Plumage polymorphism and variation in the melanocortin-1 receptor gene in the Fuscous Flycatcher, *Cnemotriccus fuscatus* (Wied, 1831)

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**ABSTRACT:** We investigated the possible mechanisms behind the variation plumage color of the Fuscous Flycatcher, *Cnemotriccus fuscatus*, by sequencing the melanocortin-1 receptor (MC1R) gene, which has been associated with the variation in plumage coloration in birds. *C. fuscatus* is widely distributed in South America and includes seven subspecies, which differ in their plumage coloration. Here we tested the hypothesis that the variation in the MC1R gene explains the plumage polymorphism found in *C. fuscatus*. We sequenced the MC1R gene in six subspecies, representing two groups: group 1 (yellow morph), with three subspecies, *C. f. duidae*, *C. f. fumosus*, and *C. f. fuscatus*, and group 2 (white morph), with the remaining subspecies, *C. f. bimaculatus*, *C. f. beniensis*, and *C. f. fuscatior*. The only variation we found among the *C. fuscatus* sequences were six non-synonymous substitutions from 22 variable sites, none of which were associated systematically with either plumage morph. The result of the neutrality test indicated that the polymorphism of the MC1R gene is not suggestive of significant selection pressure. We conclude that variation in plumage coloration in *C. fuscatus* does not appear to be determined by the MC1R gene, and that it may be related to other loci or under the influence of environmental factors.

**KEY-WORDS:** birds, MC1R gene, mutation, pigmentation, Tyrannidae.

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**INTRODUCTION**

The variation in plumage coloration has been studied from ecological, evolutionary and genetic perspectives (Hoekstra & Price 2004, Mundy 2005, Uy et al. 2016). Such diversity has been related to visual communication, and may have evolved in response to the evolution of the avian visual system (Osorio & Vorobyev 2008), although there is also some evidence that changes in plumage coloration may be a response to varying pressures in different types of habitat (Gomez & Théry 2004, McNaught & Owens 2002). Many questions remain unresolved, however, on the evolution of plumage coloration and its relation to speciation in birds (Stoddard & Prum 2011, Seddon et al. 2013), such as the mechanisms that mediate the change in coloration between juveniles and adults (Galván & Jorge 2015), and the factors determining changes in coloration despite the considerable energetic costs of this process (Legagneux et al. 2012, Mercadante & Hill 2014).

Previous studies (e.g., Robbins et al. 1993, Vidal et al. 2010, Johnson et al. 2012) have suggested that the melanocortin-1 receptor (MC1R) gene may be involved in the differentiation of the plumage in avian species, due to the association between mutations in this gene and the phenotypic variation found in a number of different groups of wild birds (Johnson et al. 2012, Ran et al. 2016). For example, single non-synonymous mutations in the MC1R gene were associated with plumage polymorphisms in the bananaquit (*Coereba flaveola*) and the chestnut-bellied monarch, *Monarcha castaneiventris* (Theron et al. 2001, Uy et al. 2009). Studies in birds have also shown that the MC1R gene controls the amount of both eumelanin (brown/black) and pheomelanin (red/yellow) produced (Takeuchi et al. 1996, Wen et al. 2015). In particular, García-Borrón et al. (2005) showed that the yellow (pheomelanin) phenotype is produced by recessive MC1R extension (e) alleles.

In this context, we investigated the variation in coloration found among the subspecies of the Fuscous Flycatcher, *Cnemotriccus fuscatus*, a monotypic genus widely distributed in South America (Fig. 1). There are seven *C. fuscatus* subspecies, which are differentiated not only on the basis of their morphological characters, but also their vocalizations and ecology (Fitzpatrick et al. 2004). These subspecies can be divided into two groups,
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Species distribution

- *C. f. duidae*
- *C. f. fuscatus*
- *C. f. fumosus*
- *C. f. bimaculatus*
- *C. f. fuscatior*
- *C. f. beniensis*

based primarily on the coloration of the belly, which is either white or yellow. These flycatchers can be found in a variety of habitats, including fluvial islands, rainforest, dry forests, riparian habitats, and lowland and secondary forests (Rasmussen & Collar 2002). It is thus important to understand which factors may influence the variation in the coloration of plumage found among the different subspecies of the Fuscous Flycatcher (Farnsworth & Lebbin 2017). In particular, if a relationship can be found between genotype and phenotype, it might represent evidence of the role of natural selection in the fixation of subspecific coloration patterns (Hewitt 1988, Chunco et al. 2007).

