The molecular basis of an avian plumage polymorphism in the wild: A *melanocortin-1-receptor* point mutation is perfectly associated with the melanic plumage morph of the bananaquit, *Coereba flaveola*

Emmalize Theron*, Kim Hawkins*, Eldredge Bermingham†, Robert E. Ricklefs‡ and Nicholas I. Mundy*

Background: Evolution depends on natural selection acting on phenotypic variation, but the genes responsible for phenotypic variation in natural populations of vertebrates are rarely known. The molecular genetic basis for plumage color variation has not been described in any wild bird. Bananaquits (*Coereba flaveola*) are small passerine birds that occur as two main plumage variants, a widespread yellow morph with dark back and yellow breast and a virtually all black melanic morph. A candidate gene for this color difference is the *melanocortin-1 receptor* (*MC1R*), a key regulator of melanin synthesis in feather melanocytes.

Results: We sequenced the *MC1R* gene from four Caribbean populations of the bananaquit; two populations of the yellow morph and two populations containing both the yellow morph and the melanic morph. A point mutation resulting in the replacement of glutamate with lysine was present in at least one allele of the *MC1R* gene in all melanic birds and was absent in all yellow morph birds. This substitution probably causes the color variation, as the same substitution is responsible for melanism in domestic chickens and mice. The evolutionary relationships among the *MC1R* haplotypes show that the melanic alleles on Grenada and St. Vincent had a single origin. The low prevalence of nonsynonymous substitutions among yellow haplotypes suggests that they have been under stabilizing selection, whereas strong selective constraint on melanic haplotypes is absent.

Conclusions: We conclude that a mutation in the *MC1R* is responsible for the plumage polymorphism in a wild bird population and that the melanic *MC1R* alleles in Grenada and St. Vincent bananaquit populations have a single evolutionary origin from a yellow allele.

Addresses: *Department of Biological Anthropology, University of Oxford, Oxford, OX2 6QS, UK. †Smithsonian Tropical Research Institute, PO Box 2072, Balboa, Panama. ‡Department of Biology, University of Missouri-St. Louis, 8001 Natural Bridge Road, St. Louis, Missouri, 63121.

Correspondence: Nicholas I. Mundy
E-mail: nick.mundy@bioanth.ox.ac.uk
Phone: +44-1865-274567; Fax: +44-1865-274699

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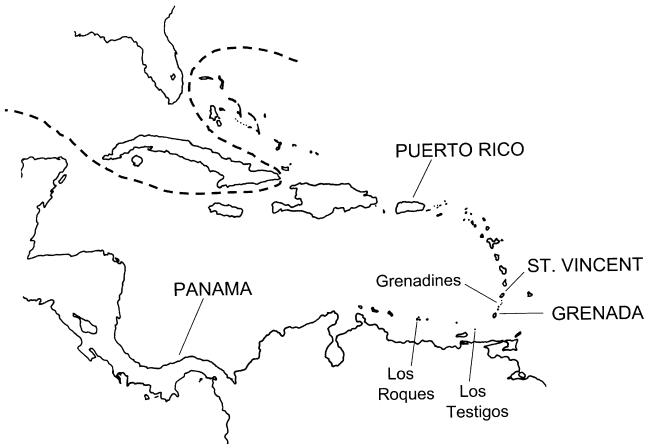
Background

The molecular basis of phenotypic diversity within species is of great interest to evolutionary biologists because adaptive morphological evolution depends on selection of genetic variants. However, there are few examples of phenotypic variation whose molecular basis is understood, especially in vertebrates. The study of avian plumage polymorphisms is attractive in this regard, as many polymorphisms have been documented [1], and recent work in domestic species has suggested candidate loci that may cause polymorphisms in wild populations [2, 3].

The bananaquit (*Coereba flaveola*) is a small passerine bird that is widely distributed in the Neotropics. In most populations, all individuals have the typical, or "yellow morph," plumage that includes a white eye-stripe and bright yellow breast, but black individuals are common on the Lesser Antillean islands of Grenada and St. Vincent (GSV) and on Los Testigos and Los Roques islands off the northern coast of Venezuela (Figure 1) [4]. Melanic birds are almost completely black due to eumelanin deposition throughout all feathers. Melanic bananaquits retain the yellow carotenoid breast pigment found in yellow morph individuals, it is simply obscured by the darker eumelanin [4]. The bananaquit populations on Grenada and St. Vincent are polymorphic, with melanic birds occupying moist forests at low to high elevations and yellow morphs occupying dry lowland habitats [5, 6]. Narrow clines occur on both islands where the yellow and melanic forms meet and mate randomly [5, 6].

