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Evolution of the Melanocortin-1 Receptor (MC1R) in Boobies and Gannets (Aves, Suliformes)

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Abstract

The melanocortin-1 receptor (MC1R) has been linked to intraspecific variation of melanin-based plumage color in several unrelated bird species. However, its involvement in interspecific variation has far less evidence. The Sulidae is a family in the Suliformes composed of 10 species of pelagic seabirds, distributed in 3 genera. There is significant variation in the amount and distribution of melanin pigments among species in the family Sulidae, and 2 species, the brown booby (*Sula leucogaster*) and the red-footed booby (*S. sula*), present plumage polymorphisms, with the latter being considered one of the most plumage polymorphic birds. We performed a survey of the MC1R evolution in 68 individuals representing all 9 species in the Sulidae, except the Abbott's booby, to determine the role played by this locus in explaining the melanic variation observed in the Sulidae. We found the amino acid substitution R112H to be in full concordance with the plumage color observed in the brown booby, which shows a unique phaeomelanin-dominant coloration. Furthermore, all amino acid residues known to be important for function at the MC1R were completely conserved in the Sulidae, except for the previously described V85M and H207R substitutions among the 2 red-footed booby's color morphs. A total of 14 substitutions were inferred from estimated ancestral nodes throughout the Sulidae phylogeny. Finally, we found evidence that the MC1R is under strong purifying selection in all Sulid species. This study provides additional evidence of the potential involvement of the MC1R in melanin-based plumage variation at the interspecific level.

Key words: *boobies, gannets, MC1R, melanin, melanocortin-1 receptor, Sulidae*

The study of phenotypic evolution within and among species is of vital importance to evolutionary biology because phenotypes are the units at which natural selection can act. However, loci underlying phenotypic variation in natural populations are rarely known, limiting our understanding of the complex evolutionary processes that govern the development of the variation observed in the wild. Patterns of plumage coloration in birds are diverse, and remarkable variation is observed both inter- and intraspecifically, and since several candidate loci for the control of plumage coloration have been identified in domestic and wild species (Takeuchi, Suzuki, Yabuuchi, et al. 1996; Theron et al. 2001; Kerje et al. 2003; Mundy et al. 2004; Baião et al. 2007; Nadeau et al. 2007, 2008; Minvielle et al. 2010), these systems provide an opportunity for understanding phenotypic evolution at the macroevolutionary level.

Among these loci, the one encoding the melanocortin-1 receptor (MC1R) has received much attention. This G-protein–coupled receptor is a 7 transmembrane receptor expressed in the melanocytes, and it plays a critical role in melanin synthesis, acting as a switch between the 2 types of melanins found in birds, eumelanin, and phaeomelanin (Mountjoy et al. 1992; Kerje et al. 2003). Eumelanin produces brown to black coloration, and phaeomelanin produces yellow to red coloration (Takeuchi, Suzuki, Hirose, et al. 1996). The type of melanin synthesized in melanocytes is determined by the level of tyrosinase, a rate-limiting enzyme (Robbins et al. 1993; Takeuchi, Suzuki, Hirose, et al. 1996); eumelanin is synthesized when tyrosinase is active, whereas phaeomelanin is synthesized when the enzyme is inactive. The binding of α-melanocyte stimulating hormone (α-MSH) activates tyrosinase, and the melanocyte switches to eumelanin synthesis (Mountjoy et al. 1992; Robbins et al. 1993). Dominant mutations at the MC1R determine the synthesis of eumelanin by generating a constitutively active receptor, whereas loss-of-function mutations are

