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SHORT-COMMUNICATION

Confirmation of the hybridization of *Chiroxiphia* Cabanis, 1847 and *Antilophia* Reichenbach, 1850 (Passeriformes: Pipridae) using molecular markers

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ABSTRACT: Events of hybridization between species of different manakin genera are well documented, although the relative contribution of the species remains unclear, as well as the factors determining the occurrence of these events. In this context, the use of molecular markers has become increasingly important for the reliable diagnosis of the species involved and the understanding of the process. In 2008 two unusual manakins were observed in the Ibiapaba Highlands of the Brazilian state of Ceará (municipality of Tianguá). Their morphological traits indicated that they were male hybrids of *Chiroxiphia* and an *Antilophia* species, despite the fact that neither of the two species of *Antilophia* had been found in the Ibiapaba region. In order to confirm the hybridization and identify the species involved, the present study was based on the analysis of samples taken from a supposed hybrid and a specimen of *C. pareola* collected in the Ibiapaba Highlands, together with two samples of the two species of the genus *Antilophia* (*A. bokermanni* and *A. galeata*) provided by research collections. Partial sequences of three mitochondrial markers (ND2/COI/16S) and a nuclear intron (I7BF) were obtained to confirm hybridization and identify the contribution of each parent species. Results confirmed that the specimen was a hybrid produced by the crossing of a male *Chiroxiphia* (*C. pareola* as most likely species) with a female *Antilophia*, although it was not possible to identify which species were involved. The confirmation that this hybridization event in the Ibiapaba Highlands involved *Antilophia* indicates that one of the two species of this genus occurs in this region, which may thus constitute a previously unknown hybrid zone between two manakin species.

KEY-WORDS: hybrid, Ibiapaba Highlands, intergeneric mating, manakin.

The hybridization of different manakin species (family Pipridae) is a phenomenon that has been well documented by ornithologists, with a multitude of cases being scientifically confirmed (Parkes 1961, Stotz 1993, Pacheco & Parrini 1995, Sick 1997, Brumfield *et al.* 2001, Marini & Hackett 2002). A special case of hybridization in this bird family involves species of the genera *Chiroxiphia* Cabanis, 1847 and *Antilophia* Reichenbach, 1850, with the hybrid being known as "King-of-the-manakins" (Sick 1979, Rezende *et al.* 2013). These two genera, while closely-related phylogenetically as sister groups (Tello *et al.* 2009, McKay *et al.* 2010, Ohlson *et al.* 2013), have very distinct reproductive behavior. *Antilophia* males do not engage in a mating dance during the breeding season, and only display flights have been observed (Marini & Cavalcanti 1992, Aquasis *et al.* 2006). By contrast, *Chiroxiphia* males form leks during the breeding season, in which they present display behavior (Foster 1981, Sick 1997). Despite these differences, a number of hybrids of these genera have been observed in the wild, especially between *Chiroxiphia caudata* (Shaw & Nodder, 1793) and *Antilophia galeata* (Lichtenstein, 1823). This hybridization occurs in the transition zone between the Atlantic Forest and the central Brazilian Cerrado savanna (Pacheco & Parrini 1995, Sick 1997, Vasconcelos *et al.* 2005, Rezende *et al.* 2013), where the geographic distributions of the two species overlap.

The pattern of hybridization observed in *C. caudata* and *A. galeata* is restricted to the marginal transition zone between the preferred biome of each species (Vasconcelos *et al.* 2005). These authors concluded that biogeographic factors may also influence the hybridization of these two species, reflecting differences in their abundance, given that the forest formations in which the hybrid was found are more characteristic of the natural habitat of *Chiroxiphia*, which is more common in this type of environment. In addition, it is possible that *Chiroxiphia* males not selected by females of this species during the mating display may remain aroused by this behavior and try to copulate with females of other species, in particular *Antilophia* (Rezende *et al.* 2013).