The *melanocortin-1-receptor* gene (*MC1R*) is expressed in the melanocytes of developing feathers and hairs and plays a critical role in the control of melanin synthesis [7]. MC1R is a seven transmembrane, G protein-coupled receptor that is activated by melanocyte-stimulating hor-

Figure 1



Map of the Caribbean showing sampling localities (in capitals) in the present study and other sites mentioned in the text. The range of bananaquits extends from the dashed line south to Southern Brazil.

mone (MSH), leading to an increase in black/brown eumelanin production in melanosomes that are then transferred to the surrounding feathers. In many domesticated vertebrate species, including chickens, gain-of-function *MC1R* variants are associated with an increase in the production of eumelanin, while loss-of-function mutations are associated with an increase in red/yellow phaeomelanin production (chickens [2,3], mice [8], cattle [9], horses [10], pigs [11], sheep [12], and dogs [13]). *MC1R* variants are also associated with dark hair color in foxes [14] and with the occurrence of red hair in humans [15, 16].

In this study, we investigated the possibility that the plumage differences between melanic and yellow bananaquits might be due to mutations in the *MC1R* gene. We show that melanic morphs carry a *MC1R* mutation that is known to lead to the constitutive activation of the MC1R protein in mice and chickens, producing dark coat color in these species. Furthermore, we demonstrated that the black morphs on Grenada and St. Vincent had a single evolutionary origin and that patterns of nucleotide substitution are suggestive of different selective forces among yellow and melanic *MC1R* alleles.

Results

Association of *MC1R* genotype with plumage color

There were 33 variable sites in the 962 bp of the *MC1R* gene among all bananaquits sampled. Thirty-one substitutions occurred in the open reading frame, of which 24 were synonymous and 7 were replacement substitutions (Figure 2). Two substitutions were observed in the 3′ noncoding region. One nonsynonymous site showed a perfect correlation with phenotype, a guanine (G) to adenine (A) transition at site 274 causing a glutamate to lysine substitution at amino acid position 92 of MC1R. All yellow bananaquits ($n = 39$) were homozygous for guanine at this position, while all melanic individuals ($n = 29$) had a homozygous adenine or were heterozygous for A/G (Fisher's exact test, $p < 0.001$). Other nonsynonymous variants occurred only in some melanic birds, sites 272 (7/29 black individuals) and 494 (13/29 black individuals), or only in some typical bananaquits, sites 16 (3/39 yellow individuals), 85 (1/39 yellow individuals), 181 (5/39 yellow individuals), and 535 (34/39 yellow individuals). Two additional associations between *MC1R* genotypes and bananaquit phenotypes are worth noting. First, the lysine at amino acid position 92 was perfectly associated with a threonine replacement at amino acid position 179 in the black bananaquits, caused by a G to A transition at nucleotide site 535. This threonine replacement was also observed in 5 out of 87 yellow alleles. Second, variation at site 960 in the 3′ noncoding region was in complete linkage disequilibrium with site 274, suggesting that no recombination between these positions within the *MC1R* gene has occurred since the melanic haploptype originated.

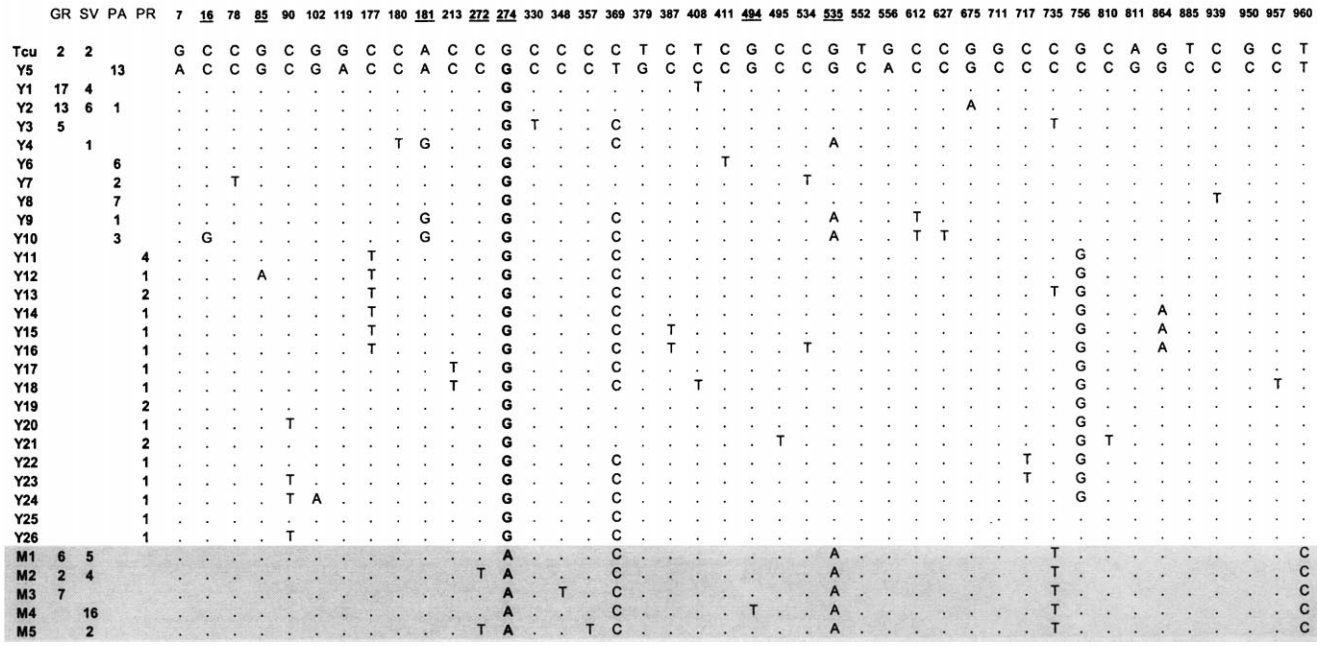
Given the known association of a lysine at position 92, or its equivalent, with melanism in chickens and mice (see Discussion), it is highly likely that this substitution is responsible for the phenotypic change in bananaquits. Accordingly, we define haplotypes with a lysine at position 92 as "melanic" and those with glutamate at position 92 as "yellow".