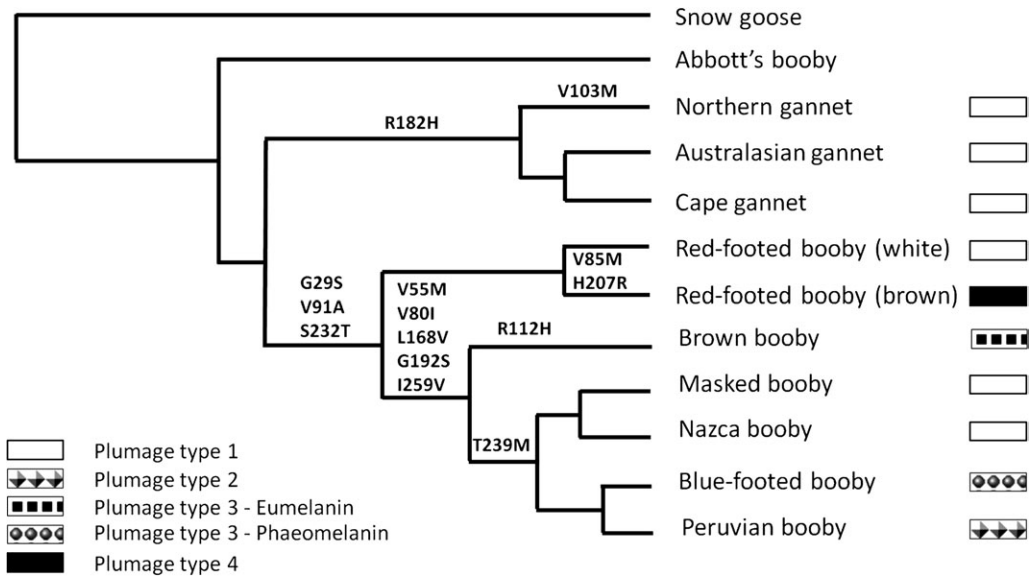

Figure 1. Reconstruction of MC1R evolution in the Sulidae based on estimated ancestral amino acid sequences. Reconstruction is over a phylogeny of the Sulidae based on independent data, using multiple molecular markers (Patterson et al. 2011). The Abbott's booby was not included in the analysis and branch lengths have no meaning. The outgroup is the snow goose (*Anser c. caerulescen*).

recessive and lead to the synthesis of phaeomelanin (Kerje et al. 2003). However, in the snow goose (*Anser c. caerulescen*), the arctic skua (*Stercorarius parasiticus*), and the red-footed booby (*Sula sula*), the MC1R gene controls the switch from the synthesis of eumelanin to the absence of melanin (Mundy et al. 2004; Baião et al. 2007). Based on the studies conducted to date on associations between MC1R variation and plumage and coat color, from both domestic and wild populations, several amino acid residues have been identified as important to changing the MC1R function among birds and mammals (Takeuchi, Suzuki, Yabuuchi, et al. 1996; Theron et al. 2001; Kerje et al. 2003; Mundy et al. 2004; Nadeau et al. 2006; Baião et al. 2007). Because the MC1R function seems to be conserved, even among lineages as distantly related as mammals and birds (e.g., Theron et al. 2001), these residues are good starting points for new association studies, and they can potentially further our understanding of the evolution of melanism.

The Sulidae is a family in the Suliformes composed of 10 species of pelagic seabirds, classified into 3 genera, *Papasula*, *Sula*, and *Morus* (Chesser et al. 2010). A phylogenetic hypothesis based on multiple nuclear markers places the gannets and the boobies in the genus *Sula* in separate monophyletic groups (Patterson et al. 2011). The Abbott's booby (*Papasula abbott*) is basal to both the gannets and the boobies, and the red-footed booby (*S. sula*) is the most divergent booby in the genus *Sula* (Patterson et al. 2011) (Figure 1).

There is significant variation on the amount and distribution of melanin pigments among species in the Sulidae (Nelson 1978) and 2 species, the brown booby (*S. leucogaster*) and the red-footed booby, present plumage polymorphisms (Nelson 1978), with the latter being considered one of the most plumage polymorphic birds (Galeotti et al. 2003). Three main color morphs were initially recognized in this species, the white, white-tailed brown, and brown, with several degrees of intermediates (Nelson 1978), and 2 linked point substitutions at the MC1R locus were found to be perfectly associated with the observed color variation in this species (Baião et al. 2007). All 10 species in the Sulidae fall within 4 broadly defined plumage color categories: 1) white dorsally and ventrally, with some black on the wings, and in some cases tail; 2) white ventrally and on the head, but brown dorsally; (3) mostly white ventrally but with head and part of throat and upper breast brown, and brown dorsally; and 4) brown dorsally and ventrally (Nelson 1978) (Table 1).