In 2008, in a fragment of rainforest in the Ibiapaba Highlands of the Brazilian state of Ceará (municipality of Tianguá), two birds with unusual morphological characteristics (in the plumage and body size) were observed. One specimen was captured, and an analysis of its morphology indicated that it was an adult male produced by an intergeneric cross between *Chiroxiphia pareola* and an *Antilophia* species, either *A. bokermanni* or *A. galeata* (Silva *et al.* 2011, Kirwan & Green 2011). This finding was somewhat enigmatic, given that the only *C. pareola* had been recorded previously in the Ibiapaba Highlands, while the nearest record of *A. galeata* is from a site approximately 550 km away, and that of *A. bokermanni* is more than 400 km distant (Silva *et al.* 2011) (Figure 1). The determination of the parental contribution of the two species involved in this hybridization event provides important insights into the reproductive patterns of the species involved and the ways in which they interact with one another in the sympatric zone. Given this, the principal objective of the present study was the confirmation of the hybrid status of the specimen captured in the Ibiapaba Highlands, and the identification of the two species that contributed genetically to this hybrid.

Samples of muscle tissue were obtained from specimens of *Antilophia bokermanni* (n = 2), *Antilophia galeata* (n = 2), and *Chiroxiphia pareola* (n = 2), as well as of a single hybrid. One of the *C. pareola* specimens (specimen 01) and the hybrid were captured by mist-netting in the municipality of Tianguá, in the Ibiapaba Highlands of Ceará (Brazil). The tissue samples of *Antilophia* species and the second *C. pareola* (specimen 02) were obtained from the collection of the Genetics and Conservation Laboratory of the Federal University of Pará (UFPA). Samples were stored in a freezer in 95% ethanol.

Total DNA was extracted using the standard phenol-chloroform method, followed by precipitation with sodium acetate and alcohol (Sambrook *et al.* 1989). The DNA was electrophoresed horizontally in 1%

