The Genetic Basis of Phenotypic Convergence in Beach Mice: Similar Pigment Patterns but Different Genes

Cynthia C. Steiner, Holger Römpler,†‡[1] Linda M. Boettger,* Torsten Schöneberg,† and Hopi E. Hoekstra‡*

*Division of Biological Sciences, University of California, San Diego; †Institute of Biochemistry Medical Faculty, University of Leipzig, Leipzig, Germany; and ‡Department of Organismic and Evolutionary Biology and The Museum of Comparative Zoology, Harvard University, Cambridge, MA

Convergent evolution is a widespread phenomenon seen in diverse organisms inhabiting similar selective environments. However, it is unclear if similar phenotypes are produced by the same or different genes and mutations. Here we analyze the molecular mechanisms underlying convergent pigment pattern among subspecies of the beach mouse (*Peromyscus polionotus*) inhabiting the Gulf and Atlantic coasts of Florida. In these two geographic regions, separated by more than 300 km, "beach mice" have lighter colored coats than do their mainland counterparts, produced by natural selection for camouflage against the pale coastal sand dunes. We measured color pattern in eight beach mouse subspecies and showed that three of the Gulf Coast subspecies are more phenotypically similar to an Atlantic coast subspecies than to their Gulf Coast neighbors. However, light-colored beach mice do not form a monophyletic group. Previous results implicated a single derived amino acid change in the *melanocortin-1 receptor* (*Mc1r*) as a major contributor to pigment pattern in the Gulf Coast beach mice; despite phenotypic similarities, the derived *Mc1r* allele was not found in the Atlantic coast beach mouse populations. Here we show that Atlantic coast beach mice have high levels of *Mc1r* polymorphism but they lack unique alleles. Functional assays revealed that single amino acid mutations segregating in Atlantic coast beach mice do not cause any change in *Mc1r* activity compared with the activity of *Mc1r* from dark-colored mice. These joint results show that convergent pigment patterns in recently diverged beach mouse subspecies—whose developmental constraints are presumably similar—have evolved through a diversity of genetic mechanisms.

Introduction

One of the most fascinating phenomena in evolutionary biology is that of phenotypic convergence, whereby unrelated species respond to similar selection pressures by evolving similar traits. Convergence has been seen between species residing at many taxonomic levels: between evolutionarily distant taxa (e.g., mimicry in members of different insect orders [Wickler 1968]) and in more closely related species (e.g., ecomorphs of *Anolis* lizards on Caribbean islands [Williams 1972; Roughgarden 1995; Losos et al. 1998]). Convergence within species, often referred to as parallel evolution, has also been demonstrated (e.g., albinism in isolated populations of cavefish [Strecker et al. 2003]). But does such phenotypic convergence imply genetic convergence—that is, does the attainment of similar forms or patterns in different species involve the same genes and/or genetic pathways? Until recently, this question could be answered only by using genetic crosses to test for complementation (e.g., Borowsky 2008), an impossibility in most groups, and which can tell you only if the same genes, but not necessarily the same mutations, are involved.

A few studies have begun to use a molecular approach to study phenotypic convergence in nature. In a surprising number of cases studied to date, the same genes are repeatedly involved in the production of similar adaptive phenotypes (Ardent and Reznick 2008). For example, the *yellow* gene is involved in the independent evolution of wing spots in several *Drosophila* species (Prud'homme et al. 2006), the ocular albinism (*Oca2*) gene in multiple cavefish populations (Protas et al. 2006), and *Pitx1* in different populations (Cresko et al. 2004; Shapiro et al. 2004; Coyle et al. 2007) as well as different species (threespine and ninespine; Shapiro et al. 2006) of stickleback fish showing pelvic reduction. Likewise, the reduced degree of armor plating seen in sticklebacks that have independently colonized freshwater lakes appears to be largely due to a single allele of the Ectodysplasin (*Eda*) gene occurring at low frequency in ancestral oceanic populations (Colosimo et al. 2005). Thus, for these species and populations, convergence has been attained by either independent mutations in the same gene or the fixation of the same allele derived from standing genetic variation in ancestral populations (Barrett and Schluter 2008). In both scenarios, convergent phenotypes share a similar genetic underpinning.

This genetic similarity is not ubiquitous, however. In *Drosophila* pigmentation, for example, convergent phenotypes in different species appear to involve different genes (Wittkopp et al. 2003; Carbone et al. 2005), as does adaptive melanism in different lava-dwelling populations of pocket mice (Hoekstra and Nachman 2003; Nachman et al. 2003).

Here we study the phenomenon of convergence in pigment pattern among populations of a single species, *Peromyscus polionotus*. Throughout most of its range in the southeastern United States, this species is called the "oldfield mouse" because it inhabits old, overgrown agricultural fields. Oldfield mice also have colonized the light-colored sandy coastal dunes and barrier islands along the Gulf Coast as well as the Atlantic coast of Florida. In both of these areas, they are called "beach mice" (Osgood 1909; Bowen 1968). Compared with the darker pigmented inland conspecifics, beach mice have a unique pigmentation pattern with reduced pigmentation on their faces, flanks, and tails (fig. 1).

This light pigmentation in beach mice on the coastal sand dunes is driven by selection for camouflage, yielding a strong correlation between the coat color of a population

[1] Present address: Rudolf-Boehm-Institute of Pharmacology and Toxicology, Medical Faculty, University of Leipzig, D-04107 Leipzig, Germany.

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E-mail: hoekstra@oeb.harvard.edu.

