

# Unmelanized Plumage Patterns in Old World Leaf Warblers Do Not Correspond to Sequence Variation at the Melanocortin-1 Receptor Locus (*MC1R*)

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Evolutionary changes in patterns and coloration of plumage are likely to represent a major mechanism for speciation among birds, yet the molecular basis for such changes remains poorly understood. Recently much attention has focused on the melanocortin-1 receptor (*MC1R*) as a candidate locus for determining the level and extent of epidermal melanin deposition. We tested the hypothesis that *MC1R* sequence variation is associated with interspecific variation in unmelanized plumage pattern elements in Old World leaf warblers (genus *Phylloscopus*). This genus is characterized by a variety of plumage patterns that nonetheless vary along similar lines. Species vary in the presence or absence of pale (unmelanized) pattern elements against a dark background, and these patterns are used in species recognition and courtship. We sequenced most of the *MC1R* coding region for eight *Phylloscopus* species, representing the full range of plumage patterns found in this genus. Although *MC1R* sequence varied among species, this variation was not related to melanin-based plumage variation. Rather, evolution of this locus in these birds appears to be conservative. Ratios of nonsynonymous to synonymous substitutions ( $d_N/d_S$ ) were consistently low, suggesting that strong purifying selection has operated at this locus, and likelihood ratio testing revealed no evidence of variable selective pressures among lineages or across codons. Adaptive evolution at *MC1R* may be constrained by the adaptive importance of plumage pattern elements in this genus.

## Introduction

Plumage patterns and coloration are extremely diverse among bird taxa, and closely related species often differ strikingly in plumage characters (e.g., Omland and Lanyon 2000), suggesting that plumage may evolve quickly relative to other traits. Plumage characters are likely to be targets of both natural and, particularly among adult males, sexual selection. Specifically, these traits are often used in species recognition (Price 1998) and/or courtship displays (Marchetti 1993; Endler and Thery 1996). Thus, evolutionary changes in plumage patterns and in particular the distribution of melanins, are likely to represent an important mechanism for avian evolution.

There are many genes known to affect vertebrate pigmentation. For example, in mice, nearly 100 such loci have been identified, many of which form complex networks of interaction with one another (Barsh 1996). However, much recent attention has focused on the locus encoding the melanocortin-1 receptor (*MC1R*) as a promising candidate locus for melanization. *MC1R* is a seven-transmembrane G<sub>s</sub>-coupled receptor expressed on melanocytes (Mountjoy et al. 1992). The receptor is activated by melanocyte stimulating hormone (MSH), resulting in increased black/brown eumelanin production. The *MC1R* receptor has been strongly implicated in determining the level and extent of melanin deposition in the hair, skin, or feathers of a wide range of taxa, including mice (Robbins et al. 1993), humans (Valverde et al. 1995), pigs

(Kijas et al. 1998), and domestic chicken (Takeuchi et al. 1996b). Naturally and artificially selected polymorphisms at this locus include loss-of-function mutations (premature termination resulting in nonfunctioning receptors and red or yellow animals) and gain-of-function mutations (point mutations resulting in constitutively active receptors and black or brown animals) (Robbins et al. 1993). In addition, *MC1R* mutations may also have conserved effects across vertebrate taxa. For example, a point mutation associated with black coat color in mice (*Glu92Lys*) is also associated with black plumage in chickens (Robbins et al. 1993; Takeuchi et al. 1996b). This substitution has recently been implicated in plumage variation in a wild population of bananaquits (*Coereba flaveola*; Theron et al. 2001). These examples represent a rare case in evolutionary biology in which relatively simple changes at the DNA level can be linked to adaptive changes in phenotype.