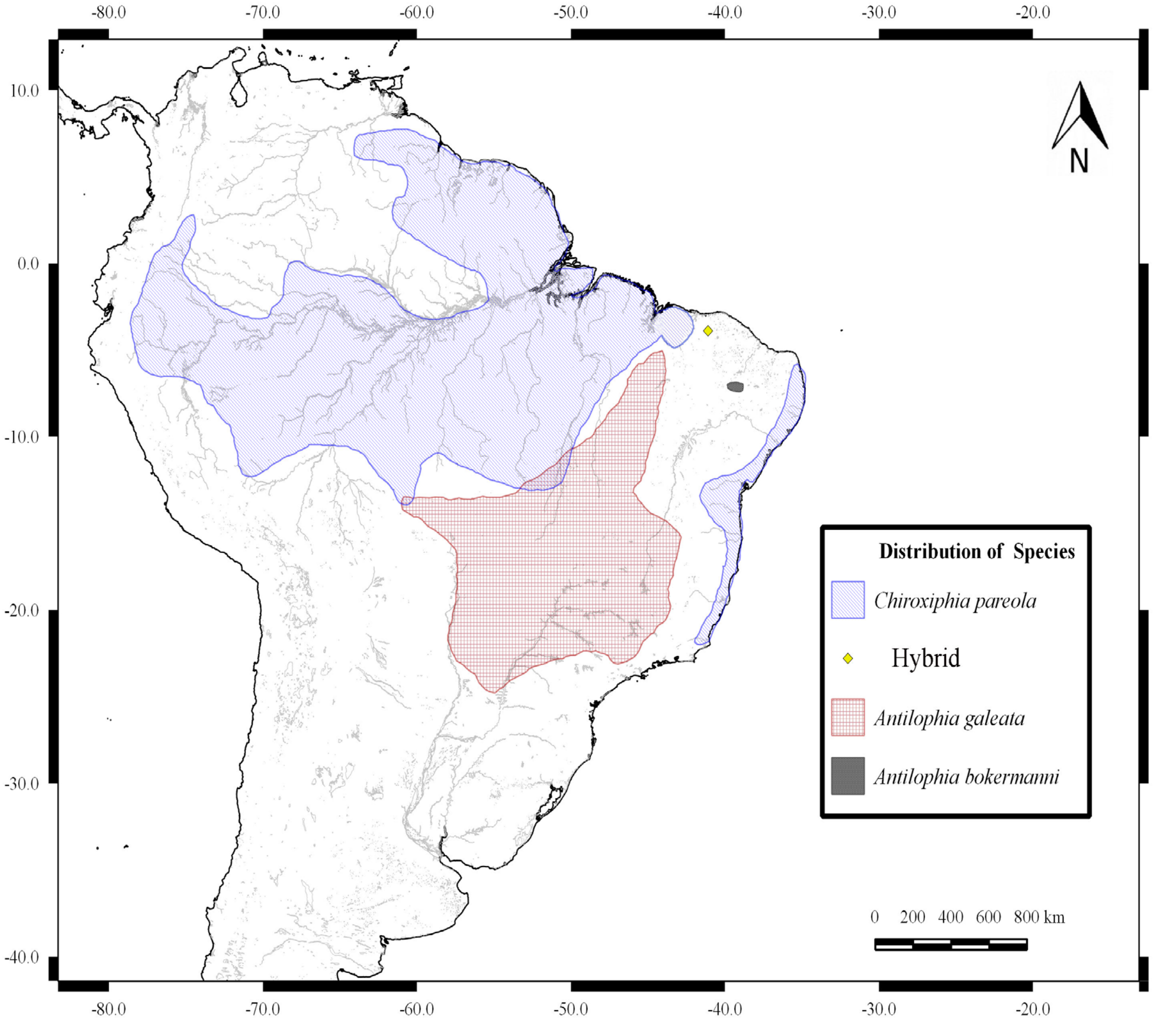

FIGURE 1. Geographic distribution of the three manakin species analyzed in the present study. The yellow dot shows the locality at which the hybrid and one specimen of *Chiroxiphia pareola* (specimen 01) were captured in the Ibiapaba Highlands of Ceará state, Brazil.

agarose gel and stained with GelRed (Uniscience) for visualization in an ultraviolet transilluminator, where the efficiency of the isolation process was evaluated. The Polymerase Chain Reaction (PCR) was used to amplify fragments of three mitochondrial genes, using available primers and reaction conditions. The genes were NADH dehydrogenase subunit 2, or ND2 (Sorenson *et al.* 1999), Cytochrome Oxidase subunit I, COI (Ward *et al.* 2005), and rDNA 16S (Palumbi *et al.* 1991). In addition to these mitochondrial markers, a fragment of the nuclear intron 7 of the β-fibrinogen gene (I7BF) was also sequenced. This fragment was chosen because it contains specific sites that can distinguish the two study genera (Prychitko & Moore 1997). Each reaction was conducted in a final volume of 25 µL, containing 4 µL of dNTP (1.25 mM), 2.5 µL of buffer (10 ×), 1 µL of $MgCl_2$ (25 mM), 0.3 µL of each primer (200 ng/µL), 1µL of the total DNA, 0.25 µL of Taq polymerase (5 U/µL - Invitrogen) and ultrapure water to complete the reaction volume. The PCR products were purified with a PEG 8000, using the protocol described by Dunn & Blattner (1987). The positive reactions were sequenced by the dideoxy-terminal method of Sanger *et al.* (1977) using the Big Dye kit (Applied Biosystems) and run in an ABI 3500XL automatic sequencer, according to standard protocol from the supplier.

The sequences were aligned and edited manually using BioEdit, version 7.1.3.0 (Hall 1999). The differences among the variable sites found in the sequences were verified using MEGA, version 5.0.5 (Tamura *et al.* 2011). This analysis consisted of comparisons between the variable points that differentiate each species and the supposed hybrid. The mitochondrial markers allowed the identification of species that contributed to the maternal lineage, given that the mitochondrial DNA is inherited matrilineally. The nuclear marker should confirm the hybridization process through the observation of species-specific sites with a double signal in the supposed hybrid.