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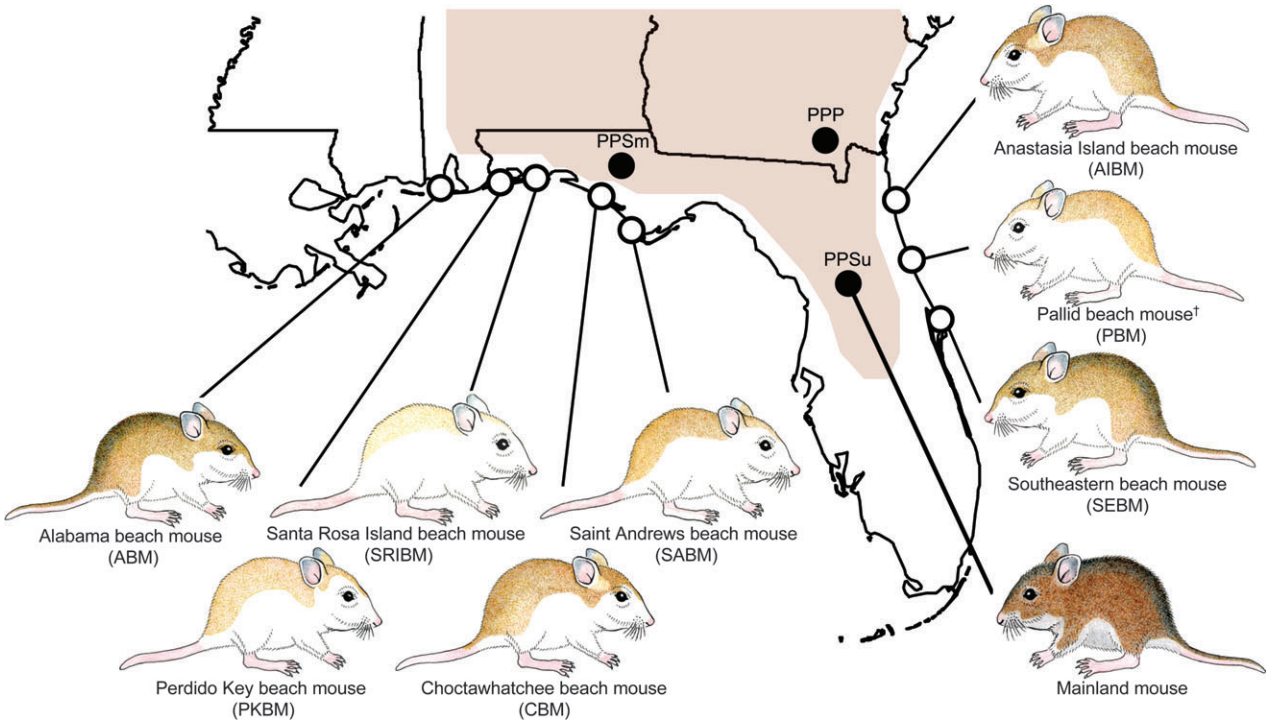

FIG. 1.—Geographic distribution of *Peromyscus polionotus*. Brown shading represents the approximate range of mainland subspecies in Florida, Georgia, and Alabama. Circles indicate collection locations for mice (filled, mainland; open, beach) used in the molecular and/or morphological studies. Abbreviations for mainland subspecies are as follows: *P. polionotus sumneri* (PPSm), *P. p. polionotus* (PPP) and *P. p. subgriseus* (PPSu). Cartoons represent the typical color pattern for each *P. polionotus* subspecies surveyed.

and the reflectance of the soil on which it lives (Blair 1951; Belk and Smith 1996) even in the face of high levels of homogenizing gene flow (Mullen and Hoekstra 2008). Major visual predators of *P. polionotus* include owls, hawks, herons, and mammalian carnivores (VanZant and Wooten 2003). Owl predation experiments in field enclosures using *P. polionotus* subspecies differing in dorsal color showed that conspicuously colored mice were captured more frequently than were their more cryptic conspecifics (Kaufman 1974).

The genetic basis of pigmentation differences between two populations of *P. polionotus* has been studied recently. Genome-wide quantitative trait analyses have implicated *melanocortin-1 receptor* (*Mc1r*) as one of three major contributors to differences between two subspecies: *subgriseus*, a mainland dark-pigmented form, and *leucocephalus*, a light Gulf Coast subspecies (the Santa Rosa Island beach mouse [SRIBM]). Although *Mc1r* shows no differences in expression level (Steiner et al. 2007), a single nucleotide difference produces a charge-changing amino acid mutation (Arg to Cys at amino acid position 65), which alters both ligand-binding and receptor-signaling potentials consistent with its involvement in light pigmentation (Hoekstra et al. 2006).

Light coloration in Atlantic coast populations could in principle be due to the identical mutation in *Mc1r* that is involved in Gulf Coast populations, to different mutations in the same gene, or to different genes altogether. Here we ask whether the similar light pigmentation of Gulf and Atlantic coast beach mice (populations separated by over 300 km) has a similar genetic basis. To address this question, we first documented phenotypic convergence in pigment pattern of Gulf and Atlantic coast subspecies. We then reconstructed an intraspecific phylogeny of *P. polionotus* subspecies to determine the relationship between Atlantic and Gulf coast beach mice. Finally, we sequenced the entire *Mc1r*-coding region in eight subspecies of beach mice and functionally tested all derived amino acid mutations individually to determine if any "new" mutations in *Mc1r* contribute to light pigmentation. Our results suggest that this adaptive convergence is based, at least in part, on different genetic mechanisms.

Methods

Sampling

We examined a total of 305 individuals of *P. polionotus* (112 individuals for molecular analyses and 193 for phenotypic analyses; table 1). We obtained samples from the Florida Museum of Natural History or from individuals caught in the field. We took tissue samples from the liver or tail tips. For samples of mainland mice, we prepared and accessioned specimens at the Museum of Comparative Zoology, Harvard University.

Phenotypic Measurements

We measured pigmentation phenotypes of both live individuals and museum specimens derived from eight beach mouse subspecies, five on the Gulf Coast and three on the Atlantic coast, as well as three mainland subspecies (table 1). We scored eight pigmentation traits using

Table 1
Sample of Beach Mouse and Mainland Populations Used in Phenotypic, Phylogenetic, and *Mc1r* Genetic Variation Analyses

Common name	Abbreviation	Subspecies	Phenotype Survey (*N*)	Phylogenetic Survey (*N*)	*Mc1r* Survey (*N*)	Location	Collecting Site
Alabama beach mouse	ABM	*Peromyscus polionotus ammobates*	14	3	10	Gulf	Alabama Island, AL
Perdido Key beach mouse	PKBM	*Peromyscus polionotus trissyllepsis*	8	2	10	Gulf	Perdido Key, FL
Santa Rosa Island beach mouse	SRIBM	*Peromyscus polionotus leucocephalus*	43	3	20	Gulf	Santa Rosa Island, FL
Choctawhatchee beach mouse	CBM	*Peromyscus polionotus allophrys*	41	2	10	Gulf	Topsail Hill State Park, FL
St. Andrews beach mouse	SABM	*Peromyscus polionotus peninsularis*	23	2	20	Gulf	St. Joseph Peninsula, FL
Mainland (Lake Lousia)	PPSu	*Peromyscus polionotus subgriseus*	22	2	20	Mainland	Lake Louisa State Park, FL
Mainland (Georgia)	PPP	*Peromyscus polionotus polionotus*	10	3	—	Mainland	Daniel Preserve, GA
Mainland (Florida)	PPSm	*Peromyscus polionotus sumneri*	5	2	—	Mainland	Panhandle, FL
Southeastern beach mouse	SEBM	*Peromyscus polionotus niveiventris*	7	3	10	Atlantic	Cape Canaveral National Seashore, FL
Pallid beach mouse[a]	PBM	*Peromyscus polionotus decoloratus*	4	—	2	Atlantic	Ponce de Leon, FL
Anastasia Island beach mouse	AIBM	*Peromyscus polionotus phasma*	16	2	10	Atlantic	Anastasia Island State Park, FL

