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Genetics of Plumage Color in the Gyrfalcon (*Falco rusticolus*): Analysis of the Melanocortin-1 Receptor Gene

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Abstract

Genetic variation at the melanocortin-1 receptor (MC1R) gene is correlated with melanin color variation in a few reported vertebrates. In Gyrfalcon (*Falco rusticolus*), plumage color variation exists throughout their arctic and subarctic circumpolar distribution, from white to gray and almost black. Multiple color variants do exist within the majority of populations; however, a few areas (e.g., northern Greenland and Iceland) possess a single color variant. Here, we show that the white/melanic color pattern observed in Gyrfalcons is explained by allelic variation at MC1R. Six nucleotide substitutions in MC1R resulted in 9 alleles that differed in geographic frequency with at least 2 MC1R alleles observed in almost all sampled populations in Greenland, Iceland, Canada, and Alaska. In north Greenland, where white Gyrfalcons predominate, a single MC1R allele was observed at high frequency (>98%), whereas in Iceland, where only gray Gyrfalcons are known to breed, 7 alleles were observed. Of the 6 nucleotide substitutions, 3 resulted in amino acid substitutions, one of which ($Val^{128}Ile$) was perfectly associated with the white/melanic polymorphism. Furthermore, the degree of melanism was correlated with number of MC1R variant alleles, with silver Gyrfalcons all heterozygous and the majority of dark gray individuals homozygous (Ile^{128}). These results provide strong support that MC1R is associated with plumage color in this species.

Key words: *arctic, color polymorphism, MC1R, melanin, mutation, falcon*

Introduction

The melanocortin-1 receptor (MC1R) gene encodes a key protein involved in regulating melanin synthesis for deposition in specialized pigment cells called melanocytes during vertebrate tissue development (Hoekstra 2006; Mundy 2006). Multiple studies have linked amino acid variability of MC1R with qualitatively different plumage color patterns in distantly related species: Bananaquit (*Coereba flaveola*; Theron et al. 2001), Lesser Snow Geese (*Anser caerulescens caerulescens*) and Arctic Skua (*Stercoarius parasiticus*; Mundy et al. 2004), Red-footed Booby (*Sula sula*; Baião et al. 2007), swans (*Cygnus* spp.; Pointer and Mundy 2008), flycatchers (*Monarcha castaneiventris*; Uy et al. 2009), Eleonora's Falcon (*Falco eleonorae*; Gangoso et al. 2011), and domestic Japanese Quail (*Coturnix japonica*; Nadeau et al. 2006), Guineafowl (*Numida meleagris*; Vidal et al. 2010) and chickens (Kerje et al. 2003; Ling et al. 2003; see also Mundy 2005).

Although the actual biochemical mechanism by which nonsynonymous substitutions in MC1R affect melanin synthesis in birds is not fully understood (McGraw 2006; Hofreiter and Schöneberg 2010), studies with mammals (Robbins et al. 1993; García-Barron et al. 2005; Steiner et al. 2009), domestic chicken (Ling et al. 2003), and lizards (Rosenblum et al. 2010) have identified altered functional properties specific to certain MC1R allelic variants and colored traits. In beach mouse (*Peromyscus polionotus*), for example, Hoekstra et al. (2007) have identified multiple nonsynonymous point substitutions in the coding region of MC1R that alter protein function and influence the degree of signaling activity associated with the production of hair pigmentation. In this case, a single point substitution influenced fur color in beach mouse providing an adaptive mechanism associated with crypsis, or reduced predation, within its environment, suggesting that MC1R represents a large effect locus associated with fitness and adaptation in some populations (Mullen et al. 2009; see also Mundy 2005). In birds, a total of 334 species (3.5%) exhibit plumage polymorphism (Galeotti et al. 2003). Selection pressures influencing plumage color polymorphisms differ based on the species or population, but studies suggest that disruptive selection through crypsis may play a large role depending on varying environmental light conditions (Galeotti et al. 2003;

see also Roulin 2004). Alternatively, mate choice may play a role (Bortolotti et al. 2008) influencing plumage color polymorphism or possibly pleitrophic effects associated with MC1R and immunity (Gangoso et al. 2011).