These previous studies demonstrate that sequence variation in *MC1R* is frequently linked to striking variation in melanism within species. Among species, however, the molecular genetic basis for changes in melanistic phenotype is much less well understood, despite the probable evolutionary importance of such variation. The selective forces governing interspecific variation in plumage patterns are likely to be similar to those operating within a species. For example, plumage differences between species may reflect differences in light environment (Marchetti 1993) or in the intensity of sexual selection (Johnson and Lanyon 2000). However, the form taken by plumage variation differs somewhat within species versus among species. Specifically, melanistic and nonmelanistic morphs within a species often differ dramatically in their coloration (e.g., Takeuchi et al. 1996b; Theron et al. 2001; Lank 2002) and known *MC1R* variants generally affect melanism in large portions of the body. Conversely, melanistic variants among closely related species may be relatively subtle, differing only in the presence or absence of certain spatially restricted pattern elements (Johnson

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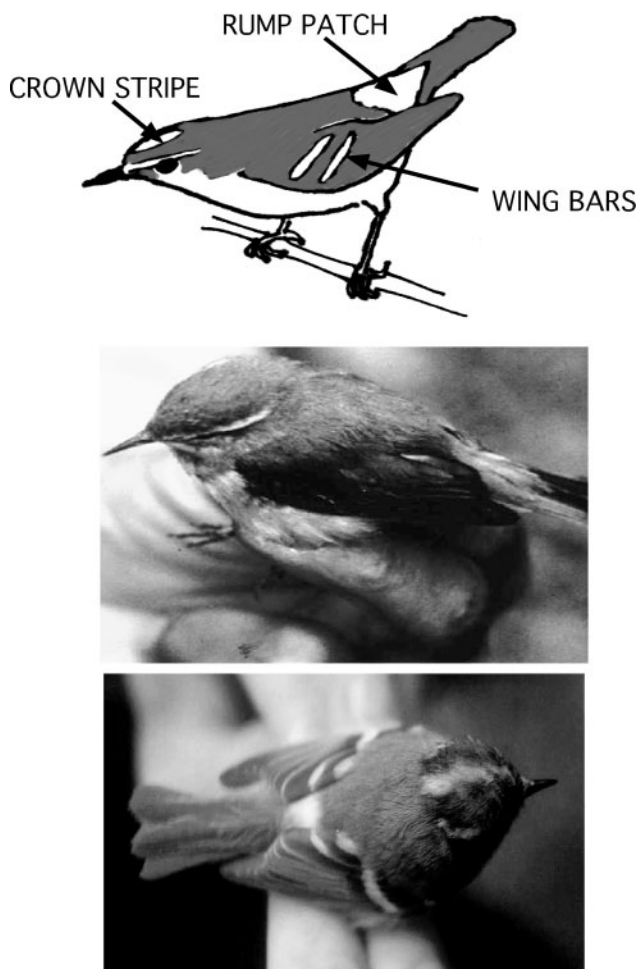


FIG. 1.—Top panel: Generalized *Phylloscopus* warbler, showing the locations of unmelanized plumage pattern elements. Center panel: *P. tytleri*, a species with no unmelanized pattern elements (except eyestripe). Bottom panel: *P. chloronotus*, a highly patterned species (note unmelanized wing bars, crown stripe, and rump patch). Photos provided by Trevor Price.

and Lanyon 2000; Price and Pavelka 1996). Sequence variation at *MCIR* has yet to be implicated in such small scale, localized patterns. However, at least one *MCIR* variant is associated with dorsal/ventral patterning in wild mice (Robbins et al. 1993), raising the possibility that smaller scale patterns might also be governed by variation at this locus. Thus, determining whether interspecific variation in melanistic pattern results from simple point substitutions at *MCIR* represents a crucial first step toward understanding the molecular bases for the evolution of plumage pattern elements.

The Old World leaf warblers (genus *Phylloscopus*) show a wide variety of melanin-based plumage patterns that nonetheless vary along similar lines. Each of the genus's approximately 40 species has dark upperparts, pale underparts, and a pale eyestripe (Price and Pavelka 1996). The species vary from this general theme in the presence or absence of additional pale pattern elements, such as wing bars, crown stripes, and rump patches (fig. 1). The dark background coloration results from eumelanin deposition in feathers (Prota 1992); pattern elements range

in color from white to yellow, due to an overlay of carotenoids, and result from a lack of melanin in the feather tip (Price and Pavelka 1996). Interspecific variation in plumage patterns is related to the birds' physical environment, with brighter, more patterned species occupying lower light habitats (Marchetti 1993). Moreover, pattern elements appear to be used in intraspecific communication. By flashing their unmelanized patterns during courtship and territorial displays, individuals make themselves temporarily more conspicuous. Experimentally altering the size of these pattern elements affects the ability of a male to defend a breeding territory (Marchetti 1993). Thus these plumage patterns are likely relevant both to speciation and to individual fitness in this group of birds.