Haplotypic diversity and evolution

MC1R haplotypes and the frequencies of the haplotypes in each population are shown in Figure 2, and genotypes are shown in Figure 3. Each population has between 6 and 16 haplotypes. Some yellow (Y1 and Y2) and melanic (M1 and M2) haplotypes are shared by both Grenada and St. Vincent, whereas others (Y3, and M3 on Grenada; and Y4, M4, and M5 on St. Vincent) are island specific. All haplotypes on Puerto Rico are unique, and, with the exception of a single Y2 haplotype, the Panamanian haplotypes are also unique within our sample. Yellow and melanic haplotypes do not deviate from Hardy-Weinberg equilibrium on either Grenada ($p = 1.00$) or St. Vincent ($p = 0.24$).

On a haplotype network, the melanic haplotypes form a cluster with 1–3 mutational steps between any pair of haplotypes (Figure 4). The root (R) of the network, as determined by the tanager outgroup sequences, is two mutational steps away from the nearest yellow haplotypes (Y1, Y11, Y17, Y18, Y19, Y22, and Y25) and is four mutational steps nearer to these yellow haplotypes than to the nearest melanic haplotype (M1). Therefore, the most parsimonious interpretation of the haplotype network is that melanic haplotypes form a derived monophyletic

Figure 2



MC1R haplotypes. Variable sites are shown, with amino acid replacement substitutions underlined. A dot in a bananaquit haplotype represents the same nucleotide as haplotype Y5, except for the substitution at site 274 that causes the glutamate to lysine replacement at amino acid 92, which is shown separately for all individuals. Tcu is the *Tangara cucullata* outgroup sequence.

group, with M1 at its base. Most (22/26 with Y4, Y9, Y10, and Y12 as the exceptions) of the yellow haplotypes have the same amino acid sequence as the predicted ancestral yellow haplotype (R).

The yellow haplotypes on Grenada and St. Vincent are not closely related to the melanic haplotypes on these islands and mostly cluster with the Panamanian alleles. The two most common yellow haplotypes on Grenada and St. Vincent, Y1 and Y2, are at least six mutational steps away from the melanic haplotypes but only differ by one substitution from the most common haplotype in Panama, Y5. The majority of Puerto Rican haplotypes (all except Y25 and Y26) are separated from the other bananaquit haplotypes by a transversion at position 756. Three pairs of haplotypes, differing by a C-T transition at position 90, are present among the Puerto Rican haplotypes (Y19 and Y20, Y22 and Y23, and Y25 and Y26), a finding that is suggestive of recombination. Nonetheless, we deduce from the lack of recombinants between the common haplotype classes that point substitution has been more frequent than recombination in generating the observed haplotype diversity.

Nucleotide diversity indices are greater for Grenada and St. Vincent than for Panamanian and Puerto Rican populations when all haplotypes are considered together (Table 1), owing to the mixture of black and yellow alleles. Comparison among just the yellow haplotypes indicates that Puerto Rico and Panama have higher nucleotide diversity than Grenada and St. Vincent. Furthermore, melanic haplotypes on Grenada and St. Vincent have about half the diversity of yellow haplotypes on those islands.

All pairwise F_{st} values between populations were significantly greater than zero (data not shown). When considered separately, melanic haplotypes on Grenada and St. Vincent were significantly differentiated from each other (F_{st} = 0.366, p = 0.00), whereas yellow haplotypes were not (F_{st} = 0.005, p = 0.32).

