specimens have haplotypes that diverge by only a few mutations, probably derived from the more abundant lineages. The presence of most of the different haplotypes in each of the two hypothesized subpopulations (east and west) is a further indication of the overall homogeneity within the species and the absence of any differentiation. The most common haplotype in the population of *A. bokermanni* (H_3) was also recorded in *A. galeata* (Fig. 2 and Table 2), albeit at a lower frequency. The second-most-common haplotype in *A. bokermanni* (H_1) was absent in *A. galeata*.

The indices of intraspecific genetic diversity were slightly lower in *A. bokermanni* (Table 3) than in *A. galeata*, even though the latter was represented by a smaller sample. Both haplotype (*h*) and nucleotide (π) diversity in *A. bokermanni* were moderate in comparison with results of other avian studies, although the values were higher than those recorded for some endangered species (Questiau et al. 1998, Asai et al. 2006, Lopes et al. 2007). No differences in diversity indices were found between the eastern and western subpopulations.

A negative, nonsignificant value was obtained for the fixation index (F_{ST} = –0.00471) between "east" and "west" samples of *A. bokermanni*, corroborating the lack of differentiation between the eastern and western subpopulations. The AMOVA analysis indicated that all the molecular variability occurred at the level of

Table 2. Haplotypes identified in *Antilophia bokermanni* (and its subpopulations; see text) and *A. galeata*. The columns marked in gray refer to the variable sites within the pseudo-control region (ΨCR)

Haplotype	Number of specimens	Position of variable sites (vertical)														Identification of the specimens		
		0	0	1	1	1	2	2	2	3	5	5	5	6	7	7	*A. bokermanni*		
		4	8	5	5	6	5	6	8	1	2	8	9	3	2	2			
		2	7	8	9	0	9	2	3	8	7	6	2	2	5	7	Western subpopulation	Eastern subpopulation	*A. galeata*
H_1	7	C	G	C	A	A	C	T	T	A	T	A	A	T	A	C	CLU_01, CVV_01, CPI_01, BME_02, BCE_01	BGA_01, MFU_01	—
H_2	3	–	–	–	–	–	T	–	C	G	–	–	–	–	–	–	CLU_02	MFU_02, MVA_02	—
H_3	18	T	–	–	–	–	T	–	C	G	–	–	G	C	–	T	CVV_02, CAA_01, CAA_02, CPI_02, BME_01, BCE_02, BFA_01	BRM_02, BSR_01, BSR_02, MHE_01, MHE_02, MCA_01, MVA_01	AGA_39, AGA_40; AGA_41, AGA_43
H_4	1	–	–	–	–	–	T	–	C	G	–	–	G	C	–	T	BFA_02	–	—
H_5	4	T	–	–	–	–	T	–	C	G	–	–	–	–	–	–	—	BRM_01, BSI_01, BSI_02, MFU_03	—
H_6	1	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	—	MVA_03	—
H_7	3	–	–	–	–	–	T	–	–	G	–	–	–	–	–	–	—	—	AGA_42, AGA_545, AGA_795
H_8	1	–	–	X	X	X	T	–	–	G	–	–	–	–	–	–	—	—	AGA_75
H_9	3	T	A	–	–	–	T	C	C	G	–	–	G	C	–	T	—	—	AGA78, AGA_131, AGA_134
H_10	4	T	A	–	–	–	T	–	C	G	–	–	–	C	–	T	—	—	AGA_410, AGA_454, AGA_519, AGA_822
H_11	3	T	A	–	–	–	T	–	C	G	A	–	G	C	G	T	—	—	AGA_612, AGA_639, AGA_761
H_12	2	–	–	–	–	–	T	–	–	G	–	T	–	T	A	–	—	—	AGA_906, AGA_1010