Phylogenies of this taxon, based on mitochondrial DNA sequence, are available (Richman 1996; Price, Helbig, and Richman 1997) and have been used to infer the evolutionary history of pattern elements in these birds (Price and Pavelka 1996). The ancestral state appears to have been unpatterned, except for the pale underparts and the eyestripe found throughout the genus, and pattern elements have apparently been gained and lost over evolutionary time. Of the four clades identified within *Phylloscopus*, two are entirely unpatterned and two contain species with varying degrees of pattern. The distribution of pattern elements is nested, such that species with less common elements such as rump patches also have more common elements such as wing bars. Species with more elaborate pattern elements also tend to have larger wing bars than do less-patterned species (Price and Pavelka 1996).

Price and Pavelka (1996) noted that the distribution of pattern elements found among *Phylloscopus* species is consistent with a threshold mechanism of melanocyte sensitivity to epidermal signals. That is, a high threshold may be associated with more active melanocytes, leading to a lack of pattern, whereas a low threshold may reflect melanocytes that are highly sensitive to epidermal signals blocking melanin deposition, resulting in pale (unmelanized) pattern elements. Thus a global shift in receptor activity could theoretically result in the appearance or disappearance of local pattern. Because *MCIR* variants have been linked to changes in melanocyte activity within species (Robbins et al. 1993), we tested this hypothesis by comparing *MCIR* sequence variation to the evolutionary distribution of plumage pattern elements among *Phylloscopus* species.

## Methods

### Study Animals and Samples

Blood samples (approximately 100  $\mu$ L) were collected from individuals of eight *Phylloscopus* species that represent a variety of plumage patterns (fig. 1, table 1), ranging from unpatterned (eyestripe only) to highly patterned (eyestripe plus crown stripe, double wing bars, rump patch, and tail patch). For comparison purposes, we also collected samples from three other species of New World (*Vermivora peregrina*) and Old World warblers (*Seicercus burkii*; *S. xanthoschistus*). We sequenced *MCIR* in one individual per species, except *P. collybita*

**Table 1**  
**Unmelanized Pattern Elements (in Addition to Eyestripe and Pale Underparts) and Amino Acid Substitutions in *MC1R* for Eight *Phylloscopus* Species and Three Outgroup Warblers**

Species	Pattern elements	Amino acid at position						
		26	88	103	120	166	173	220
<i>Phylloscopus collybita</i>	None	V	G	V	M	V	S	S
<i>P. trochilus</i>	None	*	*	*	*	*	*/T	*
<i>P. fuscatus</i>	None	I	*	*	*	*	*	*
<i>P. tytleri</i>	None	*	*	L	*	*	*	*
<i>P. trochiloides</i>	Wing bars	*	S	*	T	*	*	F
<i>P. humei</i>	Double wing bars	*	*	*	*	*/I	*	*
<i>P. chloronotus</i>	Double wing bars							
	Crown stripe							
	Rump patch	*	S	*	*	*	*	*
<i>P. pulcher</i>	Double wing bars							
	Crown stripe							
	Rump patch							
	Tail patch	*	*	*	*	*	*	*
<i>Vermivora peregrina</i>	None	D	S	*	T	*	*	*
<i>Seicercus burkii</i>	Wing bars	*	S	*	T	*	*	*
<i>S. xanthoschistus</i>	None	*	S	*	T	*	*	*

NOTE.—An asterisk indicates identity with *P. collybita*; a forward slash indicates two amino acids within a species.

where two individuals were sequenced. Blood samples were stored in lysis buffer at 4°C until genomic DNA was isolated by phenol-chloroform extraction.

#### Characterizing the 3' Flanking Sequence of *MC1R*

We used inverse polymerase chain reaction (PCR) (Ochman et al. 1990) to identify the sequence 3' of the *MC1R* coding region. For this purpose, 500 ng of genomic DNA from *P. trochiloides* was digested overnight at 37°C with 10 U each of 10 restriction enzymes in a volume of 30 µL per digestion. After digestion, the enzymes were heat-inactivated and 400 ng of digested DNA was ligated overnight at 14°C with 5 U T4 DNA Ligase (Gibco-BRL), in a total volume of 125 µL per reaction.