Here, we investigate whether MC1R allelic variants correspond with plumage color in Gyrfalcon (*Falco rusticolus*), a species that possesses extensive plumage color variation from white to silver and gray, to almost black, with multiple color variants occurring in many of the same areas (Todd and Friedmann 1947; Vaurie 1961; Ellis et al. 1992; Flann 2003; Potapov and Sale 2005). This species possesses a circumpolar arctic and subarctic distribution with latitudinal breeding range extending north to 82°N in Peary Land, Greenland, and south to 54°N on the Kamchatka Peninsula in Russia and Hudson Bay in Canada (Potapov and Sale 2005). Populations are generally distributed across 6 areas: Alaska (United States), Canada, Greenland, Iceland, Fennoscandia, and Siberia (Cade 1982; Potapov and Sale 2005), with frequencies of plumage color variants differing among areas.

Gyrfalcons in north Greenland and northeast Canada are largely white and white to silver, respectively; in mid- to south Greenland and North America, white to silver to gray to dark gray or black; Iceland, light gray to gray; Fennoscandia and Russia, mostly gray; and in Siberia, gray to light gray or white (Cade et al. 1998; Potapov and Sale 2005). White Gyrfalcons with minimal barring pattern dominate north Greenland, with only a single gray female and her chick observed from over 285 recorded Gyrfalcon sightings in the northwest near Thule (75.9–77.6°N) between 1993 and 2010, and all but 2 gray Gyrfalcons captured (n = 125 total) during 2 consecutive autumn migrations in central-east Greenland (Scoresbysund, 70.4°N; Burnham 2008; Burnham, unpublished data). Further south in central-west Greenland, near Kangerlussuaq (66.5–67.5°N; approximately 1170 km south of Thule), multiple plumage color variants exist. Between 1998 and 2006, 60 white (51%), 22 silver (19%), and 35 gray (30%) adult Gyrfalcons were observed in this area (Johnson, Burnham, unpublished data, Timing of breeding covaries with plumage colour among Gyrfalcon.). Based on previous studies investigating MC1R allelic variability and plumage color in wild birds, we predict that nonsynonymous mutations exist in MC1R that correlate with the degree of melanin variation observed among Gyrfalcon plumages.

Materials and Methods

Tissue Collection and DNA Extraction

Blood or feather samples were collected from individual Gyrfalcons at six different geographic locations: Alaska (n = 20), Greenland (Kangerlussuaq, n = 32; Thule (Pituffik), n = 32; and Scoresbysund, n = 30), north central Canada (n = 10), and Iceland (n = 22; Figure 1). The samples used in this study, including DNA extraction methods, have been described elsewhere (Johnson et al. 2007). All samples were obtained during the breeding season with the exception of

Figure 1. Geographic breeding distribution of Gyrfalcons. Sampling locations are shown with the size of each ellipse representing the approximate geographic area sampled.

Scoresbysund, where Gyrfalcons were sampled during autumn migration, presumably in transit from breeding territories in northeast Greenland (Figure 1; see also Burnham 2008). Plumage color (white, silver, and gray) was recorded for the majority of individuals with the exception of those from Canada.

MC1R Gene Sequencing

A 729-bp fragment of the 945-bp avian MC1R-coding region was amplified using primers MSHR72 and MSHR9 (Mundy et al. 2004). Polymerase chain reaction (PCR) amplifications were performed with the following cycling parameters: 94 °C for 2 min, 35× (94 °C for 30 s, 60–65 °C for 45 s, and 72 °C for 90 s), and 72 °C for 5 min. The PCR products were directly sequenced on both strands using standard cycle sequencing protocols using an ABI 3130xl Genetic Analyzer (Applied BioSystems) and edited using the program Sequencher version 4.10.1 (Gene Codes Corporation). Heterozygotes were identified by visual inspection of chromatograms and confirmed by sequence from both DNA strands. MC1R haplotypes were inferred using PHASE v2.1.1 (Stephens and Donnelly 2003) with 1000 iterations and a 1000-generation burnin. Consistent results were observed across 5 replicates using different seeds, and all ambiguous genotypes were resolved with confidence probability values more than 0.90. The association of variable sites at MC1R and plumage color variants ("white" and "silver or gray" or melanic) was obtained by visual interpretation of unambiguously aligned sequences.