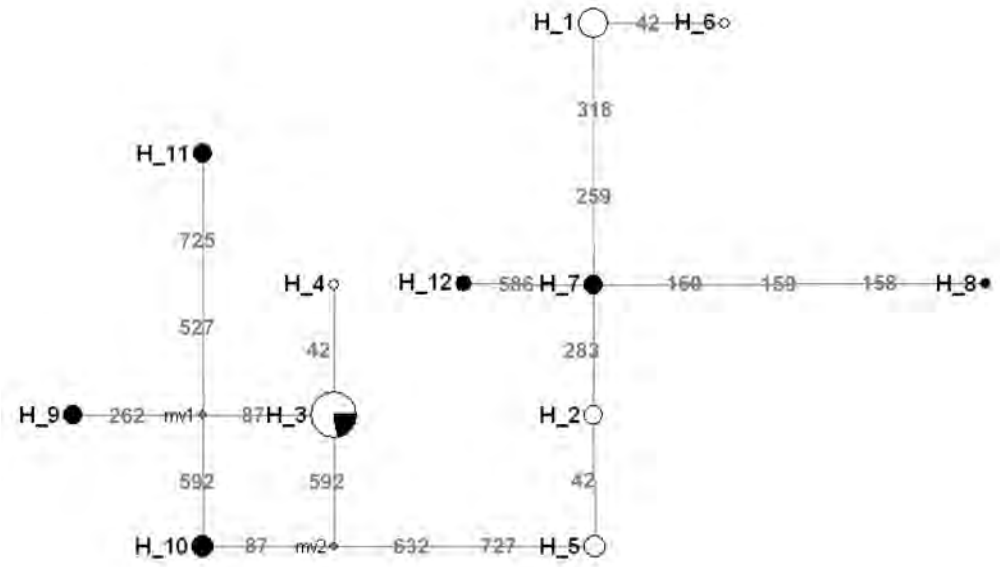

Fig. 2. The haplotype network found for *Antilophia bokermanni* and *A. galeata*. Haplotypes are represented by circles, the sizes of which are proportional to their frequencies. Numbers on the lines that join the circles correspond to the positions of the divergent nucleotides in the region studied. Samples of *A. galeata* are in black and those of *A. bokermanni* are in white.

Table 3. Indices of genetic diversity for *Antilophia bokermanni* and *A.galeata* obtained from 788 base pairs of mtDNA between the ND6 and 12S rRNA genes (*n* = number of individuals, *Nh* = number of haplotypes, *h* = haplotype diversity, and π = nucleotide diversity).

Species	Population	*n*	*Nh*	*h* ± SD	π ± SD
A. bokermanni	Western subpopulation	14	4	0.659 ± 0.090	0.0046 ± 0.0006
	Eastern subpopulation	16	5	0.758 ± 0.080	0.0037 ± 0.0006
	Whole population	30	6	0.722 ± 0.062	0.0041 ± 0.0004
A. galeata	Whole population	20	7	0.884 ± 0.031	0.0049 ± 0.0005

the whole population (100%) rather than among subpopulations, which further contradicts the hypothesis of differentiation between the eastern and western subpopulations. Monmonier's algorithm was consistent with the hypothesis that the east–west gap serves as a potential barrier to gene flow (data not shown).

The mismatch plots were multimodally distributed (Fig. 3), confirming the results of the haplotype network, which indicated two common but divergent haplotypes. Graphs with a multimodal distribution contradict the hypothesis of a process of expansion in panmictic populations and instead suggest other processes, such as hitchhiking effects or retention of ancestral polymorphisms (Rogers and Harpending 1992). The *P* value for the comparison of observed and expected values (SSD) did not differ for any analyzed group (Table 4).

The values of θ_0 and θ_1 for both the total population and the two subpopulations overlapped (Table 4), which indicates no expansion. This conclusion is further corroborated by the lack of differences in the raggedness index (Table 4). Similarly, although the values of both Tajima's *D* and Fu's *F*s were positive for all groupings (Table 4), none was significant ($P > 0.01$).

Discussion

Because little more than a decade has passed since the discovery of *A. bokermanni*, data on population structure and dynamics are still scarce. The principal, integrated study of the characteristics of this bird was conducted by the nongovernmental organization Associação de Pesquisa e Preservação de Ecossistemas Aquáticos (Association for Research and Preservation of Aquatic Ecosystems). The project was supported by prominent Brazilian and international institutions and resulted in the Araripe Manakin Conservation Plan (Associação de Pesquisa e Preservação de Ecossistemas Aquáticos 2006), which presents information on home range, population size, reproduction, and dispersal.

In the present study, we sequenced the ψCR, a small segment of ~200 bp with no repetitions or indels in either *A. bokermanni* or *A. galeata*. The utility of the ψCR for both inter- and intraspecific studies of birds remains to be determined because of (1) the limited number of species in which it has been described, (2) the lack of reliable estimates of its rate of evolution, and (3) the difficulties of sequence alignment between distinct species (Riesing et al. 2003, Kruckenhauser et al. 2004, Nittinger et al. 2005).

The indices of genetic diversity in *A. bokermanni* ($\pi = 0.0041$ and $h = 0.722$) were moderate and differed little from those in *A. galeata* (Table 3). Given the differences between the two species in sample size and geographic distribution, the relative lack of differentiation found between them is intriguing. Considering the