To amplify the flanking regions of the *MC1R* locus, we designed two outward-facing nested primer pairs based on the chicken *MC1R* sequence (Takeuchi et al. 1996a). Primer sequences and positions are provided online as Supplementary Materials. First-round PCR reactions were done in a total volume of 50 µL and included 5 µL ligation mix as template DNA, 1X PCR buffer (Gibco-BRL: 20 mM Tris-HCl pH 8.4, 50 mM KCl), 2 mM MgCl<sub>2</sub>, 200 µM of each dNTP, 0.4 µM each of primers MSH 10R and MSH 11R, and 2.5 U Platinum Taq (Gibco-BRL). Cycling conditions were as follows: denaturation for 3 min at 94°C; 40 cycles of 60 s at 94°C, 60 s at 55°C, and 120 s at 72°C; and a final extension of 5 min at 72°C. Because these conditions amplified multiple fragments, we ran a second round of PCR using internal primers (MSH 8R and 9R) to amplify 1 µL of first-round PCR product. Reaction conditions were identical to first-round conditions, except that the number of cycles was decreased to 35 and extension time was reduced from 120 s to 60 s. Of the 10 restriction enzymes tested, two yielded single fragments that could be digested with the appropriate enzyme. These two candidates were sequenced with the ThermoSequense kit (Amersham) according to the manufacturer's protocol, using primers MSH 8R and 9R. Both candidates

contained sequence matching the last 45 bp of *MC1R* (96% nucleotide identity with the 3' end of chicken *MC1R*) and about 75 bp of readable 3' flanking sequence. Neither candidate contained the complete 5' end of the gene.

#### Sequencing *MC1R*

Using primers based on the 5' end of the chicken *MC1R* sequence (MSH 1), the 3' flanking sequence obtained for *P. trochiloides* (MSH 12), and two internal primers based on chicken *MC1R* sequence (MSH 8 and 9), we were able to amplify two overlapping fragments containing most (minimum 885 of 942 bp) of the *MC1R* coding sequence for each individual. The PCR reactions (total volume 50 µL) included 100 ng genomic DNA, 1X PCR buffer (Gibco-BRL), 2 mM MgCl<sub>2</sub>, 200 µM of each dNTP, 0.4 µM of each primer (MSH 1 and 9, or MSH 8 and 12), and 2.5 U Platinum Taq (Gibco-BRL). Thermocycling conditions were as follows: denaturation for 3 min at 94°C; 40 cycles of 60 s at 94°C, 60 s at 55°C (58°C for primer pair MSH 1 and 9), and 75 s at 72°C; and a final extension of 7 min at 72°C. The PCR products were purified on a 1% agarose gel, and both strands were directly sequenced with primers MSH 1, 8, 9, and 12 on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems) according to the manufacturer's protocol.

Sequences were aligned manually using MacClade 4 (Maddison and Maddison 2001). In a small number of cases ( $n=4$  individuals) we noted the presence of double peaks at single sites along the sequence, where the height of the chromatograph peaks for two different bases were roughly equal in height. If inspection of the chromatograph for the other strand showed a similar situation for complementary bases, we considered the individual to be heterozygous at that site. For each species, the overlapping fragments generated at least 899 bp of readable sequence, from nucleotide position 58 (based on chicken *MC1R* sequence) to 11 bp 3' of the termination codon. Thus we were able to

predict the sequence of all but the first 19 amino acids of this 314 amino acid protein. *MCIR* sequences have been deposited in GenBank (accession numbers AY308747–AY308757). Only the coding portion of the sequences (885 bp) is considered in the analyses reported below.

### Phylogeny Construction

We used unweighted parsimony to build a phylogeny based on *MCIR* sequences from the eight *Phylloscopus* species and rooted with the outgroup *V. peregrina*. Parsimony analysis was done in PAUP 4.0b10 (Swofford 2002), using a heuristic search method and 10 replicates of random stepwise addition of taxa. Intraspecific variation had no effect on the relationships between species, and only one sequence per species (selected randomly) was used in the phylogeny reconstruction. We used MacClade 4.03 (Maddison and Maddison 2001) to identify the most parsimonious reconstructions for amino acid substitutions and plumage evolution.