NOTE.—*N*, number of individuals.
[a] Extinct beach mouse subspecies.

categories that are unambiguous and together give an accurate description of overall pigmentation pattern (following Hoekstra et al. 2006). For six traits (rostrum, cheek, eyebrow, earbase, ventrum, and ankle), we assigned values of 0, 1, or 2, where "0" corresponds to white or unpigmented hairs, "1" to hairs pigmented at the base but white or unpigmented on the tip, and "2" to fully pigmented hairs. One trait, rump color, was scored using five categories to describe the dorsolateral extension of pigment: scores, ranging from "0" (minimally pigmented) to "4" (fully pigmented), reflect the extent of rump pigmentation. Tail pigmentation was scored using six categories, ranging from "0" (lack of any tail stripe) to "5" (full tail stripe). We performed discriminant analyses of the combined color traits using JMP v.5.1.2 statistical software package (SAS Institute).

Molecular Methods

Using a DNeasy tissue kit (Qiagen, Valencia, CA), we extracted DNA from 11 subspecies of *P. polionotus* comprising 112 individuals (table 1) and a single individual of *Peromyscus maniculatus* to serve as an outgroup.

Mitochondrial DNA

We generated partial sequences (819 bp) of the mitochondrial gene *COIII* from 24 *P. polionotus* and 1 *P. maniculatus*. We amplified this gene using polymerase chain reactions (PCRs) in a 15 μl volume using Eppendorf Mastercycler Gradient thermal cyclers. Each reaction included 30 ng of template DNA, 10× *Taq* buffer with 1.5 mM $MgCl_2$ (Eppendorf), 0.3 μl of 10 mM deoxynucleoside triphosphates, 0.6 μM each primer, and 0.15 units *Taq* DNA polymerase (Eppendorf). PCR forward and reverse primers, PCR cycling, and sequencing conditions are given by Hoekstra et al. (2004). For Atlantic coast subspecies, we designed new primers: forward 5′-TATGTTTATTACTATCTTCTAGGTT-3′ and reverse 5′-CATGACCACTAACAGGAGCA-3′. The cycling conditions for the new primer pair were 94 °C for 3 min, followed by 29 cycles of denaturation at 94 °C for 30 s, 50 °C annealing for 45 s, and 72 °C for 1 min, and the final extension occurred at 72 °C for 10 min. We used these PCR primers in the cycle sequencing reactions.

We also generated partial sequences (919 bp) of the mitochondrial control region for the same 25 individuals. We designed specific primers for *P. polionotus*: forward 5′-TAAACTACTTCTTGTACATA-3′ and reverse 5′-GTATATGTACCACTAATGTTGA-3′. We used the following PCR cycling conditions: 94 °C for 3 min, followed by 34 cycles of denaturation at 94 °C for 30 s, 48 °C annealing for 45 s, and 72 °C for 1 min, and the final extension occurred at 72 °C for 10 min. We used these PCR primers in the cycle sequencing reactions.

Nuclear Locus Genotypes

We screened 14 nuclear (nonpigmentation) genes for polymorphisms in *P. polionotus*. For each gene, we

designed PCR primers in conserved exonic regions based on alignments of mouse, rat, and human sequences. To maximize our chance of detecting variation between subspecies, we designed amplification primers to span introns. Following PCR optimization, we amplified introns to identify polymorphisms. We then edited those sequences using Sequencher 3.1.1 (Gene Codes, Ann Arbor, MI) and identified polymorphisms by eye. PCR primers and amplification conditions are provided in supplementary table S1 (Supplementary Material online).

Using a TaqMan assay, we scored a single nucleotide polymorphism (SNP) identified in each nuclear gene in 25 individuals on an ABI 7000. In each reaction, we used 60 ng of genomic DNA and the following cycling parameters: 40 cycles of 50 °C for 2 min, 95 °C for 10 min, and 92 °C for 15 s followed by an allelic discrimination step of 60 °C for 2 min. TaqMan primer sequences are listed in supplementary table S2 (Supplementary Material online).

Mc1r Sequences

We amplified and sequenced the entire *Mc1r*-coding region (954 bp) of 112 individuals (table 1). The *Mc1r* primer sequences, the PCR cycling, and sequencing conditions follow the methods of Hoekstra et al. (2006).

Phylogenetic Reconstruction

Population Tree Estimation

Because each locus in the genome is expected to have an independent genealogical history, we considered evidence from multiple loci to obtain a "best estimate" of population history. We aligned the mitochondrial DNA (mtDNA) control region and *COIII* sequences using Sequencher. We then concatenated the mitochondrial genes and appended the 14 SNP markers genotyped in the same individuals (1,752 bp total) to maximize phylogenetic signal (Weins 1998).

We first performed a Bayesian analysis using MrBayes v3.1 (Ronquist and Huelsenbeck 2003) with the following models chosen by the Akaike information criterion in MrModeltest v2.2 (Nylander 2004): control region = general time reversible (GTR) + I + Γ, *COIII* = Hasegawa–Kishino–Yano (HKY) + I, SNPs = F81 + Γ. The analysis was partitioned by gene, model parameters were unlinked across partitions, and among-partition rate variation was accommodated using rate multipliers (see Marshall et al. 2006). Two concurrent runs consisted of four Markov chains (one cold and three heated chains with a temperature of 0.2), five million generations (sampled every 1,000 generations), and a 25% burn-in. We considered runs to have converged on stationarity when there were no trends in generation versus logL plots, potential scale reduction factors were near 1.0 for all parameters, and the average standard deviation of split frequencies was below 0.01. We also calculated posterior probabilities for each node.