Selection on yellow and melanic haplotypes

Two of 4 (50%) substitutions among melanic haplotypes are nonsynonymous, whereas only 4 of 22 (18%) substitutions among the yellow haplotypes lead to amino acid replacements, although this difference is not significant (G = 1.7, p = 0.19, df = 1). A McDonald-Kreitman test [17] comparing yellow haplotypes with the outgroup sequence (Table 2) revealed a significant excess of synonymous substitutions among yellow haplotypes. No significant differences were found in a McDonald – Kreitman test among all haplotypes or among melanic haplotypes only, although the numbers involved in the latter case are small.

Figure 3

Individual	Phe	Hap 1	Hap 2	Individual	Phe	Hap 1	Hap 2	Genotype	Phe	GR	SV	PA	PR
PA-CFA1	Y	Y8	Y10	GR-CFA2	M	M1	Y1	Y1/Y1	Y	1			
PA-CFA3	Y	Y5	Y8	GR-CFA5	M	M3	M3	Y1/Y2	Y	5	1		
PA-CFA4	Y	Y8	Y10	GR-CFA7	M	M3	Y1	Y1/Y3	Y	3			
PA-CFA5	Y	Y8	Y9	GR-CFA8	M	M3	Y1	Y2/Y2	Y	2	1		
PA-CFA6	Y	Y5	Y5	GR-CFA11	M	M1	Y2	Y2/Y3	Y	1			
PA-CFA7	Y	Y5	Y10	GR-CFA14	M	M1	Y2	Y2/Y4	Y		1		
PA-CFA8	Y	Y8	Y8	GR-CFA17	M	M2	Y3	Y2/Y5	Y			1	
PA-CFA341	Y	Y5	Y6	GR-CFA18	M	M2	Y2	Y5/Y5	Y			4	
PA-CFA1116	Y	Y7	Y7	GR-CFA19	M	M1	Y1	Y5/Y6	Y			1	
PA-CFA1135	Y	Y5	Y8	GR-CFA20	M	M1	Y1	Y5/Y8	Y			2	
PA-CFA1140	Y	Y5	Y5	GR-CFA22	M	M3	Y1	Y5/Y10	Y			1	
PA-CFA1304	Y	Y5	Y5	GR-CFA24	M	M1	M3	Y7/Y7	Y			1	
PA-CFA1339	Y	Y5	Y2	GR-CFA30	M	M3	Y1	Y8/Y8	Y			1	
PA-CFA2026	Y	Y5	Y5	SV-CFA5	Y	Y2	Y2	Y8/Y9	Y			1	
PR-CFA2	Y	Y14	Y22	SV-CFA2085	Y	Y2	Y4	Y8/Y10	Y			2	
PR-CFA3B	Y	Y11	Y19	SV-CFA2129	Y	Y1	Y2	Y11/Y18	Y				1
PR-CFA6	Y	Y15	Y23	SV-CFA1	M	M2	M5	Y11/Y19	Y				2
PR-CFA11352	Y	Y13	Y20	SV-CFA2	M	M2	M4	Y11/Y21	Y				1
PR-CFA11363	Y	Y11	Y21	SV-CFA3	M	M4	Y1	Y12/Y17	Y				1
PR-CFA11364	Y	Y21	Y25	SV-CFA4	M	M2	Y2	Y13/Y20	Y				1
PR-CFA11365	Y	Y12	Y17	SV-CFA6	M	M1	Y1	Y13/Y24	Y				1
PR-CFA11366	Y	Y11	Y18	SV-CFA2077	M	M4	M4	Y14/Y22	Y				1
PR-CFA11367	Y	Y16	Y26	SV-CFA2087	M	M4	Y1	Y15/Y23	Y				1
PR-CFA11368	Y	Y11	Y19	SV-CFA2094	M	M4	M4	Y16/Y26	Y				1
PR-CFA11372	Y	Y13	Y24	SV-CFA2109	M	M4	Y2	Y21/Y25	Y				1
GR-CFA3	Y	Y1	Y2	SV-CFA2120	M	M1	M4	Total yell		12	3	14	11
GR-CFA9	Y	Y1	Y1	SV-CFA2122	M	M2	M2	M1/Y1	M	3	1		
GR-CFA10	Y	Y1	Y3	SV-CFA2150	M	M1	M4	M1/Y2	M	2			
GR-CFA12	Y	Y1	Y3	SV-CFA2159	M	M2	M5	M2/Y2	M	1	1		
GR-CFA13	Y	Y1	Y2	SV-CFA2160	M	M1	M4	M2/Y3	M	1			
GR-CFA15	Y	Y1	Y2	SV-CFA2161	M	M1	M4	M3/Y1	M	4			
GR-CFA21	Y	Y1	Y2	SV-CFA2162	M	M4	M4	M4/Y1	M		2		
GR-CFA23	Y	Y2	Y2					M4/Y2	M		1		
GR-CFA25	Y	Y2	Y3					M1/M3	M	1			
GR-CFA26	Y	Y1	Y2					M1/M4	M		4		
GR-CFA28	Y	Y2	Y2					M2/M2	M		1		
GR-CFA29	Y	Y1	Y3					M2/M4	M		1		
								M2/M5	M		2		
								M3/M3	M	1			
								M4/M4	M		3		
								Total mel		13	16	0	0
								Total all		25	19	14	11