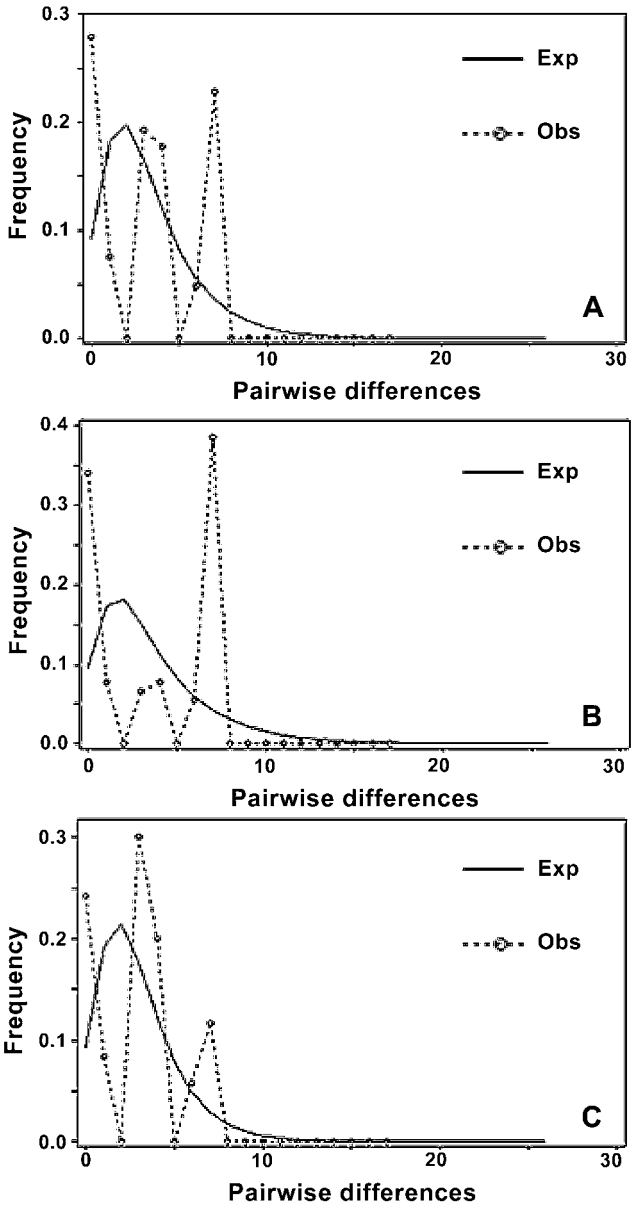

Fig. 3. Mismatch distribution of the sequences of the pseudo-control region of *Antilophia bokermanni*, based on the sudden-population-expansion model. Frequency is expressed as a fraction of all comparisons. Expected and observed values are marked "Exp" and "Obs," respectively.

Table 4. Statistics for mismatch distribution and neutrality tests in *Antilophia bokermanni* and *A. galeata* (n = number of haplotypes; τ = estimate of the time of expansion in mutational units; θ_0 and θ_1 = estimate of population size before and after the expansion, respectively; r = raggedness index).

	Statistical parameter of the mismatch distribution						Neutrality tests	
Species	n	τ	θ_0	θ_1	r	SSD	Tajima's D	Fu's Fs
A. bokermanni								
Western subpopulation	14	7.975	0.000–4.986	1.495–56.841	0.3455 [a]	0.159 [a]	2.35233 [a]	2.95544 [a]
Eastern subpopulation	16	4.702	0.000–4.399	0.991–28.922	0.1924 [a]	0.077 [a]	1.40378 [a]	1.37835 [a]
Whole population	30	7.080	0.000–6.281	1.047–109.775	0.2018 [a]	0.080 [a]	2.4732 [a]	2.05445 [a]
A. galeata								
Whole population	20	2.013	0.000–13.893	5.042–705.962	0.1020 [a]	0.035 [a]	1.2370 [a]	0.98955 [a]

[a] Not significant ($P < 0.05$).

combination of factors that have led *A. bokermanni* to the brink of extinction, these levels of diversity might be considered relatively high in comparison with other endangered bird species (Moum and Árnason 2001, Van Den Bussche et al. 2003, Martínez-Cruz et al. 2004, Asai et al. 2006). The results of the mismatch distribution (multimodal) and the haplotype network (small number of unique haplotypes) argue against a hypothesis of demographic expansion and for a hypothesis of retention of ancestral polymorphisms that predate the separation of the two species. Similarly, the results of the tests for deviations from neutrality (D and Fs) were positive, although not significant, providing further evidence against population expansion. This could also indicate a recent bottleneck. It will be necessary to use other, more sensitive markers (e.g., microsatellites) to test this hypothesis.

We also found basically the same haplotypes in both species of *Antilophia* (Fig. 2), which suggests incomplete lineage sorting. Our results therefore indicate a surprising degree of similarity between the species, given their disjunct ranges and pronounced morphological (plumage color) divergence.

We found no evidence of population differentiation within *A. bokermanni*, despite the fairly large gap in its distribution. The AMOVA and F_{ST} tests indicated that the gap has not yet resulted in any significant substructuring of the population, at least in the mitochondrial sequence analyzed here. Given that the present study is the first to use the pseudo-control region as a population marker in Passeriformes, our results should be interpreted with caution, and we advise against making comparisons between this marker and other mitochondrial markers commonly used in the literature. Additional mitochondrial or nuclear markers may help reveal additional patterns of variation within *A. bokermanni* and *A. galeata*.

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