To determine if different phylogenetic algorithms produced similar topologies, we also ran maximum likelihood (ML) and maximum parsimony (MP) analyses with Paup* v4.0b10 (Swofford 2002). Using Modeltest v3.06 (Posada and Crandall 1998), we determined the best-fit model of sequence evolution for the combined data set to be the GTR + I + Γ model. For the ML analyses, we used previously estimated optimal parameters with a Neighbor-Joining (NJ) starting trees and TBR branch swapping. We assessed support for internal nodes by bootstrap analyses with 1,000 replicates (Felsenstein 1985).

Although genetic and morphological evidence suggests that there is little gene flow between *P. polionotus* subspecies (e.g., Mullen et al. forthcoming; Degner et al. 2007), it is possible that stochastic lineage sorting nevertheless may complicate inference of population history. To account for possible gene tree–population tree discordance due to lineage sorting, we used the minimize-deep-coalescence (MDC; Maddison 1997; Maddison and Knowles 2006) method in the program Mesquite (Maddison WP and Maddison DR 2004) to identify the "best" population tree that requires the fewest deep coalescent events. We used the following options: subtree pruning and regrafting branch swapping, MAXTREES set to 100, and gene trees were rooted with *P. maniculatus*.

To statistically confirm that Gulf and Atlantic coast beach mouse subspecies are not monophyletic, we compared ML scores for a *posteriori* selected topologies (Goldman et al. 2000) in which different combinations of beach mouse subspecies were forced to be monophyletic and the best ML topology from the combined data using the SH test (Shimodaira and Hasegawa 1999) in Paup*. Significance values were determined using a RELL approximation with 10,000 bootstrap replicates.

Pigmentation Gene Tree Estimation

Unlike the nonpigmentation genes used to estimate population history, *Mc1r* may be affected by nonneutral processes because of its role in pigmentation. Therefore, we estimated the genealogy of *Mc1r* alleles separately. Using Sequencher, we aligned 25 complete *Mc1r* sequences from the same individuals used in the population-history analyses. We performed the same three phylogenetic analyses as described above: Bayesian, ML, and MP algorithms. For *Mc1r*, we used the HKY model for the Bayesian analysis and the TrN + Γ model for ML analysis. We also used the SH test to determine if the *Mc1r* genealogy differed from the population tree.

Functional Variation in the *Mc1r*

In addition to the 25 *Mc1r* sequences generated for the phylogenetic analyses, we amplified and sequenced 78 more individuals (112 individuals total; see table 1). For the extinct Pallid beach mouse (PBM), we extracted DNA from four museum skins (following Mullen and Hoekstra 2008). We were able to amplify the complete *Mc1r*-coding region in only two PBMs by generating seven overlapping PCR fragments. We sequenced each base from at least two independent PCR products to confirm any polymorphisms. Primers and PCR conditions are given in supplementary table S3 (Supplementary Material online). For all *Mc1r* sequences, we determined haplotypes using Phase (Stephens

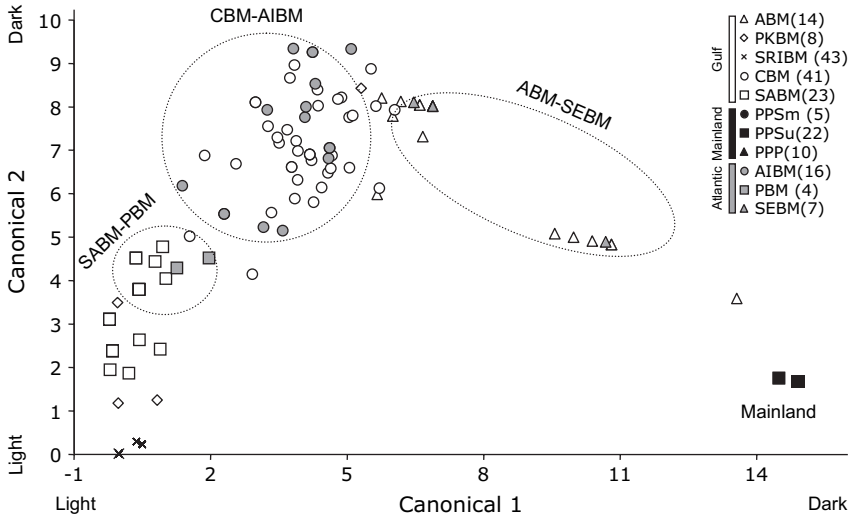

FIG. 2.—Discriminant analysis of eight combined color traits measured in 11 *Peromyscus polionotus* subspecies. The two main canonical axes are shown and together explain 90.4% of the phenotypic variance. Gulf Coast beach mouse subspecies (ABM, PKBM, SRIBM, CBM, SABM) are indicated by white symbols, mainland subspecies (PPSu, PPSm, PPP) by black, and Atlantic coast beach mice (AIBM, PBM, SEBM) by gray. Sample sizes for each subspecies are provided in parentheses (cases in which individuals have identical pigment patterns, their symbols will overlap). Dashed circles highlight phenotypic clustering between Gulf and Atlantic coast subspecies.

and Donnelly 2003) and estimated population-genetic summary statistics in DnaSP (Rozas J and Rozas R 1999).

To test the functional consequences of amino acid variation in *Mc1r*, we used cell-based cAMP accumulation assays that measure receptor signaling and serve as a proxy for pigment phenotype; for example, hyperactive receptors can result in melanic phenotypes, whereas hypoactive receptors are associated with light-colored phenotypes (e.g., Robbins et al. 1993). We first amplified the complete coding region of the most common allele observed in the mainland *Peromyscus polionotus subgriseus* (PPSu) population from genomic DNA. This "mainland allele" was inserted into the eukaryotic expression vector pcDps (Bonner et al. 1988). We then introduced seven amino acid mutations (the six new mutations and the $Arg^{65}Cys$ mutation as a control) individually using a PCR-based site-directed mutagenesis and restriction fragment replacement strategy. For all constructs, we verified sequences to ensure correct orientation of inserts and to exclude PCR-induced mutations.

We grew COS-7 cells in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified 7% CO_2 incubator. We used lipofectamine 2000 (Invitrogen, Carlsbad, CA) to transfect cells following manufacturer's protocols.