MC1R genotypes. The left hand columns show genotypes of each individual in the study. The right hand column summarizes population genotypic data.

Discussion

Genotype-phenotype association

As far as we are aware, this is the first demonstration of an association between a nucleotide polymorphism and a plumage polymorphism in a wild bird. The same glutamate to lysine substitution identified here in bananaquits is associated with melanic plumage/pelage in chickens [3] and mice [8] (see Figure 5). Furthermore, in vitro experiments in $E^{So\text{-}3J}$ mice have demonstrated that this substitution causes the melanic coat, as it results in the constitutive activation of the MC1R protein [8] (i.e., an MC1R that is continually active in the absence of MSH stimulation). In the chicken and mouse models, both normal and constitutively active variants of the *MC1R* have a threonine at position 179, as in all melanic haplotypes in bananaquits. We cannot, therefore, rule out a permissive role of this mutation in generating the melanic haplotype. Also, although we consider it unlikely, the possibility that the noncoding mutation at position 960 plays a role in altering transcriptional regulation in melanic bananaquits cannot be formally ruled out.

Our model of the constitutive activation of the MC1R protein coupled to the distribution of black feathers in the black and yellow color morphs of bananaquits warrants some preliminary speculation on the control of eumelanin production in this species. According to the model, the production of eumelanin throughout the plumage of melanic birds is solely dependent on the constitutive activation of the MC1R protein, and not changes in *MC1R* gene expression. Accordingly, the *MC1R* gene is also expressed throughout the plumage of yellow bananaquits. Therefore, control of regional eumelanin production in yellow birds must be due to the local variation in concentration of MSH and/or an MC1R antagonist, such as agouti protein.

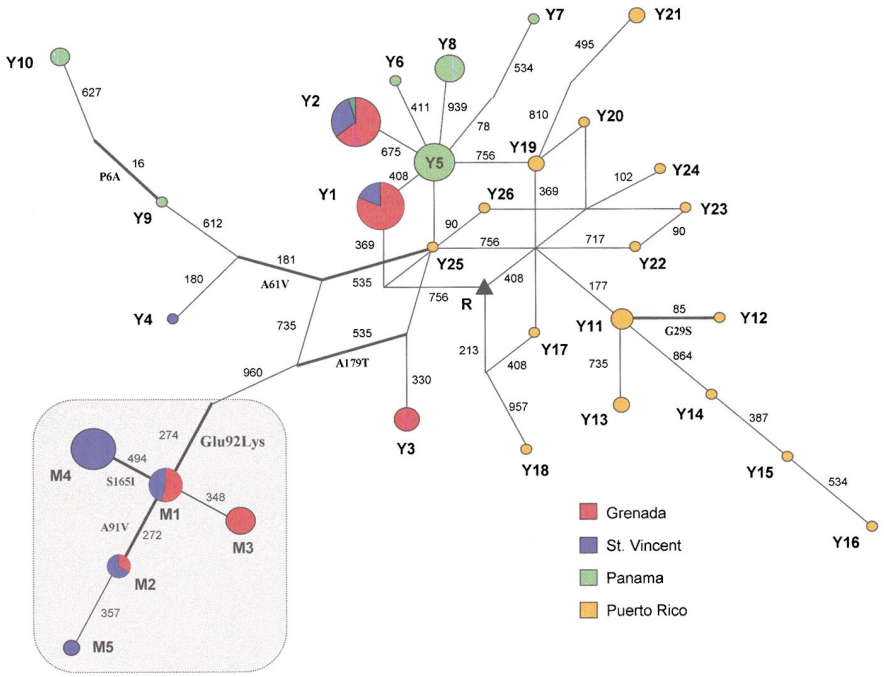
The bananaquit *MC1R* data are consistent with the dominant inheritance of the melanic alleles, a finding further supported by pedigree analysis of *MC1R* inheritance in domestic animals [7]. Wunderle [5] had previously deduced the dominant inheritance of melanism from observations of plumage color of wild bananaquit pairs and their offspring. Considering all the data, it is likely that the Glu92Lys mutation causes a constitutively active MC1R protein that results in melanic plumage in bananaquits. In vitro expression experiments to test this hypothesis are currently underway.