Melanin-based coloration in feathers is, in all cases analyzed to date, the result of the relative amount of each type of melanin, eumelanin, and phaeomelanin, rather than just the total melanin concentration (McGraw 2006). Detailed plumage color descriptions of the species in the Sulidae allow a tentative classification of the relative amount of the 2 types of melanin that might be involved in the determination of plumage color in these species because the 2 shades of brown observed are quite different (Table 1). We have performed preliminary biochemical analyses on the relative amounts of the 2 types of melanin in red-footed and brown boobies' feathers, and the results corroborate our assumption that brown boobies have greater amounts of phaeomelanin when compared with red-footed boobies (JJN, unpublished data).

Intraspecific polymorphism such as that observed in red-footed boobies provides important evidence of the link

Table 1 Description of color categories, types of melanin, sampling location and sample sizes for nine species in the Sulidae family

Plumage color category	Description	Species	Type of melanin	Sampling location	*N*
1	White D and V, black wing tips and tail	*M. bassanus*	Restricted melanin	Skarvklakken, Norway	3
		M. serrator		Pedra Branca, Australia	2
		M. capensis		South Africa	4
		S. granti		Isla Genovesa, Galápagos	5
		S. dactylatra			5
		White morph *S. sula*		Isla Genovesa, Galápagos	10
2	White V and on head, brown D	*S. variegata*	Predominant eumelanin	Mazorca, Peru	10
3	White V head, upper breast and throat and brown D	*S. nebouxii*	Predominant eumelanin	Isla Española, Galápagos	5
		S. leucogaster	Predominant phaeomelanin	Panama (A) and Mexico (B)	5
4	Brown D and V	Brown morph *S. sula*	Predominant eumelanin	Isla Genovesa, Galápagos	10

D = dorsally; V = ventrally; *M* = *Morus*; *S* = *Sula*

between micro- and macroevolution. However, to understand the scope of the involvement of the MC1R locus in the evolution of plumage variation, analyses at broader phylogenetic levels (i.e., among closely related species) are necessary (Mundy 2006). To address this issue, variation at the MC1R locus and differences in melanin-based coloration among bird species have been investigated in Old World leaf warblers of the genus *Phylloscopus* (MacDougall-Shackleton et al. 2003). Their results show no association between variation in the coding region of the MC1R locus and changes in melanization among species. However, Pointer and Mundy (2008) show that MC1R is related to melanism among species of the genus *Cygnus*. Additional analyses at this phylogenetic level are necessary in order to expand our understanding of the role of the MC1R locus in plumage color evolution to other bird groups. The Sulidae represents a good candidate to perform such analyses, since a well-supported phylogenetic hypothesis has been determined for the family, and because of the observed variation in the amount and distribution of melanins among species. Furthermore, the established involvement of the MC1R locus in the observed polymorphism in red-footed boobies (Baião et al. 2007) may indicate further involvement of this locus in the plumage variation of the other species in the family.

In this study, we performed a survey of the MC1R evolution in the Sulidae, in order to address the following questions: 1) do substitutions at the MC1R locus among closely related species coincide with plumage color? 2) Have important amino acid residues been conserved throughout sulid evolution? And 3) is the MC1R locus under selection in the Sulidae?

Materials and Methods

Molecular Laboratory Protocols

A total of 68 individuals representing all 9 species in the Sulidae, except the Abbott's booby, were sampled (Table 1). Different color morphs of red-footed booby were included, but only one color morph of the Brown booby was included. Samples were stored in lysis buffer (Longmire et al. 1988), and total DNA was extracted using standard phenol: chloroform extraction protocols. A 817 bp segment of the 945-bp coding region of the avian MC1R was amplified, using primers MSHR72 (5′ ATGCCAGT-GAGGGCAACCA 3′) and MSHR9 (5′ CTGGCTC-CGGAAGGCATAGAT 3′) (Mundy et al. 2004). This segment includes most sites in the coding region known to be important for function. It is possible that nonsequenced sites could have importance for function. For PCR amplification of the MC1R locus, the following reagents were combined in a 25 μl reaction: ~30 ng of genomic DNA; 16 mM $(NH_4)_2SO_4$, 671 mM Tris–HCl (pH 8.8 at 25 °C, 0.1%); 1.5 mM $MgCl_2$; 0.6 mM of each dNTP; 0.3 μM of each primer; 2% DMSO; and 0.5 U of *Taq* DNA polymerase (Biolase Red; Integrated DNA Technologies). PCR cycling protocols were initial denaturation at 94 °C for 2 min; 35 cycles of 94 °C for 30 s, 61 °C for 30 s, 72 °C for 30 s; and final extension of 94 °C for 5 min. PCR products were directly sequenced on both strands using sequencing primers MSHR73 (5′ GGCGTAGAAGATGGTGATG-TAGC 3′) and MSHR74 (5′ GTGGACCGCTACATCAC-CAT 3′) (Mundy et al. 2004) under standard conditions, and run on an ABI Prism 3100 genetic analyzer. Heterozygous sites were identified by visual inspection of chromatograms, and confirmed by sequences from both DNA strands. A total of 24 sites presented heterozygosity. Only 15 individuals presented heterozygosity at more than 1 site, and therefore we did not attempt to resolve haplotype phase experimentally. Sequences with heterozygous sites were not included in analyses. Sequences were edited using Seqman (Lasergene software, DNAStar Inc.) and were unambiguously aligned by eye using MacClade version 4.06 (Maddison and Maddison 2003).