To assay signaling, we measured the cAMP content of cell extracts by a nonradioactive cAMP assay based on the ALPHAScreen technology (PerkinElmer; Stäubert et al. 2007). We split cells into 50 ml cell culture flasks (1×10^6 cells/flask) and transfected each with 5 μg of plasmid. Following transfection, we seeded cells in 48-well plates (5×10^4 cells/well) and 24 h later, performed cAMP accumulation assays. We washed cells once and then incubated them in serum-free DMEM containing 1 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma, St Louis, MO) in the absence or in increasing amounts of the agonist α-MSH (Sigma) for 1 h at 37 °C. We terminated the reactions by aspirating media and then lysed cells in 50 μl lysis buffer containing 1 mM IBMX. From each well, we transferred 5 μl of lysate to a 384-well plate. We then added acceptor beads (in stimulation buffer w/o IBMX) and donor beads according to the manufacturer's protocol. We analyzed cAMP accumulation data using GraphPad Prism software.

Results

Beach Mouse Pigmentation Patterns

To compare patterns of pigment variation between beach mice from the Gulf and the Atlantic coasts, we performed discriminant analyses using eight combined color traits (fig. 2). The two main canonical axes explain 90.4% of the phenotypic variance. The canonical scores show that the mainland populations are phenotypically distinct from all beach mouse subspecies: mice from the three mainland populations ($N = 37$; Georgia, Panhandle, and Lake Louisa) all cluster together. Using Tukey–Kramer HSD tests, comparisons between the mainland subspecies and each beach mouse subspecies revealed that the mainland mice are statistically unique, and two traits, ventral and tail pigmentation, showed the most divergence between mainland and beach forms (all comparisons Tukey–Kramer HSD, alpha = 0.05, $q^* = 3.26$).

In the Gulf Coast, the SRIBM and St Andrews beach mouse (SABM) are distinct from all other Gulf Coast beach mice, consistent with their overall lighter coat color, particularly for cheek and rostrum traits (HSD, $q^* = 3.26$). Some individuals of a third Gulf Coast subspecies, Perdido Key beach mouse (PKBM), show significant phenotypic overlap with individuals of SABM (for eyebrows, earbase, rump, ankle, and tail traits; HSD, $q^* = 3.26$), but PKBM mice are quite variable.

In most cases, however, Gulf Coast beach mice are more similar in pigmentation to Atlantic coast beach mice

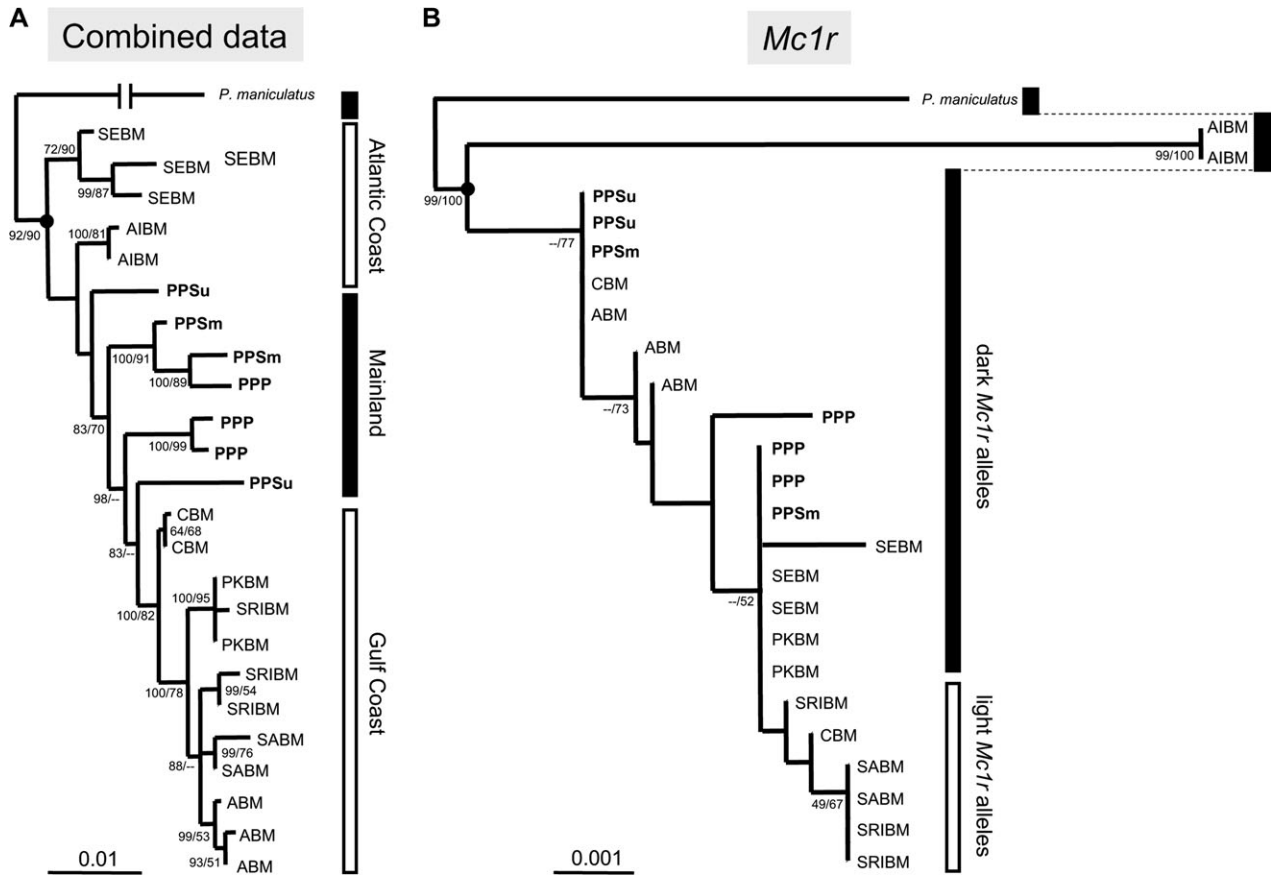

FIG. 3.—Phylogenetic reconstruction for the combined data set (mtDNA and SNPs) and separately the *Mc1r* gene. For both trees, *Peromyscus maniculatus* was used as the outgroup. Mainland mouse samples are in bold. Support values above 50% are given at each node (Bayesian posterior probabilities/ML bootstrap values). (*A*) ML topology of *Peromyscus polionotus* individuals based on mitochondrial loci, the control region and *COIII* sequences, and 14 SNPs in nuclear loci (a total of 1,752 bp). Individuals are labeled by subspecies. Gulf Coast, mainland, and Atlantic coast subspecies are highlighted using vertical bars: white bars represent light pigmentation (beach mouse subspecies) and black bar dark pigmentation (mainland subspecies). (*B*) ML genealogy of 24 *Mc1r* alleles (954 bp) labeled by subspecies. White bar highlights the light *Mc1r* allele defined by the $Arg^{65}Cys$ mutation.

