



Mutations in the melanocortin-1 receptor (*MC1R*) gene have no influence on the distinct patterns of melanic plumage found in the manakins of the genus *Antilophia* (Aves: Pipridae)

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ABSTRACT

The melanocortin-1 receptor gene is the most widely-used marker for the investigation of the genetic determination of melanic plumage patterns. Studies of a number of wild bird species have shown an association between non-synonymous mutations of the *MC1R* gene and the presence of melanic variants. The genus *Antilophia* (Pipridae) includes only two manakin species (*A. galeata* and *A. bokermanni*), which are distinguished primarily by the differences in the pattern of melanic coloration of the plumage of the mantle in the adult males. In *A. galeata*, this plumage is black, while in *A. bokermanni*, it is predominantly white. This study investigates the possible association between mutations of the *MC1R* marker and the variation in plumage coloration observed in the two species. The *MC1R* sequences of the two species was analyzed, and the observed nucleotide variation was compared. Six polymorphic sites were identified, representing seven distinct genotypes. Five of these polymorphic mutations were non-synonymous, but were not related to the different phenotypes. Neutral evolution and the absence of any systematic association between the variants of the *MC1R* and plumage coloration in the *Antilophia* species indicate that alternative mechanisms regulate the expression of the coloration of the plumage in the adult males.

Key words: candidate gene, manakin, melanism, neutral evolution, plumage color.

INTRODUCTION

The evolution of plumage coloration in birds may be driven by a range of factors related to both natural

and sexual selection, constituting an important speciation mechanism in many taxonomic groups (Dunn et al. 2015). Part of the variation in plumage color within and between species is known to have a strong genetic component (Mundy 2005,

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Roulin and Ducrest 2013), although the genetic mechanisms involved in this process are still poorly-understood, especially in wild species.

The melanocortin-1 receptor (*MC1R*) gene is the most popular genetic marker for the analysis of melanic patterns in bird plumage (Uy et al. 2009, Bourgeois et al. 2012). In most cases, mutations of the *MC1R* gene that cause an increase in its receptor activity are dominant, and are associated with black coloration (Bourgeois et al. 2012, Roulin and Ducrest 2013). However, a growing number of studies have shown that variations in the *MC1R* sequence may not always be associated with distinct patterns of coloration in birds (Haas et al. 2009, Bourgeois et al. 2012).

The genus *Antilophia* is formed by two species of manakin (Pipridae, Passeriformes). The principal difference between the two species – *Antilophia galeata* and *Antilophia bokermanni* – is observed in the coloration of the plumage of the adult males. In *A. galeata*, the cover feathers are black, whereas in *A. bokermanni*, these feathers are white (Coelho and Silva 1998), contrasting with the black tertial and rectrice flight feathers (Figure 1).

Recent studies have shown that the genetic profiles of the two species are virtually indistinguishable (Rêgo et al. 2010, Luna et al. 2017), and that the mixture of genetic features observed in the two species is likely the result of the incomplete separation of the two lineages following a very recent process of diversification (Luna et al. 2017). In this context, a single mutation in the *MC1R* gene might have resulted in a change in the melanin levels of the feathers (Uy et al. 2009), resulting in a distinct phenotype, which would be fixed rapidly in small populations, such as that of *A. bokermanni*.

In the present study, we investigated whether possible mutations in the codifying region of the *MC1R* gene are associated with the process that determines the difference in the principal phenotypic characteristic that distinguishes the two *Antilophia*

species. To this end, we attempted to resolve two principal questions: 1) Is the inter- and intra-specific variation in the *MC1R* sequences associated directly with the different melanic patterns observed in the plumage of the two *Antilophia* species? and 2) Does the variation in the *MC1R* nucleotide sequences reflect selection pressures driving the fixation of the non-synonymous mutations that determine the phenotypes of the two species?

MATERIALS AND METHODS

SAMPLING AND GENETIC SEQUENCING

Samples of the tissue of *A. bokermanni* (n = 16) were obtained from specimens captured in the northeastern portion of the Chapada do Araripe, in the state of Ceará, Brazil (Figure 2). Samples of the sister species *A. galeata* were obtained from researchers at the Federal University of São Carlos (UFSCar, n = 9) and the University of Brasília (UnB, n = 5).