All target sequences selected for the present study were successfully sequenced in the six specimens, resulting in a total of 2630 base pairs (bps). A total of 1900 bps were obtained for the mitochondrial sequences, with 121 sites varying among the species (Table 1). The nuclear fragment (I7BF) rendered 730 bps, with nine variable sites (Table 1). GenBank accession numbers for the sequences of the different molecular markers analyzed: ND2 (KX394495–KX394501), COI (KX394509–KX394515), 16S (KX394516–KX394522) and I7BF (KX394502–KX394508).

The same mitochondrial sequences were observed in *Antilophia* (*A. galeata* and *A. bokermanni*) and the hybrid, whereas the *Chiroxiphia* (*C. pareola*) samples had 106 different sites in the three fragments (Table 1). This evidence confirms conclusively that the hybrid was produced by a female *Antilophia*.

In the electropherogram of the nuclear sequences of the hybrid, it was possible to observe double peaks at nine sites. These heterozygous signals coincided with the sites of the I7BF marker that distinguish the genera *Antilophia* and *Chiroxiphia*, confirming the contribution of species of both genera to the production of the hybrid. The degenerated bases found in the nuclear markers obtained from the hybrid coincide with the nucleotides that differentiate the two genera, confirming its hybrid status (Table 1).

However, it was not possible to determine precisely which *Antilophia* species was involved in the hybridization, given that the molecular markers used in the present study were unable to differentiate between the two species. This lack of differentiation was observed in all four molecular markers, with only a single haplotype found in each mitochondrial sequence for the two *Antilophia* species, and a single genotype for the nuclear sequence. Based in the geographic distribution, *C. pareola* is the most likely second parental.

Most reports of hybrids between *Chiroxiphia* and *Antilophia* refer to *C. caudata* and *A. galeata* in southeastern Brazil, in the states of Minas Gerais (Pacheco & Parrini 1995, Vasconcelos 2005, Rezende *et al.* 2013) and São Paulo (Gussoni *et al.* 2005). In all these cases, hybridization was confirmed on the basis of morphological characteristics and field observations. Prior to the present study, the only molecular confirmation of intergeneric hybridization in manakins was that of Marini & Hackett (2002) for *Ilicura militaris* and *Chiroxiphia caudata*. Results of the present study provide the first evidence of hybridization between *Chiroxiphia*, most likely *C. pareola*, and a member of the genus *Antilophia* in northeastern Brazil, confirmed by the genetic evidence.

No *Antilophia* manakin have been recorded in the Ibiapaba Highlands, where the hybrid analyzed in the present study was captured, while a number of records of *C. pareola* have now been confirmed for the region. Nascimento *et al.* (2005) conducted an extensive bird survey in the region, and found no evidence of the occurrence of either *A. galeata* or *A. bokermanni*. The majority of manakin species – including those of the genera *Chiroxiphia* and *Antilophia* – the plumage of the female is predominantly olive green, making them difficult to distinguish. This may account for the difficulty of recording *Antilophia* in the region, given that the molecular data confirmed that the hybrid was generated by a female *Antilophia*. The greater abundance of female *Antilophia* may be related to differences between males and females in the use of habitats and movements among areas, as observed in many piprids, in which the females tend to occupy larger ranges than males (Théry 1992, Durães *et al.* 2007).

TABLE 1. Variable sites observed in the mitochondrial (**A**) ND2, (**B**) COI, and (**C**) 16S fragments, and the nuclear (**D**) I7BF fragment. The letters R, Y, and K represent the degenerated bases for the pairs of nucleotides, A/B, C/T, and G/T, respectively, found in the nuclear fragment. The fragment length of each marker is shown between parentheses next to its acronym.