than to other Gulf Coast subspecies. For example, the Atlantic coast Anastasia Island beach mouse (AIBM) and the Gulf Coast Choctawhatchee beach mouse (CBM) cluster together (fig. 2); in fact, 23% of the AIBM individuals are statistically indistinguishable from CBM individuals by discriminant analysis. AIBM and CBM subspecies both have light brown pigmentation on their dorsal surface and white pelage on their face, although CBM individuals sometimes have a lighter rostrum. In addition, the Atlantic coast Southeastern beach mouse (SEBM) cluster with the Gulf Coast Alabama beach mouse (ABM); 33% of SEBM individuals are indistinguishable from members of the ABM subspecies. SEBM and ABM both have light brown facial and dorsal coats and partially striped tails. Finally, the now extinct Atlantic coast PBM cluster with a third Gulf Coast subspecies, SABM. Two of the three PBM individuals are statistically indistinguishable from SABM mice. These results show that populations of beach mice from the Gulf and the Atlantic coasts share similar pigmentation patterns and are often phenotypically indistinguishable, particularly the CBM–AIBM, ABM–SEBM, and PBM–SABM pairs.

Phylogenetic Analyses of Convergent Light Pigmentation

Using data from mtDNA sequences and SNPs in nuclear loci, we reconstructed the evolutionary history of *P. polionotus* subspecies. Different phylogenetic algorithms (Bayesian, ML, MP, and NJ) produced similar topologies, and consistent (but less well resolved) topologies were recovered from smaller data sets (e.g., mtDNA alone). In all but one case, beach mouse subspecies were recovered as monophyletic groups. However, based on this multilocus topology, the beach mice from the Gulf and the Atlantic coasts are not monophyletic (fig. 3*A*). Likewise, the population tree recovered from the MDC analysis was consistent with the topology shown in figure 3*A* and shows that beach mice do not form a monophyletic group (data not shown).

On the Atlantic coast, the SEBM and AIBM populations are basal and form a paraphyletic group with respect to mainland mice, consistent with current taxonomy as distinct subspecies. Paraphyly of Atlantic coast beach mice also suggests that these two Atlantic coast subspecies may have originated from different ancestral populations or alternatively from the same ancestral population but at different

Table 2
Comparison of the Best Phylogenetic Trees (from ML analysis) versus Alternative Phylogenetic Hypotheses about Monophyly of Beach Mouse Subspecies

Comparisons	−ln L Best	−ln L Hypothesis	Difference −ln L	*P*
Best versus monophyly	3680.29	3697.83	17.53	0.029
Best versus AIBM + Gulf Coast	3680.29	3696.57	16.28	0.035
Best versus SEBM + Gulf Coast	3680.29	3696.26	15.97	0.032

NOTE.—Likelihood scores of the best and alternative topologies, the difference in likelihood scores, and *P* values are given.

times. However, given the recent origin of the subspecies and the possibility of gene flow among them, additional loci and individuals are needed to confirm these phylogenetic patterns.

In contrast, the five Gulf Coast subspecies cluster together in a monophyletic group with strong support and distinct from the Atlantic coast beach mice, suggesting a single evolutionary origin of Gulf Coast beach mice. To statistically demonstrate that Gulf and Atlantic coast beach mice have independent origins, we tested alternative phylogenetic hypotheses about their evolutionary history, which include the monophyly of 1) all beach mouse subspecies, 2) only AIBM and Gulf Coast subspecies, and 3) only SEBM and Gulf Coast subspecies. All these alternative topologies are significantly less likely than the best combined data ML topology (SH test, $P < 0.05$; table 2).

A comparison of this population history (derived from presumably neutral loci) to a genealogy of *Mc1r* alleles reveals several patterns (fig. 3*B*). First, the *Mc1r* genealogy, although less robust than that from combined data, shows that the Atlantic coast *Mc1r* alleles do not form a monophyletic group—the AIBM alleles are basal and distinct from all other *Mc1r* alleles, whereas SEBM alleles cluster with mainland and some Gulf Coast alleles. By contrast, the light *Mc1r* allele ($Arg^{65}Cys$) is monophyletic, derived, and found in only Gulf Coast beach mice, although not all Gulf Coast mice have the light *Mc1r* allele. In fact, the *Mc1r* genealogy is statistically different from the population tree topology (SH test, $P = 0.045$).

Genetic Variation in *Mc1r*

To determine whether convergent light pigmentation among beach mouse populations has a similar genetic or molecular basis, we examined patterns of *Mc1r* nucleotide variation in mainland, Gulf Coast, and Atlantic coast subspecies (table 3). These comparisons show that the mainland population has the highest genetic variability (PPSu; $\pi = 0.70$, $Hd = 0.92$). Compared with the mainland subspecies, all beach mouse subspecies show reduced genetic variation, consistent with the hypothesis that beach mouse populations were founded by only a few individuals, have maintained small population sizes, and/or experienced selection at *Mc1r*. The lowest variability is seen in the Gulf Coast subspecies; in particular, SRIBM has the lowest nucleotide diversity ($\pi = 0.01$; $Hd = 0.05$). On the Atlantic coast, levels of *Mc1r* genetic diversity are similar between the two beach mouse subspecies for which we had large population samples (AIBM and SEBM), and both have roughly half the diversity observed in the mainland population. Overall, these Atlantic coast beach mice have an order of magnitude more genetic diversity than Gulf Coast subspecies (with the exception of CBM).