Genetic material was extracted using the Wizard® Genomic DNA Purification kit (Promega) according to the maker's protocol. Part of the *MC1R* of each specimen was obtained using PCR reactions based on the following primers, described by Cheviron et al. (2006). We used standard methods described elsewhere (Carneiro et al. 2012, Luna et al. 2017) to amplify and obtain sequences for *MC1R* in an ABI 3500 XL automatic sequencer (Applied Biosystems).

The alignment and possible correction of the codification of the observed insertions and deletions were run in the Bioedit sequence editor (Hall 1999). All the sequences generated in the present study were deposited in GenBank (www.ncbi.nlm.nih.gov) under access numbers MG601527 through MG601534 (Table I).

GENETIC ANALYSIS

The *MC1R* sequences obtained were aligned with those available in GenBank for the species

TABLE I

List of the species analyzed in the present study with the identification code of each specimen, the variable sites identified in the genus *Antilophia* and the sites (marked in gray) that have been associated with melanic phenotypes in birds. The asterisks indicate sequences equal to the initial sequence (specimen AboCAA01). Heterozygous sites are denominated by a standardized letter code established by IUPAC (R = C / T). A genotype is a combination of two alleles. Genotype codes used in the Network program. GenBank access numbers for the samples analyzed.

Species (plumage)	Sample codes	Position sites ¹ (vertical)										Genotype	Genotype codes for Network	Access number GenBank
		1	2	2	2	2	3	5	6	6	6			
<i>A. bokermanni</i> (white)	AboCAA01	G	A	G	G	G	A	C	C	A	G	1/1	H_1	MG601527
	AboCAA02	*	*	*	*	*	*	*	*	*	*	1/1	H_1	MG601527
	AboCLU01	*	*	*	*	*	*	*	*	*	*	1/1	H_1	MG601527
	AboCLU02	*	*	*	*	*	*	*	*	*	*	1/1	H_1	MG601527
	AboCLU03	*	*	*	*	*	*	*	*	*	*	1/1	H_1	MG601527
	AboCLU04	*	*	*	*	*	*	*	*	*	*	1/1	H_1	MG601527
	AboCLU05	*	*	*	*	*	*	*	*	*	*	1/1	H_1	MG601527
	AboCVV01	*	*	*	*	*	*	*	*	*	*	1/1	H_1	MG601527
	AboBCE01	*	*	*	*	*	*	*	*	*	T	2/2	H_3	MG601529
	AboBCE02	*	*	*	*	*	*	*	*	*	*	1/1	H_1	MG601527
	AboBCE03	*	*	A	*	*	T	*	*	*	*	3/3	H_2	MG601528
	AboBME01	*	*	*	*	*	*	*	*	*	*	1/1	H_1	MG601527
	AboBME02	*	*	*	*	*	*	*	*	*	*	1/1	H_1	MG601527
	AboBME03	*	*	*	*	*	*	*	*	*	*	1/1	H_1	MG601527
	AboMHE02	*	*	*	*	*	*	*	*	*	*	1/1	H_1	MG601527
	AboMFU01	*	*	*	*	*	*	*	*	*	*	1/1	H_1	MG601527
<i>A. galeata</i> (black)	Aga39	*	*	*	*	*	*	*	*	*	*	1/1	H_1	MG601530
	Aga40	*	*	*	*	*	*	*	*	*	*	1/1	H_1	MG601530
	Aga41	*	*	*	*	*	*	*	*	*	*	1/1	H_1	MG601530
	Aga42	*	*	*	*	*	*	*	*	*	*	1/1	H_1	MG601530
	Aga43	A	*	*	*	*	*	*	*	*	*	4/4	H_4	MG601531
	Aga410	*	*	*	*	*	*	*	*	*	*	1/1	H_1	MG601530
	Aga519	*	*	*	*	*	*	*	R	*	*	1/5	H_6	MG601533
	Aga545	*	*	*	*	*	*	*	*	*	*	1/1	H_1	MG601530
	Aga761	*	*	*	*	*	*	*	T	*	*	5/5	H_5	MG601532
	Aga795	*	*	*	*	*	*	T	*	*	*	6/6	H_7	MG601534
	Aga822	*	*	*	*	*	*	*	*	*	*	1/1	H_1	MG601530
	Aga906	*	*	*	*	*	*	*	*	*	*	1/1	H_1	MG601530
	Aga1010	*	*	*	*	*	*	*	*	*	*	1/1	H_1	MG601530
	Aga1130	*	*	*	*	*	*	*	*	*	*	1/1	H_1	MG601530

¹Nucleotide sequences numbered according to their position in the *MC1R* sequence of the chicken (*G. gallus* – access number NM_001031462).