### Testing for Selection

Adaptive or diversifying selection at a locus can be inferred when the rate of nonsynonymous substitutions per nonsynonymous site ( $d_N$ ) exceeds that of synonymous substitutions per synonymous site ( $d_S$ ). To investigate whether *MCIR* has undergone adaptive selection within *Phylloscopus*, we first estimated  $d_N/d_S$  within this genus. We used a maximum likelihood model of codon substitution, implemented by the program PAML 3.13 (Yang 1997), to generate pairwise measures of  $d_N$  and  $d_S$  between *Phylloscopus* species. Codon frequency parameters were calculated from the average nucleotide frequencies at the three codon positions.

Because most proteins are under purifying selection most of the time, in practice  $d_N/d_S$  rarely exceeds 1 even if some adaptive substitutions have occurred (e.g., Sharp 1997). Another powerful approach is to compare  $d_N/d_S$  ratios among lineages (Messier and Stewart 1997; Yang 1998). Episodic molecular evolution (suggesting variable selective pressure in some lineages, due either to adaptive selection or relaxed constraint) is inferred when  $d_N/d_S$  varies significantly among lineages. We used a likelihood ratio test (Yang 1998) to compare a one-ratio model of sequence evolution (where  $d_N/d_S$  is identical for all branches) to a free-ratio model (where  $d_N/d_S$  is independent for each branch) to test the constancy of  $d_N/d_S$  at *MCIR* among evolutionary lineages. The models were tested using the three most-parsimonious independent phylogenies (not shown), based on cytochrome B sequence data previously reported for these species (Richman 1996; Price, Helbig, and Richman 1997). We constructed the cytochrome B phylogenies in the same way as the *MCIR* phylogeny described above, with two exceptions. First, *V. celata* was used as an outgroup rather than *V. peregrina*, because cytochrome B sequence was not available for the latter; second, only transversions were considered because of the high saturation of transitions at this locus (Richman 1996). Likelihood values were calculated using PAML 3.13 (Yang 1997), and a Bonfer-

roni correction was applied as multiple phylogenies were tested. To address the possibility that selection pressure (and thus  $d_N/d_S$ ) may vary across codons, we ran additional likelihood ratio tests comparing a neutral model of several models of heterogeneous selection pressure. The models we tested corresponded to the one-ratio model (all sites have the same  $d_N/d_S$ ), the neutral model (sites are either conserved or neutral), and the selection model (sites are either conserved, neutral, or positively selected) described by Yang et al. (2000).

### Results

The majority-rule consensus sequence obtained for *MCIR* in the eight *Phylloscopus* species surveyed is available online as Supplementary Materials. Overall, the *Phylloscopus MCIR* sequence shows 96%–97% nucleotide identity to that reported for other songbirds (bananaquits, and hooded tanagers *Tangara cucullata*; Theron et al. 2001) and 91% identity to chicken *MCIR* sequence (Takeuchi et al. 1996a). Alignment of this sequence with chicken *MCIR* indicates that the region we sequenced contains all seven putative transmembrane domains.

The 885 bp we analyzed contained 36 sites that varied within *Phylloscopus*. Of these 36 variants, 29 were synonymous and seven were nonsynonymous. Several polymorphisms occurred only in presumed heterozygotes. Excluding these variants, which leaves 22 synonymous and six nonsynonymous polymorphisms among species, does not alter the results presented below.

### *MCIR* Variation and Plumage Patterns

None of the seven amino acid substitutions showed a pattern with respect to plumage pattern elements. That is, we observed no variants unique to either the least-patterned or most-patterned species (table 1). Likewise, none of the species we surveyed contained either a Glu92Lys substitution, associated with melanism in other birds and mammals, or a premature termination variant, associated with loss of *MCIR* function and pale coloration.

Figure 2 shows one of the two parsimony trees obtained from *MCIR* sequences. Another equally parsimonious tree differs slightly in the placement of *P. collybita* and is less well resolved. Overall, the *MCIR* phylogeny is very similar in topology to those generated from cytochrome B for this genus (Richman 1996; Price, Helbig, and Richman 1997). For example, both identify *trochiloides* as relatively distant from other taxa, and both identify *pulcher/chloronotus/humei* and *trochilus/collybita* as clades, although the specific order of some branches differs between *MCIR*-based and cytochrome B-based phylogenies. The most parsimonious reconstructions of amino acid substitutions and plumage pattern changes are marked in figure 2. Again, we observed no clear pattern between amino acid substitutions and changes in plumage pattern elements.