Statistical Analyses

Pairwise comparisons within (when more than one species were in the same category) and among the 4 plumage color

categories (Table 1) were performed in order to assess associations between variations at the MC1R and differences in plumage color categories. The brown booby was treated separately because this species is characterized by a unique relative amount of eumelanin and phaeomelanin; pairwise comparisons between this species and all 4 plumage color categories were performed. Based in previous findings that the white allele is ancestral in red-footed boobies, snow geese, and Arctic skuas (Mundy et al. 2004; Baião et al. 2007) and the sulid phylogeny (Patterson et al. 2011), we assume that plumage category 1 (Table 1) is ancestral in the evolution of this group. Therefore, we expect that point substitutions at the MC1R could potentially be involved in the evolution of phenotypes in this family leading to 3 lineages: Peruvian booby, blue-footed booby, and brown booby. Point mutations leading to the brown morph red-footed booby have been previously described.

Ancestral state reconstruction is a useful tool since it makes it possible to infer the evolutionary pathway of amino acids in the sequence, permitting identification of specific amino acid changes that may have caused a functional change in the gene. Furthermore, it can aid in the detection of positively selected sites at the amino acid level (Yang et al. 1995). Ancestral amino acid sequences were reconstructed over a phylogeny obtained from independent data (Patterson et al. 2011) using PAML version 4 (Yang 2007), following the method outlined by Yang et al. (1995).

Comparisons of nonsynonymous (dN) and synonymous (dS) substitution rates in protein-coding genes provide an important means for detecting selection at the protein level (Yang et al. 2000). Specifically, $dN/dS = 1$ when nonsynonymous substitutions have no effect on the fitness of the protein and occur at the same rate as synonymous substitutions; $dN/dS < 1$, when nonsynonymous substitutions are selected against, and therefore appear at a lower rate than synonymous substitutions (purifying selection); and finally $dN/dS > 1$, when nonsynonymous substitutions are beneficial and appear at a higher rate than synonymous substitutions (positive selection). Because selection at the molecular level is likely to target only a small number of amino acid residues in a protein, we estimated dN/dS (ω) using the codon-based maximum-likelihood method (CODEML) implemented in the program PAML version 4 (Yang 2007). Variation in the dN/dS ratio among sites was modeled using both discrete (M1a and M2a) and continuous (M7 and M8) distributions. To test for positive selection, we performed comparisons between M1a (nearly neutral) and M2a (positive selection) and between M7 and M8. These nested models allow for different ω values (M1a vs. M2a and M7 vs. M8) and were compared using a likelihood ratio test (LRT) (Yang et al. 2000; Yang and Nielsen 2002).

Results

Association Studies

The predicted amino acid sequences of the Sulid MC1R are shown in Figure 2. We found the amino acid substitution R112H to be exclusive to the brown booby and therefore perfectly associated with the plumage color category observed in this species. All 5 brown boobies sampled had the substitution, whereas none of the other 49 boobies and gannets sampled had it. This substitution is located in the extracellular region, which is known to be functionally important because it is likely to interact with external ligands. Moreover, we found that brown boobies from Panama differed from those from Mexico by one base pair synonymous substitution (C441T). Because this substitution does not change the amino acid, it is not likely involved in phenotypic differences, but it might suggest that these populations are genetically isolated from each other. No further associations were found in the comparisons among all the other color plumage categories.