Results

Genetic variability existed among individual Gyrfalcons for the MC1R gene. Six nucleotide substitutions in the coding

	* 268	300	310	648	668	696	Greenland: Thule	Kanger.	Scoresby.	Iceland	Canada	Alaska
allele 1	G	C	C	G	T	C	63	44	60	10	8	18
allele 2	A	T	.	A	.	.	1	7	-	18	2	12
allele 3	A	.	.	.	.	.	-	6	-	12	7	9
allele 4	A	.	T	.	.	.	-	-	-	-	-	1
allele 5	A	.	.	A	C	.	-	-	-	1	3	-
allele 6	A	.	.	.	.	.	-	5	-	1	-	-
allele 7	A	T	.	.	.	.	-	-	-	1	-	-
allele 8	A	T	.	.	.	T	-	-	-	1	-	-
allele 9	.	.	.	.	C	.	-	2	-	-	-	-

Figure 2. Distribution of 9 MC1R alleles observed among Gyrfalcons populations. Vertical numbers indicate the positions of variable nucleotides within 729 bp of sequence. The 3 gray columns indicate nonsynonymous nucleotide substitutions; * indicates the nucleotide position, or $Val^{128}Ile$, perfectly associated with plumage color variants. Dots under nucleotide positions indicate an identical nucleotide as given with allele 1. The total number of each allele is given for sampled populations. MC1R haplotype sequences were deposited in GenBank (accession numbers JQ290354–JQ290362).

region of MC1R resulted in 9 alleles that differed in their overall geographic distribution with at least 2 MC1R alleles observed in almost all sampled populations (Figure 2). Gyrfalcons from Scoresbysund possessed a single allele, whereas all Gyrfalcons sampled in Thule, with the exception of one gray female, were homozygous for the same allele observed in Scoresbysund (Figure 2). The Iceland population possessed the highest number with 7 alleles.

Of the 6 observed nucleotide substitutions in MC1R, 3 resulted in amino acid substitutions (i.e., nonsynonymous): valine to isoleucine at amino acid position 128 ($Val^{128}Ile$), arginine to cysteine at amino acid position 142 ($Arg^{142}Cys$), and threonine to isoleucine at amino acid position 261 ($Thr^{261}Ile$). Amino acid residue positions are based on the wild type consensus sequence (GenBank accession number AF326275; see also García-Barron et al. 2005). All 3 amino acid substitutions occurred in transmembrane regions where mutations have been shown to have important functional consequences (García-Barron et al. 2005; Weis and Kobilka 2008). The single nucleotide substitution at nucleotide 268 resulting in an amino acid variant $Val^{128}Ile$ was perfectly associated with "white" vs. melanic variants from Kangerlussuaq and Alaska (Figure 3; Fisher's exact test, $P < 0.001$). This was further supported with Gyrfalcons from north Greenland (Thule and Scoresbysund) where the majority of birds sampled were white color variants (Figure 3). With the exception of one gray female in Thule, all Gyrfalcons in north Greenland were homozygous for the "white" allele (Val^{128}), whereas the gray female was heterozygous at this position ($Val^{128}Ile$).

In north central Canada, 2 of 10 (20%) sampled Gyrfalcons were homozygous for the "white" allele, although we do not have plumage color information to confirm. The "white" allele was also observed in Iceland where only gray Gyrfalcons are observed during the breeding season (Nielsen, personal communication). Of the 22 Gyrfalcons from Iceland, 10 possessed the "white"