### Testing for Selection

Nonsynonymous substitutions at *MCIR* appear to have occurred at a slower rate than synonymous

substitutions. Pairwise  $d_N/d_S$  ratios between *Phylloscopus* species were all less than 1 (range 0–0.115; mean 0.029). Likelihood ratio tests comparing a one-ratio model to a free-ratio model of codon evolution indicated no significant variation in  $d_N/d_S$  ratios within *Phylloscopus* ( $2\Delta L = 16.24, 13.57, 10.78$  for the three phylogenies tested; 15 df; all  $P > 0.05$ ). Likelihood ratio tests comparing one-ratio or neutral models to a model incorporating selection also revealed no significant variation in  $d_N/d_S$  across sites (one-ratio versus selection,  $2\Delta L = 5.70, 2$  df,  $P > 0.05$ ; neutral versus selection,  $2\Delta L = 0.04, 2$  df,  $P > 0.05$ ). No codons under any of the models tested were found to have  $d_N/d_S > 1$  (range 0.019–0.351; mean 0.035).

## Discussion

### MC1R Is Not Related to Plumage Variation

We found no relationship between variation in *MC1R* coding sequence and plumage variation among *Phylloscopus* species (table 1). This finding is in contrast to many studies of other birds and mammals (reviewed above) that have linked *MC1R* mutations to dramatic changes in melanistic phenotype within species. In rock pocket mice (*Chaetodipus intermedius*), *MC1R* genotype correlates perfectly with melanistic phenotype in some populations but not others (Hoekstra and Nachman 2003; Nachman, Hoekstra, and D'Agostino 2003), indicating that melanism has arisen independently through variation at *MC1R* and at other loci. Melanistic plumage variation in *Phylloscopus*, similarly, appears to be achieved through one or more alternative molecular mechanisms, possibly including *MC1R* regulatory variation (Moro, Ideta, and Ifuku 1999) or variation at *Agouti*, an antagonist to *MC1R* (Lu et al. 1994).

None of the warblers we surveyed showed the Glu92Lys mutation associated in other taxa with melanism and a constitutively active receptor (Robbins et al. 1993; Takeuchi et al. 1996b; Theron et al. 2001). Conversely, we found no premature termination mutants such as that associated in dogs with yellow coat color and apparent loss of *MC1R* function (Everts, Rothuizen, and van Oost 2000; Newton et al. 2000). Presumably, then, the *MC1R* locus encodes a functionally normal receptor throughout *Phylloscopus*. Because we did not sequence the first 17 codons of *MC1R*, we cannot conclusively dismiss the possibility that we may have missed important variants upstream of the sequenced portion. However, no other studies to our knowledge have reported a relationship between melanism and sequence variation in this initial part of the coding region.

Of the seven amino acid substitutions we observed within *Phylloscopus*, three (Ser88Gly, Thr120Met, Ser220Phe) were nonconservative, involving the substitution of hydrophobic for polar residues or vice versa. The Ser88Gly variant is of particular interest, as serine in this position is generally conserved across vertebrate species (Takeuchi et al. 1996a; Kijas et al. 1998; Theron et al. 2001). This mutation occurs in the second transmembrane domain, where several gain-of-function mutations have been identified in other species (Takeuchi et al. 1996b; see Table 3 in Newton et al. 2000; Theron et al.