Ancestral State Reconstruction

Several amino acid residues have been identified as important for function in the MC1R locus from previous association studies between MC1R variation and plumage and coat color (Robbins et al. 1993; Takeuchi, Suzuki, Yabuuchi, et al. 1996; Kerje et al. 2003; Mundy and Kelly 2003; Mundy et al. 2004; Mundy 2005; Baião et al. 2007). From these studies, residues of particular interest are: **C33,** V58, **M71, V85, E92**, L98, D115, D119, C123, D139, R140, Y141, R149, R158, **F177, H207, C213, H215, R230, L244,** H257, D291, and C312 (bold represents important sites from association studies in birds), numbered from the chicken (*Gallus gallus*) MC1R. All of these residues are completely conserved in the sulid MC1Rs sequenced, except for the previously described V85M and H207R substitutions among the 2 red-footed booby's color morphs. C312 was not sequenced in the present study. A total of 14 substitutions were inferred from estimated ancestral nodes and the reconstructed evolution of these different residues is shown in Figure 1. Different parts of the MC1R-encoded receptor have variable roles, such that the extracellular regions are known to be important for ligand binding and regulation of MC1R activity, the transmembrane regions are hydrophobic and known to maintain the structural integrity of the protein, and last, the intracellular regions are important sites for protein signal transduction. Of the 14 substitutions inferred throughout the sulid phylogeny, 5 occurred in the extracellular region, 8 occurred in the transmembrane region, and 1 occurred in the intracellular region (Table 2). Substitutions with functional changes to MC1R activity have been found in multiple regions of the receptor; however, a disproportionate number of these substitutions have been found at the second transmembrane domain (Mundy 2005), including the V85M substitution involved in plumage color variation in red-footed boobies, as well as at the second extracellular region. Therefore, these changes from ancestral states could potentially have promoted phenotypic differences among species, even though no clear pattern was identified.