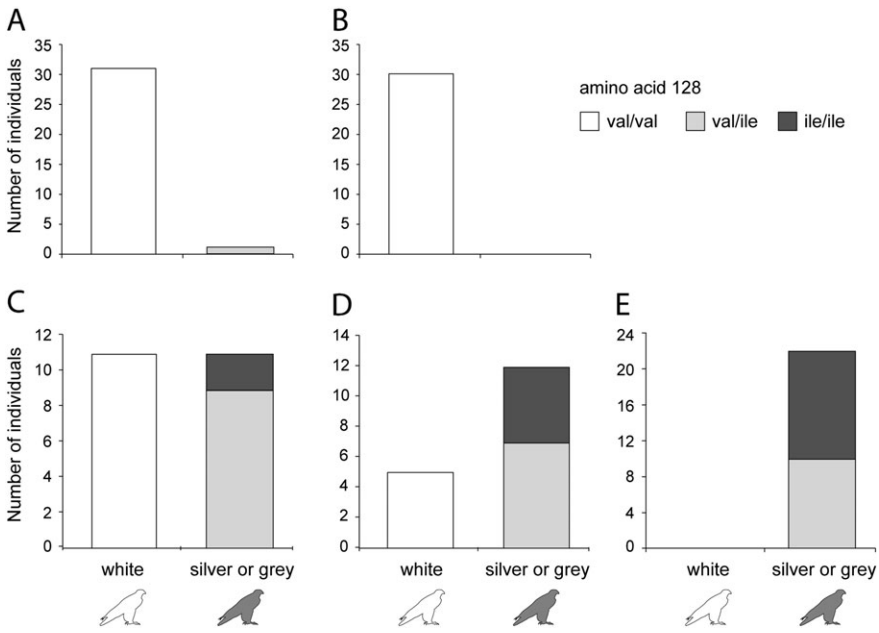

Figure 3. Frequency of MC1R genotypes and Gyrfalcon plumage color phenotypes at (**A**) Thule, Greenland; (**B**) Scoresbysund, Greenland; (**C**) Kangerlussuaq, Greenland; (**D**) Alaska, United States; and (**E**) Iceland.

allele (45%), but they were all heterozygous ($Val^{128}Ile$). In Kangerlussuaq and Alaska, all silver Gyrfalcons ($n = 9$) were heterozygous ($Val^{128}Ile$) and gray Gyrfalcons had either heterozygous ($Val^{128}Ile$; $n = 7$) or homozygous "non-white" (Ile^{128}; $n = 7$) genotypes. No obvious association between plumage color and the remaining 2 nonsynonymous point substitutions ($Arg^{142}Cys$ and $Thr^{261}Ile$) was observed; although all Gyrfalcons that possessed alleles 4, 5, or 9 (Figure 2) where either heterozygous at MC1R ($n = 2$; silver and gray phenotypes) or their plumage color was not recorded at time of sampling ($n = 5$).

Discussion

Using a candidate gene approach, we identified a nonsynonymous point substitution ($Val^{128}Ile$) in the third transmembrane domain of the MC1R gene that was perfectly associated with Gyrfalcon color variation with respect to "white" and "nonwhite" (melanic) individuals. A recent study investigating plumage color inheritance based on pedigree analysis of a captive Gyrfalcon population further support our results linking MC1R variability with plumage color in this species. Chang et al. (2010) concluded that plumage color was best described by an inheritance pattern based on 2 genes, 1 gene involved in pigment production following Mendelian dominance inheritance, and another gene, most likely codominant, controlling actual pigment deposition that influenced barring pattern. Based on our results, for example, when a gene such as MC1R is homozygous recessive (e.g., Val^{128}), pigment production would not occur, and thereby make the genotype at a second gene controlling pigment deposition irrelevant (Chang et al. 2010). Although, the gene, or set of genes, influencing barring pattern remains unknown, all of the Gyrfalcon samples from multiple populations support an association between MC1R and pigment production ("white" and "melanic"; Figure 3). The association between MC1R and plumage color is further supported by population genetic analyses based on neutral genetic markers, such as microsatellite loci and mitochondrial control region sequence data, showing significant differentiation (Johnson et al. 2007), independent of plumage color, among multiple populations included in this study.