Variable Amino Acid Sites in Beach Mouse Subspecies

Consistent with previous results, we did not find the light *Mc1r* allele (defined by the $Arg^{65}Cys$ mutation) in Atlantic coast populations. However, this result does not preclude the possibility that different coding mutations in *Mc1r* contribute to convergent pigment pattern in the Atlantic coast mice. To identify new mutations in *Mc1r* that could potentially contribute to light pigmentation in the Atlantic coast subspecies, we first looked for derived amino acid sites that were present in the light-colored beach mice but absent in the mainland forms. We identified six new mutations (amino acid positions 38, 120, 164, 203, 230, and 294; table 4) in addition to the previously described polymorphism at position 65. Four of these amino acid changes occur within conserved transmembrane domains of the protein, whereas two of the mutations (at positions 38 and 230) occur in extracellular and intracellular domains, respectively. All the new substitutions are conservative with respect to hydrophobicity and charge, except the mutation at position 230, which changes a positively charged Arg to a noncharged Gly amino acid. Position 230 is evolutionarily conserved among most vertebrates—mammals most often have a basic amino acid (i.e., Arg or His) at this position. However, nonbasic amino acids are found in some species causing no obvious effect on coat color (Gln: Lemuridae, Lorisidae; Gly: Muridae). None

Table 3
***Mc1r* Nucleotide Variation among *Peromyscus polionotus* Subspecies**

	Gulf Coast					Mainland	Atlantic Coast		
	ABM	PKBM	SRIBM	CBM	SABM	PPSu	SEBM	PBM	AIBM
No. of alleles	20	20	40	20	20	40	20	4	20
S	2	2	1	6	1	21	13	0	15
H	2	3	2	6	2	14	8	1	6
Hd	0.51	0.64	0.05	0.76	0.27	0.92	0.65	0	0.63
π (%)	0.11	0.11	0.01	0.21	0.03	0.70	0.34	0	0.41
θ (%)	0.06	0.06	0.03	0.18	0.03	0.52	0.38	0	0.44

NOTE.—Number of alleles, segregating sites (*S*), number of haplotypes (*H*), haplotype diversity (*Hd*), and nucleotide diversity (π and θ) are shown.

Table 4
Variable *Mc1r* Sites in *Peromyscus polionotus* Subspecies

Amino Acid Site	*N* Alleles	38	65	120	164	203	230	294
Nucleotide position		118	**199**	364	496	613	694	886
Location in protein		ECL1	**ICL1**	TM3	TM4	TM5	ICL3	TM7
Amino acid charge		0	**+/0**	0	0	0	+/0	0
ABM	20	Val/Tyr (0.40)	**Arg**	Val	Val	Ile	Arg	Leu
PKBM	20	Val	**Arg/Cys** (0.35)	Val	Val	Ile	Arg	Leu
SRIBM	40	Val	**Arg/Cys** (0.98)	Val	Val	Ile	Arg	Leu
CBM	20	Val/Tyr (0.70)	**Arg/Cys** (0.05)	Val/Met (0.45)	Val	Ile	Arg	Leu/Ile (0.20)
SABM	20	Val	**Arg/Cys** (0.85)	Val	Val	Ile	Arg	Leu
PPSu	40	Val/Tyr (0.75)	**Arg**	Val	Val/Met (0.18)	Ile/Val (0.23)	Arg/Gly (0.50)	Leu
SEBM	20	Val/Tyr (0.15)	**Arg**	Val	Val/Met (0.15)	Ile/Val (0.15)	Arg/Gly (0.10)	Leu
PBM	4	Val	**Arg**	Val	Met	Ile	Arg/Gly (0.75)	Leu
AIBM	20	Val/Tyr (0.80)	**Arg**	Val	Val/Met (0.80)	Ile/Val (0.80)	Arg/Gly (0.10)	Leu

NOTE.—Location in the *Mc1r* nucleotide sequence, in the protein (ECL = extracellular loop, ICL = intracellular loop, TM = transmembrane region; the number [ECL1, TM3, etc.] refers to the domain number), and any change in amino acid charge is provided (ancestral/derived). The $Arg^{65}Cys$ mutation identified in Gulf Coast beach mice (Hoekstra et al. 2006) is indicated in bold. Frequency of the derived amino acid is shown parenthetically next to each variable site.

of these mutations are fixed differences between the mainland population and any beach mouse subspecies.

Among the five Gulf Coast subspecies, there are only two high frequency–derived polymorphisms (table 4): position $Val^{38}Tyr$ (70% in CBM and 40% in ABM) and $Arg^{65}Cys$ (35% in PKBM, 98% in SRIBM, 5% in CBM, and 85% in SABM). In addition, one Gulf Coast subspecies, CBM, has two unique and low intermediate derived polymorphisms, $Val^{120}Met$ (45%) and $Leu^{294}Ile$ (20%).

By contrast, the Atlantic coast subspecies have four new amino acid mutations (table 4), all of which are shared and polymorphic in both mainland and the Atlantic coast subspecies; amino acids 38, 164, 203, and 230 (only position 38 is also polymorphic in the Gulf Coast). Thus, when compared with the mainland subspecies, there are no new mutations that are either fixed in the Atlantic coast populations or perfectly correlated with light pigmentation. Only two amino acids mutations, $Val^{38}Tyr$ and $Val^{164}Met$, are at high frequency (80%) in AIBM, but both are conservative changes. However, because the genetic background in which alleles are expressed (e.g., the genotype at other pigmentation loci) can influence *Mc1r*'s effect on pigmentation (Steiner et al. 2007), these shared amino acid mutations may still contribute to light coloration on the Atlantic coast. In vitro assays are required to rule out the role of these amino acid mutations on receptor function.

Functional Tests of *Mc1r* Alleles

To determine whether the *Mc1r* amino acid polymorphism observed in Atlantic coast beach mice contributes to differences in receptor function—and thus possibly pigmentation phenotype—we heterologously expressed eight *Mc1r* alleles in COS-7 cells (table 5). Together, these alleles test the individual effects of the six new mutations on receptor function compared with the mainland allele. As a positive control, we tested the previously characterized Gulf Coast allele ($Arg^{65}Cys$; Hoekstra et al. 2006). Consistent with previous results, we found a significant decrease in basal and agonist-induced cAMP formation for the $Arg^{65}Cys$ allele. In contrast, we found that all other alleles showed high levels of cAMP accumulation and were statistically indistinguishable from the mainland allele (fig. 4) in potency (EC_{50}), efficacy (E_{max}), and basal activity (basal cAMP; supplementary table S4, Supplementary Material online). These data suggest that the *Mc1r* polymorphisms found in the Atlantic coast beach mice do not contribute to their light pigmentation, although it is possible that more than one derived mutation is necessary to alter receptor function.