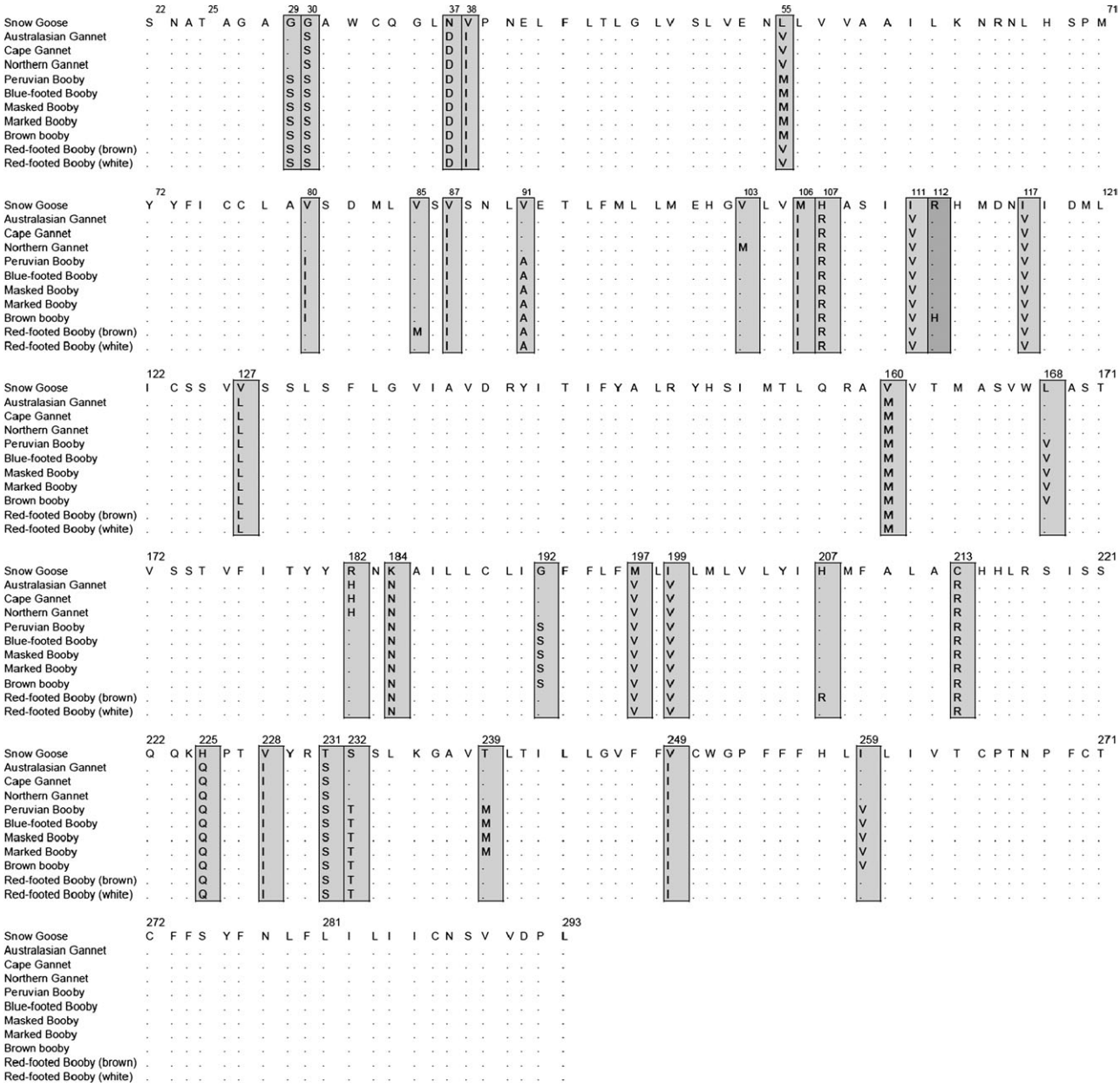

Figure 2. MC1R alignment of proteins for 9 species of the Sulidae. The outgroup sequence, snow goose (*Anser c. caerulescens*), is listed on top with single letter abbreviations for amino acids. Dots represent identity with the outgroup sequence. Numbering refers to the chicken MC1R sequence. Gray boxes evidence variable sites.

Selection on MC1R in the Sulidae

We estimated the selection parameter ω (d*N*/d*S*). The ω parameter ranged from 0.03 to 0.07, depending on the model applied. The LRTs performed between the discrete and the continuous models produced consistent results; we found the LRT for the comparisons between the discrete models (M1a vs. M2a) to be equal to 0 ($P = 1$) and between the continuous models (M7 vs. M8) to be equal to 0.02 ($P = 0.99$) (Table 3). Taken all together, these data suggest strong purifying selection for all sites at the MC1R locus in these species.

Discussion

In this study, we explored the evolution of the MC1R locus among species in the seabird family Sulidae. We found that, generally, variation at the MC1R is not correlated with the different phenotypic patterns of plumage coloration among the species in this family. This general conclusion is based on comparisons among extant species and from reconstructed changes from ancestral amino acid sequences. However, we found a nonsynonymous substitution (R112H) to be exclusive to the brown boobies, which present a unique plumage color

Table 2 Amino acid changes from estimated ancestral amino acid sequences of the MC1R locus in 9 species of the family Sulidae

Substitution	Location	Function
G29S	Extracellular	Ligand binding and regulation
V103M		
R112H		
R182H		
G192S		
V55M	Transmembrane	Structural integrity
V80I		
V85M		
V91A		
L168V		
H207R		
T239M		
I259V		
S232T	Intracellular	Signal transduction

phenotype among the members of the Sulidae, suggesting that a small change in the MC1R coding region could be responsible for the observed phenotype of this species, providing another potential example of association of genotype/phenotype at the interspecific level. Moreover, we found the MC1R locus to be under strong purifying selection, when rates of nonsynonymous to synonymous substitutions (dN/dS) were calculated among sites.