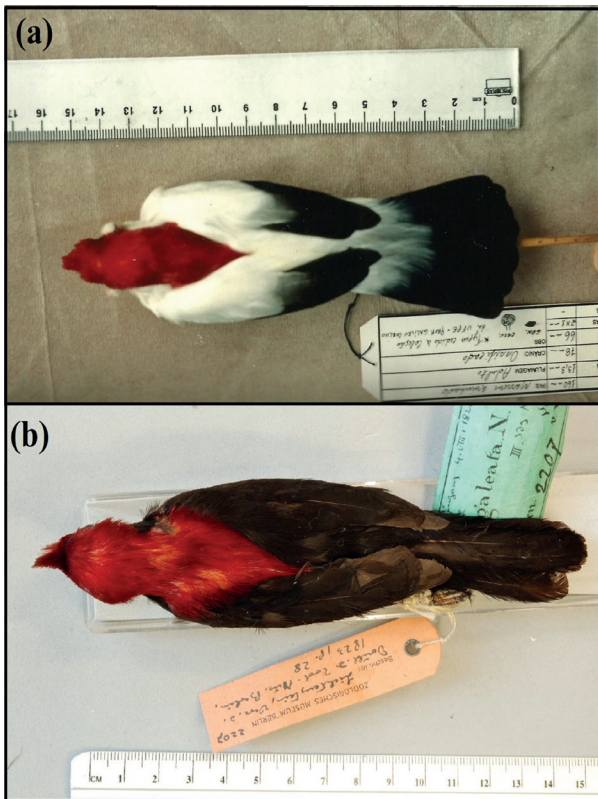


Figure 1 - Dorsal view of the holotypes of (a) *Antilophia bokermanni* (No. 1130 – Ornithological Collection of the Universidade Federal do Pernambuco, Recife) and (b) *Antilophia galeata* (ZMB#2207 – Ornithological Collection of the Zoologisches Museum, Berlin).

Lepidothrix coronata (access numbers DQ388308 and DQ388310), *L. serena* (DQ388331), and *Gallus gallus* (NM_001031462). This comparative analysis permitted the identification of variation in the sites of nucleotides known to be associated with melanic plumage in birds.

The genotype network was prepared using the maximum likelihood method in Haploviewer (Salzburguer et al. 2011). Fu’s *F_s* test (Fu 1997) was applied to evaluate the distribution of the genotype frequencies in comparison with that expected according to a neutral model, while Tajima’s *D* (Tajima 1989) was used to assess the difference between the number of segregating sites and the mean number of nucleotide differences, which were calculated in DNAsp 5.1 (Librado and Rozas

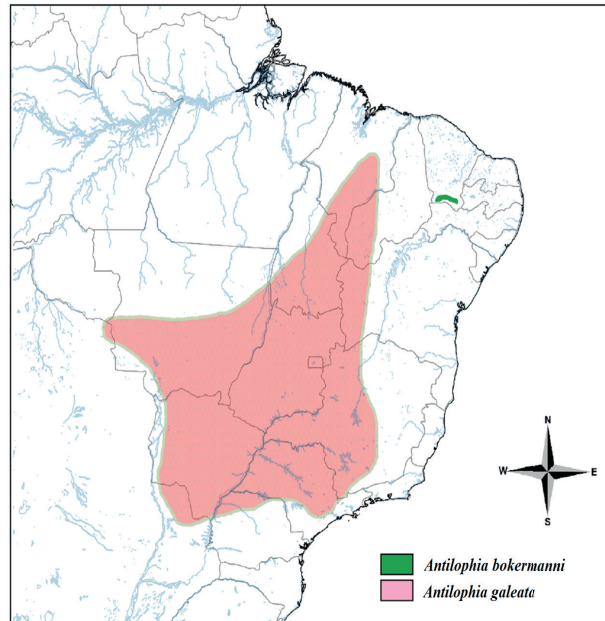


Figure 2 - Geographic distribution of *A. bokermanni* and *A. galeata*. The geographic ranges of the two species were adapted from NatureServe.

2009). The McDonald-Kreitman test (McDonald and Kreitman 1991) was used to examine the possible occurrence of positive selection, and to identify putative sites of functional importance in the two species (these analyses were also run in DnaSP).