Here we investigated the possible mechanisms that determine differentiation in plumage amongst the subspecies of *C. fuscatus*. Specifically, we tested whether non-synonymous mutations in the sequence of the melanocortin-1 receptor (*MC1R*) gene were associated systematically with variation in plumage coloration amongst the six subspecies, and whether these mutations are suffering selection pressures.

**METHODS**

We sequenced 27 samples of *Cnemotriccus fuscatus* muscle tissue (Table 1), representing six of the seven described subspecies. The samples were provided by the Goeldi Museum (MPEG: Museu Paraense Emilio Goeldi) in Belém, and the National Museum (MNRJ) in Rio de Janeiro. We followed the classification of Fitzpatrick et al. (2004) to allocate the subspecies to two groups (yellow and white morphs). Group 1 (yellow morph) was composed of *Cnemotriccus fuscatus duidae* (*n* = 5 specimens), *Cnemotriccus fuscatus fumosus* (*n* = 7), and *Cnemotriccus fuscatus fuscatior* (*n* = 5), which are ventrally yellow to light yellow. Group 2 (white morph) contained the other three subspecies, *Cnemotriccus fuscatus bimaculatus* (*n* = 5), *Cnemotriccus fuscatus beniensis* (*n* = 3), and *Cnemotriccus fuscatus fuscatior* (*n* = 2), which are ventrally white or light gray. Both male and female specimens were included, as *C. fuscatus* is not dichromatic (Fitzpatrick et al. 2004).

Total DNA was isolated from the muscle tissue using the Wizard® Genomic DNA purification kit (Promega), following the manufacturer’s instructions. To obtain a partial sequence of the *MC1R* gene, we amplified the samples by PCR using the primers described by Cheviron et al. (2006): lcorMSHR9 (5’ – CTG GCT CCG GAA GGCTA Gat – 3’) and lcorMSHR72 (5’ – AYG CGY GYG GCA ACC A – 3’). The PCR conditions were the same as those used by Cheviron et al. (2006), and the PCR products were sequenced by Sanger’s dideoxiterminal method (Sanger et al. 1977), using an ABI 3500 automatic sequencer.

The DNA sequences were aligned and their nucleotides were compared to those from the bananaquit, *Coereba flaveola* (GenBank access numbers AF362598 and AF362601) and *Gallus gallus* (AB201631) using Bioedit.

**Figure 1.** Map showing the distribution of samples of *Cnemotriccus fuscatus* sequenced in this study. Yellow points represent specimens with yellow bellies and white points denote specimens with white bellies.
Table 1. Location, coordinates, subspecies, voucher number, identification code, and source of tissue used in this study.