Most of the phenotypic variation observed among the species in the Sulidae can be described in terms of differences in the distribution of melanin pigments, or in other words, differences in pattern. Even though some of the estimated substitutions from the reconstructed ancestral nodes in the sulid phylogeny could potentially have influenced the phenotypic differences observed in the extant species due to their location in the MC1R-encoded receptor, we did not find any simple correlation between these substitutions and phenotype. This result was also true for the association studies among extant species. Mutations at the MC1R locus associated with phenotypic changes described to date are located at the MC1R coding region and are usually responsible for dramatic changes in phenotype involving a switch from eumelanin synthesis to phaeomelanin synthesis, or to the absence of melanin. In fact, it would be surprising if MC1R substitutions at the coding region had a strong effect in patterning, since these are fine-scale changes (Mundy 2005), and therefore more likely to be controlled by mutations at regulatory regions of the genes involved in coloration. Moreover, our overall finding is in agreement with other studies performed at this phylogenetic scale in the Old World leaf warblers of the genus *Phylloscopus* (MacDougall-Shackleton et al. 2003), that did not find a clear involvement of the MC1R with the observed phenotypic variation. However, the occurrence of these substitutions, in particular those at the second transmembrane region as well as those at the second extracellular region, given these regions' previously identified involvement with several changes in phenotype, should not be ignored because these could represent just one piece in a complex puzzle involving multiple genes know to be involved in plumage color, and their interpretation in the absence of more data is at best incomplete.

Table 3 Likelihood analysis of dN/dS ratios using PAML

Model	lnL	LRT	*P*
M1a	−1091.058	0	1
M2a	−1091.058		
M7	−1091.065	0.002	0.99
M8	−1091.066		

lnL = log likelihood.

In accordance with the prediction that major phenotypic changes can be created by simple substitutions at the MC1R coding region, in both cases where dramatic changes in plumage color are observed in the Sulidae, namely among the color morphs in the red-footed boobies (Baião et al. 2007) and in the brown boobies (present study), non-synonymous point substitutions at the MC1R are perfectly associated with the color variation. In the first case, an extreme switch from eumelanin to the absence of melanin occurs, while in the latter, a switch from the synthesis of eumelanin to phaeomelanin presumably occurs. The V85M substitution found in the brown morph red-footed booby was also found to be perfectly associated with the color variation in the lesser snow goose (Mundy et al. 2004), and it is located at the second transmembrane region, which is known to be important for the function of the MC1R-encoded receptor. Taken all together, these data suggest that the V85M substitution is causative of the phenotypic change. In the brown boobies, the R112H is located at the second extracellular region, which has also been described to be an important region for the function of the receptor. This substitution is only 5 amino acids away from the R109W substitution known to have phenotypic consequences in the pocket mouse (*Chaetodipus intermedius*) (Nachman et al. 2003). However, the change from an Arginine (R), which is a positively charged hydrophilic amino acid, to a Histidine (H), which is also a positively charged and hydrophilic molecule, is a conservative change, and it may not cause functional changes to the receptor. Moreover, identifying genotype–phenotype associations at the interspecific level can be challenging because different species will inevitably have accumulated nucleotide substitutions across their genome, which could increase the likelihood of spurious associations. In the present study, the R112H substitution is a fixed change in the brown booby lineage, and it is possible that it is not associated with the observed color phenotype. Further tests comparing genetic differentiation at the MC1R and at neutral loci will be necessary to resolve this issue.

Our findings of strong purifying selection is in agreement with similar studies that have investigated the role of selection at the MC1R locus among species in different taxa, such as reptiles (Rosenblum et al. 2004) and nonhuman primates (Mundy and Kelly 2003). However, given that very small changes in the MC1R coding region

can generate dramatic changes in phenotypes, it is possible that positive selection on this locus would be hard to detect using this methodology, since the information content of the molecular data in this situation will not reflect the phenotypic change and possible selective forces acting on them. Additionally, alternative modes of evolution such as strong positive selection occurring in a short burst followed by long periods of purifying selection could confound the results of these analyses. Thus, the gathered evidence of strong purifying selection does not necessarily preclude the possibility that individual amino acids could be involved in adaptive color variation, such as that observed in pocket mice (Nachman et al. 2003).

This study has expanded the investigation on the scope of the role of the MC1R locus in the evolution of phenotypes to seabirds, adding to the accumulating body of knowledge about this issue. Birds present remarkable variation in plumage color and over 300 avian species exhibit plumage polymorphisms (Galeotti et al. 2003). Even though we are still far from a holistic comprehension of this complex process, understanding the genetic basis of this dazzling variation will be important for the understanding of the general patterns of evolution of phenotypes.

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