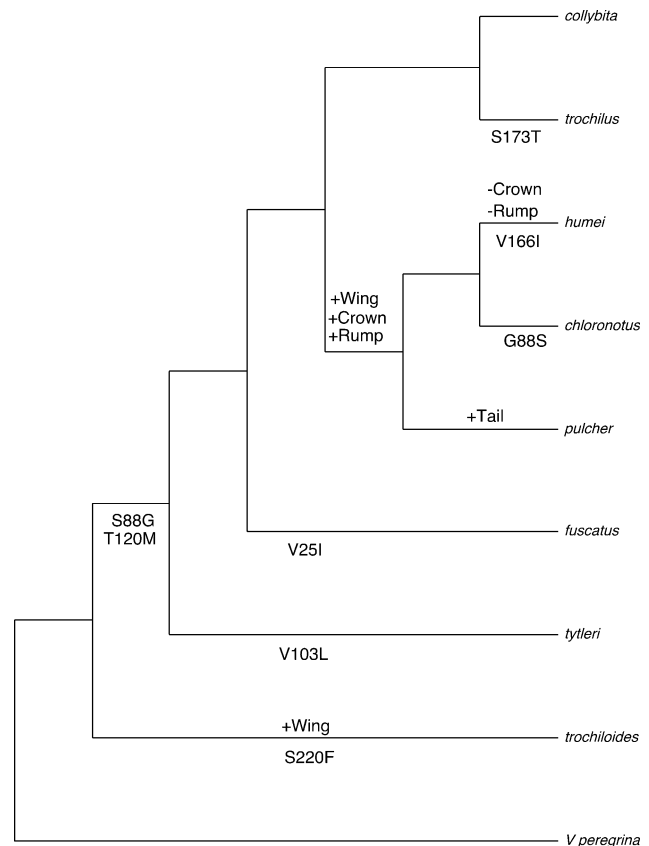


FIG. 2.—Parsimony tree based on *MC1R* sequence for eight *Phylloscopus* species, and rooted with the outgroup *Vermivora peregrina*. Most parsimonious reconstructions of pattern elements being gained (+) and lost (–), and amino acid substitutions, are shown above and below branches, respectively. “Wing” = wing bars, “Crown” = crown stripe, “Rump” = rump patch, and “Tail” = tail patch. An equally parsimonious reconstruction is, for both crown stripes and rump patches, to appear twice, along the lineages leading to *pulcher* and *chloronotus*.

2001). The equivalent substitution in dogs (Ser90Gly) is associated with a black or brown coat (Newton et al. 2000). In *Phylloscopus*, however, this variant does not appear to enhance *MC1R* activity. First, it occurs in the most-patterned species we surveyed (*P. pulcher*) as well as in the four least-patterned (*collybita*, *trochilus*, *tyleri*, and *fuscatus*; table 1). Second, these four least-patterned species are not more melanistic than the inferred ancestral state (Price and Pavelka 1996). Conversely, no variant occurred exclusively in the more-patterned species (table 1); thus we cannot explain the appearance of pattern elements through reduced activity at *MC1R*. The *Phylloscopus* are old species, with speciation events thought to have occurred as long ago as 12 MYA (Price et al. 2000). These very long time spans provide opportunities for convergent substitutions at other loci that might obscure any associations between *MC1R* sequence and plumage patterns.

### Nature of Selection at *MC1R*

We found no evidence that *MC1R* has undergone adaptive selection in *Phylloscopus*. The ratios of nonsynonymous changes per nonsynonymous site to

synonymous changes per synonymous site ( $d_N/d_S$ ) are well below 1, suggesting that *MC1R* has evolved under strong purifying selection within this genus. Selection pressures at *MC1R* did not vary significantly among lineages, suggesting that none of the species we surveyed have undergone either adaptive evolution or relaxation of purifying selection at this locus. Similarly, we found no evidence of heterogeneous selection pressure among amino acid sites, or of positive selection at any sites. Testing for positive selection is admittedly difficult, especially without extensive within-species data. Although we observed no variation in selection pressure either among lineages or among sites, due to the conservative nature of our tests, we cannot completely dismiss the possibility that a small number of adaptive substitutions may have occurred in some lineages. Finally, the general concordance between *MC1R*-based and cytochrome B-based phylogenies also suggests that sequence divergence at *MC1R* reflects evolutionary relationships rather than a different trajectory via adaptive evolution.

Our findings indicate that variation in *MC1R* is not associated with variation in plumage patterns among *Phylloscopus* species. Rather, purifying selection may have stabilized *MC1R* expression at a certain level, perhaps due to the adaptive importance of pattern elements across species. Continued research on other candidate loci and the regulatory processes affecting the expression of these loci and possibly *MC1R* will clarify the genetic basis for plumage variation among species in this and other birds.

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