Reconstruction of the ancestral phenotype and population history of the polymorphism

Identification of a molecule-based, genotype-phenotype association in extant populations creates the possibility of reconstructing phenotypic evolution. In bananaquits, the derived position of the melanic alleles in the haplotype network supports the hypothesis that there is a single origin of the black phenotype from a yellow ancestor. Furthermore, the restricted geographic distribution and lower diversity of melanic alleles is consistent with a relatively recent origin of the melanic alleles on one of the islands where black bananaquits are currently found. Although we cannot exclude the possibility that the melanic alleles originated elsewhere and are relictual at high frequency on Grenada and St. Vincent, the putative dominance of the melanic alleles suggests that one would observe the occasional melanic bananaquit in other locations even if the melanic alleles were in low frequency. We are not aware of any observations of melanic bananquits outside the populations on Grenada, St. Vincent, Los Testigos, and Los Roques.

The significant F_{st} values between the melanic bananaquit haplotypes sampled on Grenada and St. Vincent indicate that these populations are exchanging few, if any, melanic alleles at the present time. This is not surprising

Figure 4



MC1R haplotype network. This is a minimum-spanning network, except for substitutions at sites 534 and 735 that each occur at two places on the network. Circle areas are proportional to sample sizes. Each line segment represents a single substitution (with nucleotide positions numbered as in Figure 2), and thicker segments are replacement substitutions, with changes from the putative ancestral amino acid sequence of the *MC1R* gene identified and numbered. R represents the root represented by the outgroup sequences of the tanager *Tangara cucullata*, which differs from the bananaquit *MC1R* root sequence by eight substitutions (Figure 2). Melanic haplotypes are enclosed by the gray box.

given that melanic bananaquits are absent from the low-lying Grenadine islands between Grenada and St. Vincent. Given the relationship between the melanic alleles pictured in Figure 4, we presume that melanic bananaquits have previously occurred in the Grenadines and that sea level highs and/or habitat changes have caused their extirpation from these islands. In contrast, our population genetic analysis of the yellow *MC1R* alleles and previous analyses of mtDNA haplotypes [18] indicate recent or continuing gene flow between Grenada and St. Vincent, and the modern-day presence of yellow birds in the Grenadines provides a mechanism for the interisland movement of *MC1R* yellow alleles and mtDNA. Thus, the absence of gene flow of melanic *MC1R* alleles between Grenada and St. Vincent and the apparently restricted dispersal of melanic bananaquits across the Grenadines cannot be attributed to a lack of movement of bananaquits per se, but are caused by the dominant inheritance of melanism.

Two molecule-based perspectives on the evolutionary history of bananaquit populations

In addition to elucidating the molecular basis for melanism in bananaquits, the *MC1R* data permit insight into the evolutionary relationships of GSV, Puerto Rico, and Panama bananaquit populations. Furthermore, the network of *MC1R* yellow alleles can be compared to previously published mtDNA gene genealogies [18, 19] in order to provide independent perspectives on the phylogenetic history of bananaquit populations. The mtDNA-based bananaquit phylogeny separates birds from GSV, Puerto Rico, and Panama into three reciprocally monophyletic mtDNA clades that suggest relatively long periods of independent evolutionary history. In contrast, there

Table 1

***MC1R* nucleotide diversity**

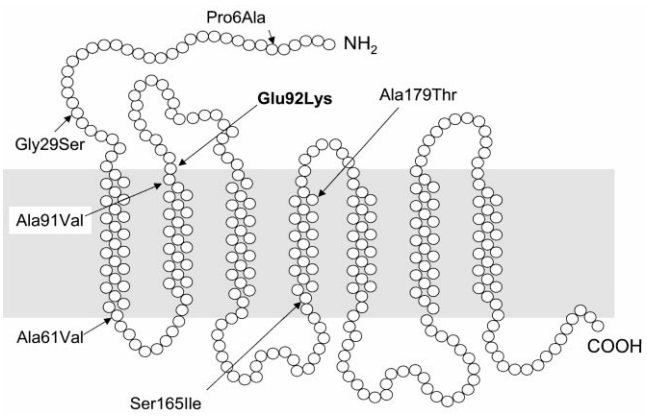
		π	S. E.
Grenada	Total	0.0038	0.0022
	Yellow	0.0018	0.0012
	Melanic	0.0008	0.0007
St. Vincent	Total	0.0037	0.0021
	Yellow	0.0019	0.0013
	Melanic	0.0010	0.0008
Panama		0.0023	0.0015
Puerto Rico		0.0032	0.0019

Table 2

McDonald–Kreitman test on bananaquit versus tanager haplotypes.