Species	ND2 (750 bps)																																																					
								1	1	1	1	1	1	1	2	2	2	2	2	2	2	3	3	3	4	4	4	4	4	4	4	4	4	4	5	5	5	5	5	5	5	5	5	5	6	6	6	6	6	7	7	7	7	7	
	1	2	2	3	5	9	9	0	1	4	5	8	9	9	3	5	6	6	6	8	9	1	6	7	1	2	2	3	4	6	6	7	8	8	0	4	4	5	5	8	8	8	9	9	0	4	4	5	6	1	1	1	2	4	(**A**)
	2	3	9	5	6	2	5	1	5	0	2	2	1	7	4	2	0	4	7	1	0	1	8	2	3	2	9	4	9	4	7	3	2	5	7	5	6	1	4	4	5	7	1	9	0	4	8	7	5	3	4	6	8	4	
A. galeata 01	T	A	C	A	A	A	C	C	C	C	C	A	T	C	A	G	T	C	C	C	A	C	T	A	C	T	G	G	T	A	G	T	A	G	G	G	A	T	C	T	G	T	T	C	G	T	T	T	A	C	A	G	T	C	
A. galeata 02	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
A. bokermanni 01	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
A. bokermanni 02	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
HIB-01	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. pareola 01	C	G	T	G	T	G	T	T	G	T	T	T	C	T	T	A	C	T	T	T	G	T	C	G	T	C	A	A	A	C	A	C	G	A	A	A	G	C	T	C	A	A	C	T	T	C	C	C	T	T	G	T	C	T	
C. pareola 02	C	G	T	G	T	G	T	T	G	T	T	T	C	T	T	A	C	T	T	T	G	T	C	G	T	C	A	A	A	C	A	C	G	A	A	A	G	C	T	C	A	A	C	T	T	C	C	C	T	T	G	T	C	T	

Species	COI (627 bps)																																						
								1	1	1	1	1	2	2	2	2	2	2	2	2	3	3	3	3	4	4	4	4	4	5	5	5	5	5	5	5	5	5	6	
			1	2	3	5	8	1	1	1	7	8	0	0	2	2	5	5	6	6	1	2	4	7	0	0	2	3	9	1	2	2	4	6	7	7	8	9	2	(**B**)
	2	5	7	6	5	3	3	0	3	6	0	0	0	9	1	4	1	8	0	3	1	6	7	4	4	7	5	7	4	5	1	4	5	6	2	5	1	3	7	
A. galeata 01	A	T	T	G	C	G	G	T	C	C	G	T	C	T	C	C	C	C	T	C	A	T	G	T	C	G	T	T	A	A	C	T	T	T	A	T	A	T	T	
A. galeata 02	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
A. bokermanni 01	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
A. bokermanni 02	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
HIB-01	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. pareola 01	G	C	C	A	T	A	A	C	T	T	A	C	T	C	T	T	T	T	A	T	G	C	A	C	T	A	C	C	G	T	A	C	C	A	G	C	C	C	C	
C. pareola 02	G	C	C	A	T	A	A	C	T	T	A	C	T	C	T	T	T	T	A	T	G	C	A	C	T	A	C	C	G	T	A	C	C	A	G	C	C	C	C	

Species	16S (510 bps)														I7BF (730 bps)									
		1	1	2	2	2	2	2	2	2	3	3	4			1	2	3	3	3	3	4	5	
	1	6	7	0	2	3	4	5	6	6	2	4	2	(**C**)		5	7	6	6	7	8	3	4	(**D**)
	5	8	0	6	9	5	3	7	2	7	2	7	9		2	8	1	4	5	3	4	2	3	
A. galeata 01	T	C	A	T	C	C	C	T	T	T	C	C	C		A	C	T	C	T	G	T	C	C	
A. galeata 02	.	.	.	.	.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
A. bokermanni 01	.	.	.	.	.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
A. bokermanni 02	.	.	.	.	.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	
HIB-01	.	.	.	.	.	.	.	.	.	.	.	.	.		R	Y	K	Y	K	R	Y	Y	Y	
C. pareola 01	A	T	C	C	T	T	T	C	C	C	T	T	T		G	T	G	T	G	A	C	T	T	
C. pareola 02	A	T	C	C	T	T	T	C	C	C	T	T	T		G	T	G	T	G	A	C	T	T	