Given that our data suggest that individuals that are heterozygous ($Val^{128}Ile$) and homozygous dominant (Ile^{128}) for MC1R synthesize melanin, yet, barring pattern also exists on white birds but at reduced intensity, additional gene(s) other than MC1R are likely to influence localized melanin production (e.g., Minvielle et al. 2009) during feather development separate from "background" plumage color (Potapov and Sale 2005; Chang et al. 2010). "White" Gyrfalcons possess a small degree of dark barring pattern, particularly on the tips of primaries, despite a homozygous recessive MC1R genotype. Dark tips on white primaries are thought to offer increased structural support and durability or antimicrobial and parasite resistance (McGraw 2006), and therefore, the presence of dark tips may be under different selection pressures compared with overall "background" plumage color (Roulin 2004). In fact, even within species, genes shown to influence melanin pigmentation can differ between populations. A recent study with beach mice (*Peromyscus polionotus*), for example, documented differences in MC1R activity relative to 2 populations that possessed similar color phenotypes suggesting that differing genes influenced similar colors in the studied populations (Steiner et al. 2009). Additional candidate genes that have been shown to influence melanin plumage color include the agouti stimulating protein (*ASIP*) gene (Nadeau et al. 2008), the microphthalmia-associated transcription factor (*MITF*) gene (Minvielle et al. 2010), and members of the tyrosinase gene family that include tyrosinase (*TYR*), tyrosinase-related protein 1 (*TYRP1*), and dihydroxyphenylalanine-chrome tautomerase (*DCT* or *TYRP2*; Buggiotti 2007; Nadeau et al. 2007).

Following melanin production, additional genes may also be involved with endocrinological pathways in Gyrfalcon that influence pigment deposition during feather development. For example, up-regulation of extracellular alpha melanin-stimulating hormone (α-MSH) may produce variation in the level of melanism as shown with vertebrates, including birds (Rasmussen et al. 1999; Takeuchi et al. 2003). Genetic analyses with a larger number of silver and gray Gyrfalcon samples with varying degrees of barring pattern will be necessary to discern these relationships in greater detail. This will also allow a more focused study on the degree of melanism relative to the number of allelic variants at MC1R. Among our samples, all silver individuals were heterozygous ($Val^{128}Ile$), whereas gray individuals were both heterozygous ($Val^{128}Ile$) and homozygous (^{128}Ile). Previous studies have documented similar results showing strong correlations between the degree of plumage melanism and allelic copy number at MC1R with lesser Snow Goose, Arctic Skua (Mundy et al. 2004), and Red-footed Booby (Baião et al. 2007).

Furthermore, predominantly light gray to gray Gyrfalcons are observed in Iceland (Cade et al. 1998). On occasion, however, white Gyrfalcons have been reported during the winter, presumably as migrants from Greenland (Pétursson et al. 1993; Nielsen and Pétursson 1995; Burnham and Newton 2011). In this study, none of the sampled Iceland Gyrfalcons were homozygous for the "white" allele (Val^{128}) despite identifying the allele in relatively high frequency as heterozygotes (10/22 individuals). Although no white Gyrfalcons have been documented breeding in Iceland to date despite ringing over 1500 offspring (Nielsen and Pétursson 1995; Nielsen, personal communication), the expected frequency of the white phenotype under Hardy–Weinberg equilibrium (HWE) is 5.2% or 1.1 of 22 individuals. At such a low frequency, a much larger sample size is required to determine if selection may play a role in influencing plumage color frequency in this population, particularly the lack of white Gyrfalcons observed during the breeding season.

In Kangerlussuaq and Alaska, HWE also cannot be rejected for the observed MC1R homozygous "white" allele

(or white Gyrfalcon) frequencies. Given the observed number of white alleles, the expected frequency of the white phenotype under HWE in Kangerlussuaq and Alaska was 49% (~11 of 22 individuals) and 25%, (~4 of 17 individuals), respectively, which is very similar to the number of white Gyrfalcons sequenced at MC1R in the 2 populations (Figure 3). However, when comparing plumage color frequency in Kangerlussuaq between the total number of male and female Gyrfalcons ($n = 51$ and 66, respectively), a significantly higher frequency of white males (67%) were observed compared with females (39%; $\chi^2 = 8.62$, degrees of freedom = 2, $P < 0.025$). Although sex-specific sample sizes are not sufficient to explore HWE conditions relative to MC1R, these results do suggest that demographic differences exist between color variants within this population, and selection, along with the effects of genetic drift and small population size, may influence plumage color distribution within Greenland.