RESULTS AND DISCUSSION

No evidence was found of selection or association between the mutations of the *MC1R* gene and the melanic variation in *Antilophia*. This reinforces the findings of previous studies, which found no association between phenotypic differences and the variation in the *MC1R* gene (Cheviron et al. 2006, Haas et al. 2009, Bourgeois et al. 2012), which rejects our first hypothesis, that changes in this codifying region may determine the differences in the melanic coloration of *A. galeata* and *A. bokermanni*.

In the present study, 702 base pairs, corresponding to positions 156-858 of the 945

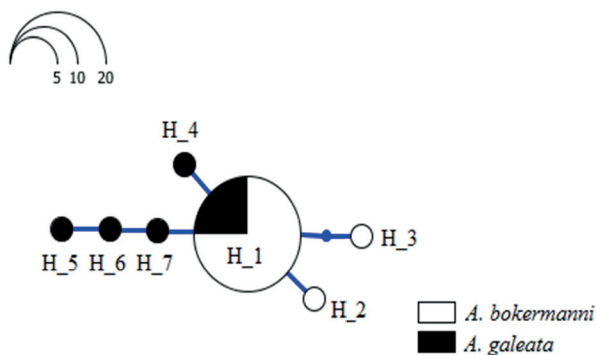


Figure 3 - Genotype network generated from the sequences of the *MC1R* generated in the present study for *A. bokermanni* and *A. galeata*. The genotypes are represented by circles, the size of which is proportional to their frequency. The white shading represents the samples of *A. bokermanni* and black shading, the samples of *A. galeata*.

base pairs of the *MC1R* sequence of *G. gallus*, were sequenced (Kerje et al. 2003). Ten sites were polymorphic, providing a total of six *MC1R* alleles in *Antilophia*. All the variable *MC1R* sites known to be associated with melanic patterns were conserved in the *Antilophia* specimens analyzed here (see the gray shaded column in Table I: the numeration corresponds to that of the *MC1R* sequence of *G. gallus*: Kerje et al. 2003). While non-synonymous mutations were recorded in some specimens, they were not shared by other individuals of the same species. In addition, the fact that the H_1 genotype was found at high frequencies in both species (Figure 3) confirms the lack of any systematic influence of the *MC1R* on the distinct plumage patterns. These findings indicate that the changes in the *MC1R* gene may not have resulted in functional changes in the expression of the melanic coloration found in *Antilophia*. A similar situation was observed by Uy et al. (2009) in the Chestnut-bellied monarch, *Monarcha castaneiventris*, from the Solomon Islands.

In the case of *Antilophia*, this alternative mechanism may have arisen from changes in the interaction of the *MC1R* gene with associated regulator pathways. The alterations in these mechanisms may have resulted in the differential

expression of the coloration pattern in the closely-related *Antilophia* species (Cone et al. 1996, Steiner et al. 2007, Manceau et al. 2011). In the specific case of *A. bokermanni*, this would account for the restriction of the black pigment to the flight feathers (Figure 1).

Due to the intense sexual selection that is characteristic of the manakins, the coloration of the male plumage tends to evolve rapidly and independently, with little convergence among taxa (Prum 1997). In the context of the very recent divergence of the two *Antilophia* species (Luna et al. 2017), a small population, such as that of *A. bokermanni*, would be subject to intense selection pressures due to its isolation, which may have contributed to the rapid fixation of a new plumage coloration pattern. The achromatic variation and that in the brilliance of the plumage may be one of the most important attributes determining female mate choice and competition between males (Doucet et al. 2005, Stein and Uy 2006, Laczi et al. 2011). However, it is still necessary to determine the role of these plumage characteristics in the reproductive success of these species, in particular in *A. bokermanni*.

Even so, the changes in the pattern of melanic coloration may be associated with a number of different selection pressures. In the present study, the results of the neutrality tests for the *MC1R* sequences did not contradict the hypothesis that the gene may be evolving in a neutral fashion. The values of Fu's F_s and Tajima's D , calculated for each species, found no evidence of non-neutral variation in either *A. bokermanni* (D : -1.6965 and F_s : -0,5109; $p > 0.05$) or *A. galeata* (D : -1.2782 and F_s : -0,5109, $p > 0.05$).

The result of the McDonald-Kreitman (MK) test did not indicate any evidence of evolutionary adaptation, given that none of the mutations were fixed, even though the majority were non-synonymous in relation to the amino acids codified. This reinforces the conclusion that the variability

found in the *MC1R* gene was not the result of functional changes, but rather that there has been some differential modification in the regulatory mechanism of its interaction with the melanin receptors it controls in the two *Antilophia* species.

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