Understanding the geographic distribution of the species is a crucial aspect of the study of hybridization events in birds (Marini & Hackett 2002). Vasconcelos *et al.* (2005) concluded that forests in the area where the hybrid was found are more characteristic of the habitats occupied by *Chiroxiphia*, supporting the conclusion that manakins of the genus *Antilophia* are colonizing areas outside their natural range. Gallery forests of the tributaries of the Parnaíba River, located less than 125 km west of the area in which the hybrid was found, may contain small populations of *A. galeata* (Silva *et al.* 2011), which would increase the likelihood of this species being involved in the production of the hybrid. The Araripe Plateau, also in Ceará state, where the Araripe Manakin (*A. bokermanni*) is found, is located approximately 400 km east of the southern portion of the Ibiapaba Highlands. The westernmost portion of the Araripe Plateau has similar climatic conditions and vegetation to those found in the Ibiapaba Highlands, although no continuous forest connects the areas (Silva *et al.* 2011).

It was not possible to determine which *Antilophia* species was involved in this hybridization, due to the considerable genetic similarities between species, given that no species-specific sites were found in their nucleotide sequences. A shared haplotype in a mitochondrial marker had already been reported by Rêgo *et al.* (2010), precluding the discrimination among the two species. The absence of reciprocal monophyletism in these two *Antilophia* species has been attributed to a relatively recent speciation process, despite the existence of phenotypic features that clearly distinguish both species, such as the color of the plumage in adult males. The uncertainty regarding the identification of which *Antilophia* species was involved in the hybridization event will continue, either until a species-specific marker can be identified or an adult male is observed in the Ibiapaba region. Despite this limitation, the fact that an *Antilophia* species does occur in the Ibiapaba Highlands reinforces the need for further studies to evaluate the exact geographic distribution and dispersal patterns of the species of this genus. Newly matured individuals usually seek new areas for establishment of their territories. This may result in the constant dispersal of young adults into new areas, at least where appropriate habitats exist (Silva *et al.* 2011).

A second factor that may have also contributed to the hybridization process is the mating behavior of *Chiroxiphia*. Rezende *et al.* (2013) reported that male *Chiroxiphia* involved in the lek display, which were not selected by females, would remain in a state of arousal for a time afterwards, and would try to copulate with females of other species, in the present case, *Antilophia*. However, this conclusion is contradicted by the predictions of intersexual selection, which is determined by female choices (Freeman & Herron 2009). Previous studies on manakins have shown that the females discriminate males actively (Brumfield *et al.* 2001, Anciães *et al.* 2009), and the presence of a female *Antilophia* in a display area used exclusively by male *Chiroxiphia* may favor mating due to the sensory predisposition of females.

Natural hybridization events require systematic monitoring in order to identify their key factors, especially where the influence of anthropogenic disturbances is suspected. The persistence of first generation hybrids that continue to breed with one of the paternal species may result in secondary introgression, which may be especially deleterious when combined with a tendency for only one of the sexes of each species to contribute to the reproductive process. This progressive introgression may result in individuals with phenotypes very similar to the parental species, but with the exclusion of the haplotype of the paternal lineage following the second generation, where the hybrid is female. In the present case, it would be important to determine whether female hybrids exist, and whether they are able to produce viable offspring. In this case, continuing unidirectional introgression would lead to the existence of a population with a phenotype similar to that of *Chiroxiphia*, but with the mitochondrial DNA of *Antilophia*. Brumfield *et al.* (2001) evaluated the effects of hybridization events involving manakins of the genus *Manacus*, and emphasized the role of sexual selection in the asymmetrical introgression of plumage coloration. Given this, female choice may be the main factor determining the existence of hybridization zones in manakins, given that females may either prefer "novel" males (when males of both species are present) or be flexible enough to accept "novel" males in the absence of males of its own species.

Rezende *et al.* (2013) described the morphology of a female hybrid of *A. galeata* and *C. caudata*, which is denominated "queen-of-the-manakins". These authors emphasized the need for additional molecular studies in areas where hybridization events occur, in order to support the effective monitoring and conservation of these species. Results of the present study indicate that a hybrid zone between *Chiroxiphia* (most likely *C. pareola*) and an as yet unspecified *Antilophia* species exists in the region of the Ibiapaba Highlands, in the Brazilian state of Ceará, with a pattern of parental contribution that requires further investigation.

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