No evidence of assortative pairing based on plumage color was observed in the Kangerlussuaq population; however, a significant sex-specific correlation between egg lay date and plumage color was observed among breeding males (Johnson and Burnham in review). White male Gyrfalcons nested earlier than gray males and therefore fledged young earlier in the breeding season. In contrast, no significant difference was observed among female color variants with respect to lay date. Among breeding pairs, a significant difference in lay date was shown with white–white pairs nesting earlier than gray–gray pairs. The number of young observed at nests was also significantly correlated with plumage color. Nests with gray–gray pairs had significantly fewer offspring (Johnson and Burnham in review). More work is needed to investigate the differences observed between the sexes relative to lay date and plumage color and to quantify the level of selection that may exist or differ among color variants in Gyrfalcon.

These results provide strong support that MC1R is associated with plumage color in Gyrfalcons. However, additional work is required to investigate whether the nonsynonymous point substitution ($Val^{128}Ile$) reported here modifies function and melanin synthesis at MC1R during feather development. Both valine and isoleucine are neutral nonpolar branch-chained amino acids that possess structural similarities differing only by an addition of CH_3 on the side chain of isoleucine. Therefore, to what degree this amino acid substitution influences MC1R function is not known, and no other study to our knowledge has reported a point substitution at this specific position influencing color. Alternatively, $Val^{128}Ile$ may be linked to a mutation elsewhere in the genome or to a regulatory region that controls melanin synthesis at MC1R (e.g., Rouzaud and Hearing 2005). Although $Val^{128}Ile$ in MC1R is in close proximity to other amino acid substitutions that have been reported to cause dominant melanistic phenotypes in mammalian taxa (Våge et al. 1997, 1999; Kijas et al. 1998), a follow-up study functionally verifying the statistical associations between the documented MC1R mutation and color patterns should be conducted similar to work done with mammals (Steiner et al. 2009), reptiles (Rosenblum et al. 2010), and domestic chicken (Ling et al. 2003).

In addition, the two nonsynonymous substitutions at $Arg^{142}Cys$ and $Thr^{261}Ile$ located in the third and sixth transmembrane domains of the MC1R gene, respectively, require further study because insufficient data existed to assess their association with plumage color in Gyrfalcon. With the exception of a single sample from Iceland, no plumage color information was available for the majority of Gyrfalcons ($n = 5$) that possessed the Thr^{261} amino acid substitution. The single Gyrfalcon sample possessing the MC1R Thr^{261} substitution (allele 5) with known plumage color was heterozygous with allele 2 (Figure 2). All sampled Gyrfalcons that possessed allele 2 at MC1R were either silver or gray in plumage color, thereby providing no evidence to suggest that $Thr^{261}Ile$ is not correlated with plumage color.

Similarly, insufficient phenotypic variation existed among Gyrfalcons that possessed the $Arg^{142}Cys$ substitution to determine its correlation with plumage color. Although heterozygous with MC1R allele 1 ("white" allele; Figure 2), the only Gyrfalcon that possessed the Cys^{142} substitution was silver in color suggesting that melanin was produced. In humans, however, the $Arg^{142}Cys$ substitution has been documented as a natural MC1R variant associated with red hair and fair skin (RHC phenotype; Matichard et al. 2004; García-Barron et al. 2005) influencing response to α-MSH in relation to cAMP production and subsequent melanocyte proliferation, differentiation, and melanogenesis (Buscà and Ballotti 2000). Additional samples with the $Arg^{142}Cys$ and $Thr^{261}Ile$ MC1R substitutions are required to assess their association with plumage color in Gyrfalcon. It should also be noted that the amplified fragment (729 bp) of MC1R used in this study did not include the entire coding region (945 bp), and therefore other nonsynonymous point substitutions may exist in the nonsequenced portions of the gene, including polymorphisms within the MC1R promoter region.

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