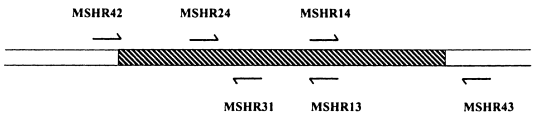
Yellow haplotypes	(n = 96)	Syn	NS
	Fixed	3	5
p = 0.02	Polymorphic	22	4
Melanic haplotypes	(n = 42)	Syn	NS
	Fixed	5	7
p = 1.0	Polymorphic	2	2
All haplotypes	(n = 138)	Syn	NS
	Fixed	3	5
p = 0.08	Polymorphic	24	7

Figure 5



Two-dimensional model of the bananaquit *MC1R* in the melanocyte membrane, with replacement substitutions from the ancestral *MC1R* haplotype indicated. The amino terminus is extracellular.

Figure 6



Positions of PCR and sequencing primers used in this study in relation to the *MC1R* open reading frame (hatched).

are no *MC1R* yellow-allele synapomorphies that permit the phylogeographic separation of the three regional populations. Nevertheless, some evolutionary independence of the three populations is strongly suggested by the *MC1R* haplotype network, which establishes that most yellow alleles are connected to haplotypes representing the same geographic population. This result is further supported by F_{st} analysis of *MC1R* yellow haplotypes, demonstrating that regional populations are significantly differentiated from one another (GSV-Panama; GSV-PR; Panama-PR).

In addition to establishing the evolutionary independence of GSV, PR, and Panama bananaquit populations, the mtDNA data also identify a significantly closer phylogenetic relationship between GSV and Puerto Rico bananaquits [18, 19]. In contrast, the *MC1R* yellow alleles from GSV cluster more closely with Panama *MC1R* haplotypes. Two explanations for this discrepancy are reasonable and cannot be distinguished based on the *MC1R* and mtDNA data in hand. First, yellow morph bananaquits may have colonized GSV relatively recently from a continental source population similar to Panama in terms of its *MC1R* and mtDNA allele complement. For this scenario to be correct, it is necessary to posit the loss of the colonists' mtDNA (due to either stochastic sampling processes, selection, or male-biased gene flow). Support for this view is bolstered by historical records from the late 1800s and early 1900s indicating that yellow morph bananaquits were uncommon on both islands but have increased in frequency since the turn of the century ([5, 6], and references therein).

Second, the apparent discrepancy noted between the phylogenetic signals carried by *MC1R* and mtDNA may simply indicate that slower rates of *MC1R* nucleotide substitution and allele coalescence have yielded an unresolved polytomy between GSV, PR, and Panama that is fully resolved by mtDNA data. This explanation presumes that the rate of *MC1R* nucleotide substitution is slow relative to the rate of mtDNA diversification of bananaquit lineages. Support for this scenario is provided by the 10-fold greater difference in substitution rate for mtDNA in comparison to *MC1R*, as measured between bananaquits and the *Tangara cucullata* outgroup. Furthermore, the branch that connects the GSV/PR/Panama common mtDNA ancestor to the GSV/PR ancestor on the mtDNA phylogeny has seven substitutions, suggesting that the rate of this lineage divergence exceeded the *MC1R* substitution rate. Compounding the difference in the evolutionary rate across the *MC1R* and mtDNA markers is their 4-fold difference in effective population size and the correspondingly slower rate of *MC1R* coalescence.

Role of selection in *MC1R* evolution

The significant excess of synonymous to nonsynonymous substitutions among yellow haplotypes in comparison with the tanager outgroup demonstrate that the *MC1R* gene has been under strong selective constraint in yellow morph birds. Although the number of substitutions involved is small, the relatively high proportion of nonsynonymous substitutions among melanic haplotypes suggests that they may not be under such strong selective constraint. One of the substitutions (alanine to valine at position 91 in alleles M2 and M5) is in the second transmembrane domain and is located close to a region implicated in agonist binding [21] (Figure 5). As gain-of-function, constitutively active melanic *MC1R* haplotypes do not need to bind MSH, it is reasonable to suppose that they have fewer structural requirements than yellow haplotypes. A contrasting situation is found in humans, in which ancestral African *MC1R* haplotypes are under purifying selection, whereas selective constraint is reduced or lost in loss-of-function *MC1R* haplotypes in European populations [20].