| Locations (Coordinates)          | Subspecies      | Voucher number | Identification of the tissue | Institutions |
|---------------------------------|-----------------|----------------|-------------------------------|--------------|
| Óbidos - PA (00°37’50”N; 55°43’40”W) | *C. f. fumosus*  | CN1410, CN1378,  | Cfu1410, Cfu1378,              | MPEG*        |
| Chaves - PA (00°12’29.2”S; 49°58’39.2”W) | *C. f. fumosus*  | MARJ117, MARJ118 | Cfu118, Cfu117                | MPEG         |
| Marajó - PA (00°59’21”S; 49°56’24”W) | *C. f. fumosus*  | MAYA008         | Cfu008                        | MPEG         |
| Oriximiná - PA (1°45’36.89”S; 55°51’30.28”W) | *C. f. fumosus*  | ORX336, ORX359   | Cfu336, Cfu359                | MPEG         |
| Porto Walter - AC (08°20’35.7”S; 72°36’19.7”W) | *C. f. duidae*   | UFAC1021        | Cfu1021                       | MPEG         |
| Japura - AM (02°02’31.5”S; 67°17’16.6”W) | *C. f. duidae*   | JAP225, JAP267,  | Cfu225, Cfu267, Cfu270        | MPEG         |
| Porto Walter - AC (08°20’35.7”S; 72°36’19.7”W) | *C. f. duidae*   | UFAC0976        | Cfu0976                       | MPEG         |
| Uruçuí - PI (07°14’2.00”S; 44°33’1.55”W) | *C. f. bimaculatus* | URC171        | Cfu171                        | MPEG         |
| Curimatá - PI (09°41’284”S; 44°14’200”W) | *C. f. bimaculatus* | SRV005        | Cfu005                        | MPEG         |
| Redenção do Gurgueia - PA (09°38’022”S; 44°08’807”W) | *C. f. bimaculatus* | SRV042        | Cfu042                        | MPEG         |
| Borba, Puruzinho, Ilha - AM (04°07’42”S; 59°21’55.4”W) | *C. f. bimaculatus* | MAD500        | Cfu500                        | MPEG         |
| Autazes, Uricurituba, Ilha - AM (03°34’47”S; 59°07’50”W) | *C. f. bimaculatus* | MAD608        | Cfu608                        | MPEG         |
| Santa Catarina - SC (27°14’32.42”S; 50°13’7.88”W) | *C. f. fuscatus*  | TERNA210, TERNA398, TERNA1068, TERNA1349, Cachimbo470 | Cfu210, Cfu398, Cfu1068, Cfu1349, Cfu470 | MNRJ†        |
| Rio Branco - AC (09°57’32.3”S; 67°43’57.2”W) | *C. f. beniensis* | UFAC1199, UFAC1297, UFAC273 | Cfu1199, Cfu1297, Cfu273      | MPEG         |
| Monte Alegre - PA (2°3’14.72”S; 54°10’24.49”W) | *C. f. fuscator*  | PEMA042, PEMA037 | Cfu042, Cfu037                | MPEG         |

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A total of 744 base pairs were sequenced for each of the 27 *C. fuscatus* samples, representing nucleotides 129–873 of the *MC1R* gene of *Gallus gallus*, which includes all the sites known to be associated with plumage polymorphisms in birds (Theron et al. 2001). Only 21 samples were considered (pp >0.6) after the resolution of the gametic phases (Harrigan et al. 2008, Table 2). In the BLAST (NCBI: National Center for Biotechnology Information) analysis, the sequences were 95% similar to that of *Gallus gallus* (Kerje et al. 2003) and 97% similar to that of *Coereba flaveola* (Theron et al. 2001).

We identified 22 variable sites in the 21 *C. fuscatus* samples (Table 2), including all six subspecies. These variable sites of the *MC1R* locus determined six non-synonymous mutations for the codification of amino acids, A8G, S9R, S10N, S89N, V226I, and L240I (Table 3). None of these sites were associated with the coloration patterns of either the two groups or any of the subspecies. Tajima’s D was not significant (-1.603, *P* > 0.05), indicating that the variation found in the study locus in *C. fuscatus* is neutral, with a signal of recent demographic expansion, against the constant demographic model. All the sequences generated in the present study were deposited in GenBank (www.ncbi.nlm.nih.gov) under access numbers MK102986 through MK103006 (Table 3).

**DISCUSSION**

Our study of *Cnemotriccus fuscatus* indicates that there is no clear association between the plumage polymorphism found in this species and mutations of the *MC1R* gene. As in previous studies of bird species such as *Phylloscopus toutinegras* (MacDougall-Shackleton et al. 2003), *Lepidothrix coronata* (Cheviron et al. 2006), *Dendrocolaptes platyrostris* (Corso et al. 2013), *Philomachus pugnax*...
(Farrell et al. 2014) and the genus Antilophia (Luna et al. 2018), our findings reinforce the conclusion that this gene does not always play a role in the variation in plumage coloration found among populations or species. In this case, other genes or mechanisms may determine this variation, as observed in a number of birds (McLean & Stuart-Fox 2014).

A number of new genes associated with plumage coloration have been identified in recent years, although they have been analyzed in only a few species (Oribe et al. 2012, Bourgeois et al. 2016). Miwa et al. (2007), for example, found an association between mutations of the endothelin receptor B2 (EDNRB2) gene and the coloration of Cortunix japonica, with a non-synonymous substitution that alters an amino acid (R332H) being associated with the “panda” pattern, in contrast with the standard “dotted white” pattern. Other genes that may be involved in pigmentation in birds include the tyrosinase-related protein 1, TYRP1 (Xu et al. 2013, Bourgeois et al. 2016), SRY-Box containing 10, SOX10 (Gunnarsson et al. 2011), Agouti protein, ASIP (Oribe et al. 2012, Zhang et al. 2013), and Corin (Bourgeois et al. 2016) genes, and the proopiomelanocortin (POMC) gene cluster, which includes MC1R (Kang & Kim 2015).

In addition to genetics, the variation found in the coloration of C. fuscatus may be related to environmental factors, given the diversity of habitats occupied by the species (Fig. 1). Uy et al. (2009), for example, found that natural selection may favor distinct coloration in different habitats based on the existence of several population patterns, with habitats dominated by short-wavelength light (e.g., shaded woodland) favoring darker birds, and habitats rich in long-wavelength light (e.g., forest clearings with direct sunlight) favor lighter-colored species.

Furthermore, the studied part of the gene MC1R includes all the main sites that were showed in previous research with plumage polymorphism of birds (Mundy 2005, Cheviron et al. 2006). Overall, our results reinforce

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**Table 3. Position of non-synonymous variations within the amino acid Cnemotriccus fuscatus. GenBank access numbers for the samples analyzed.**

| Voucher – Subspecies | Amino acid positions | Belly Plumage | Access number |
|---------------------|----------------------|---------------|---------------|
| Cfu008 - C. f. fumosus | A S S S V L | Yellow | MK102986 |
| Cfu117 - C. f. fumosus | . . . . . . | Yellow | MK102987 |
| Cfu118 - C. f. fumosus | . . . N . . | Yellow | MK102988 |
| Cfu225 - C. f. duidae | . . . . . . | Yellow | MK102989 |
| Cfu0976 - C. f. duidae | . . S/N . . | Yellow | MK102993 |
| Cfu1021 - C. f. duidae | . . . . . . | Yellow | MK102994 |
| Cfu270 - C. f. duidae | . . . . . . | Yellow | MK102995 |
| Cfu005 - C. f. fuscatior | . . . . . . | White | MK103006 |
| Cfu273 - C. f. beniensis | G R S/N . . | White | MK102999 |
| Cfu1297 - C. f. beniensis | . . . . . . | White | MK103000 |
| Cfu500 - C. f. bimaculatus | . . . . . . | White | MK103001 |
| Cfu005 - C. f. bimaculatus | . . . . . . | White | MK103002 |
| Cfu171 - C. f. bimaculatus | . . . . . . | White | MK103003 |
| Cfu042 - C. f. bimaculatus | . . . . . . | White | MK103004 |
| Cfu042 - C. f. bimaculatus | . . . . . . | White | MK103005 |
the conclusion that understanding the evolution of plumage coloration in *C. fuscatus* with varying patterns of eumelanin/pheomelanin pigmentation requires a more profound investigation of the genes in the melanocortin pathway and their potential variation, as well as other loci and environmental factors. Unlike many other bird species (see e.g., Cheviron et al. 2006, Corso et al. 2013, Farrell et al. 2014, Luna et al. 2018), the variation in the plumage coloration of *C. fuscatus* does not appear to be related to mutations of the *MCIR* gene.

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