Are melanic birds on GSV currently under selection? Wunderle [5, 6] considered it unlikely that extensive eumelanin evolved as an antipredator mechanism or for thermal adaptation. However, thermal consequences of melanism may restrict black morphs to more shaded, moist

habitats. This does not explain, however, why yellow morphs are not distributed throughout higher-elevation, moist forest, as they are on other islands in the Lesser Antilles. Yellow and melanic morphs form narrow hybrid zones in northeastern and southwestern Grenada and southwestern St. Vincent. There is no evidence of assortative mating within these hybrid zones, as indicated by Wunderle's [5] observation of random mating and our failure to detect departure from Hardy-Weinberg equilibrium. As noted above, the limited geographic presence of yellow birds on Grenada and St. Vincent may be due simply to their recent arrival at the turn of the century. Following this argument, we would expect yellow bananaquits to expand their ecological distribution on these islands in the future unless the melanic form is selectively advantageous in the upland zone.

Conclusions

Our study shows that a candidate-gene approach to evolutionary genetics can be fruitful. In the case of bananaquits, the dramatic phenotypic difference in plumage appears to have been produced by a relatively simple genetic change. It would now be useful to determine whether the *MC1R* gene has played a general role in the evolution of avian plumage, e.g., in polymorphic snow geese [22] and skuas [23], similar to the one played in mammalian pelage evolution as demonstrated by studies of *MC1R* in humans [16], foxes [14], and desert mice (M. Nachman, personal communication). Furthermore, studies of domestic animals [8] indicate that the consequences of *MC1R*-dependent melanism are not always manifest throughout the plumage/pelage. Thus, it may turn out that the evolutionary dynamics of apparently complex phenotypic transitions in plumage/pelage coloration are due to relatively simple genetic switches like the *MC1R* glutamate to lysine substitution identified in bananaquits.

Materials and methods

Samples

Samples from the following bananaquits were included in the study: 14 yellow morph birds from Panama, 11 yellow morph individuals from Puerto Rico, 25 birds from Grenada (12 yellow morphs, 13 black morphs; 24 out of 25 from a single locality in the southwestern cline), and 19 individuals from St. Vincent (3 yellow morphs, 16 black morphs; from 5 separate localities) (Figure 1). A tanager species, *Tangara cucullata* (samples GR-TCU1 from Grenada, and SV-TCU3 from St. Vincent), was used as the outgroup.

PCR, cloning, and sequencing

Genomic DNA was isolated using commercial kits (Qiagen Tissue Kit) according to the manufacturer's instructions. Polymerase chain reaction (PCR) amplifications were performed in a thermal cycler (MJR DNA engine) in 25 μl total volume containing 0.5–1.0 units Taq polymerase (Advanced Biotechnologies), 1× PCR buffer, 50 mM dNTP, and 1.5 mM $MgCl_2$. Cycling parameters were as follows: 1× 94°C, 3 min; 40 × 94°C, 45 s, 57–62°C, 60 s, 72°C, 90 s; and 1 × 72°C, 5 min.

Primers and dNTPs were removed from PCR products using Qiaquick columns (Qiagen). TA cloning was performed using the pGEM-T easy vector system (Promega) according to manufacturer's instructions. Direct sequencing of PCR products and sequencing of plasmid clones were performed on both strands by cycle sequencing using Big Dye terminators (PE Biosystems), and reactions were run on ABI 377 sequencing apparatus. Sequences were edited using Sequence Navigator and Sequencher.

Primers

Primers and their positions are shown in Figure 6. The entire *MC1R* was amplified by PCR using primers MSHR42 (5′- GGACTGAGGAGG GGCTCAT -3′) and MSHR43 (5′- CCATCCCGTCTGTCCATTTCA -3′). Sequencing was performed with MSHR42, MSHR43, and the internal sequencing primers MSHR24 (5′-CCCACCTACTACTTCATCTG CTG-3′), MSHR14 (5′- CCTCTTCATGCTGGTCCTCA -3′), MSHR 31 (5′- CCAGCAGCAGCATGAAGAGC -3′), and MSHR13 (5′- GAGGAC CAGCATGAAGAGGA -3′). These primers were designed from bananaquit sequences and have not been tested on other species, except for *Tangara cucullata*.

Sequence analysis

Sequences were unambiguously aligned by eye. A 962 bp alignment consisting of the entire 945 bp *MC1R* coding region and 17 bp of the 3′ noncoding sequence was used for further analyses. Haplotypes of individuals with two or more heterozygous sites were inferred by a combination of TA cloning and parsimony. All sequence variants occurring only in heterozygotes were confirmed by TA cloning. The haplotype network was obtained by inspection. Standard diversity-index calculations, F_{ST} estimations, and deviations from Hardy-Weinberg expectations were performed using Arlequin version 2.000 [24], with significance of F_{ST} estimations being determined by randomization tests in Arlequin. McDonald-Kreitman tests [17] were performed using R × C [25], an implementation of the metropolis algorithm method for contingency tables of Guo and Thompson [26].

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