Table 5
***Mc1r* Alleles Tested in In Vitro cAMP Assays**

Amino Acid Site	38	65	120	164[a]	203	230	294	Population
Nucleotide position	118	199	364	496	613	694	886	
Mainland allele	Tyr	Arg	Val	Val	Ile	Arg	Leu	ABM, CBM, PPSu, PBM
	Val	Arg	Val	Val	Ile	Arg	Leu	ABM, PKBM, SRIBM, CBM, SABM, PPSu, SEBM, AIBM
	Tyr	***Cys***	Val	Val	Ile	Arg	Leu	PKBM, SRIBM, CBM, SABM
	Tyr	Arg	***Met***	Val	Ile	Arg	Leu	CBM
	Tyr	Arg	Val	***Met***	Val	Arg	Leu	PPSu, SEBM, AIBM
	Tyr	Arg	Val	Val	***Val***	Arg	Leu	PPSu, SEBM, AIBM
	Tyr	Arg	Val	Val	Ile	***Gly***	Leu	PPSu, SEBM, AIBM
	Tyr	Arg	Val	Val	Ile	Arg	***Ile***	CBM

NOTE.—Each of seven polymorphic amino acid sites (in bold italics) is tested individually. Mainland allele represents the most common allele observed in the PPSu population. The population in which each allele is found is given.

[a] The ^{164}Met mutation has only been detected on the ^{203}Val background in our samples.

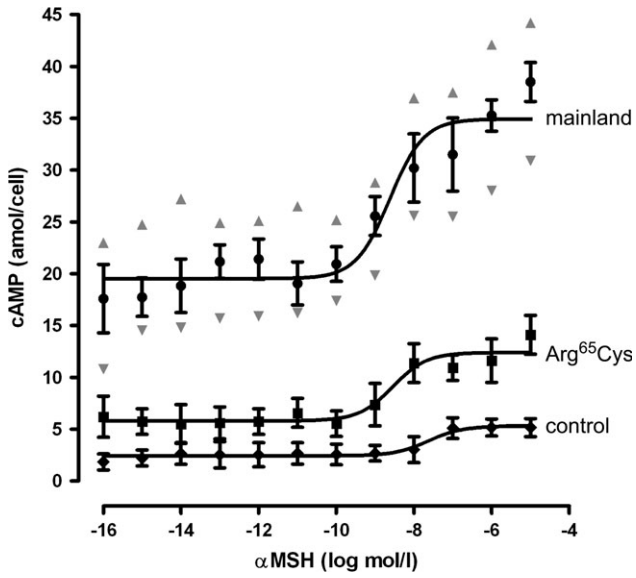

FIG. 4.—Functional analysis of *Mc1r* alleles surveyed in *Peromyscus polionotus*. Intracellular cAMP accumulation was measured in response to increasing concentrations of the agonist α-MSH. COS-7 cells were transiently transfected with empty expression plasmid (control) and the previously characterized *Mc1r* allele ($Arg^{65}Cys$) for comparison. The sigmoidal curve (mainland) represents the most common allele in the PPSu population shown in table 5. Gray triangles indicate the minimum (▲) and maximum (▼) cAMP levels observed in the mainland allele and the six new alleles each with a single *Mc1r* mutation. All constructs were tested in three independent experiments, each carried out in duplicate. The mean (±standard error of the mean) is shown.

Discussion

Beach mice represent an exciting species in which to examine the genetic basis of convergence because of the diversity in pigment patterns driven by selection for crypsis. Here, we demonstrate that although *Mc1r* contributes to adaptive light-colored phenotypes on the Gulf Coast of Florida, this same gene does not contribute to light pigmentation on the Atlantic coast. Using a phylogenetic approach, we show that light-colored beach mice do not form a monophyletic group, raising the possibility that light pigmentation has evolved at least twice independently. Moreover, we show that actual molecular changes contributing to light coloration in Gulf Coast beach mice are absent in the Atlantic coast, and there are no new *Mc1r*-coding mutations on the Atlantic coast that alter receptor activity in a cell-based functional assay. Together, these data suggest that there can be different molecular solutions to arrive at the same phenotype in similar environments.

Beach mice present us with several levels of phenotypic convergence. The first level is the generally light pigmentation observed in both Atlantic and Gulf coast populations compared with dark mainland mice, undoubtedly due to similar selection pressures acting in similar environments. The second level of convergence is based on more fine-scale differences in pigment pattern: beach mice can be grouped in pairs based on the similarity of their precise pigmentation patterns, with each Atlantic coast population most closely resembling a Gulf Coast population.

Our multilocus analysis of subspecies shows that these similarities in phenotype are not mirrored in overall genetic similarity. First, all light-colored beach mice do not form a monophyletic group. Second, Atlantic coast beach mice cluster independently from the Gulf Coast subspecies that form a separate monophyletic group. This topology, however, raises several possibilities about the evolution of light coloration: light pigmentation evolved 1) once ancestrally in *polionotus* but was subsequently lost in each mainland population, 2) once in one beach mouse population followed by gene flow to other beach mouse subspecies, or 3) at least twice in *polionotus*: once in the Atlantic coast beach mice followed by a loss in ancestor of mainland and Gulf coast mice and again in the ancestor of Gulf Coast subspecies. The most robust test of these hypotheses is to identify the genes responsible for light pigmentation phenotype and ask if they are similar in the Atlantic and Gulf coasts (scenarios 1 and 2) or different (scenario 3).

In the case of general convergence, that is, the overall lighter color of beach mice versus their mainland counterparts, the difference in pigmentation of the two coastal beach mice from their presumed ancestor is clearly based on at least some different mutations. The *Mc1r* light allele is not found in any Atlantic subspecies but occurs at high frequencies in three Gulf Coast subspecies (SRIBM, PKBM, and SABM).

The second form of convergence involves similarities in pigment pattern among pairs of Gulf Coast and Atlantic coast subspecies. The two darkest populations of Atlantic coast beach mice (SEBM and AIBM) each are phenotypically similar to the two darkest populations of Gulf Coast beach mice (ABM and CBM, respectively) that also lack or have a low frequency (CBM, 5%) of the light *Mc1r* allele. From this result alone, we cannot conclude whether these populations used similar or different genes in evolving light pigmentation, only that none use the light *Mc1r* allele.

We can say something, however, about the genetics of convergence between the now-extinct PBM subspecies and populations on the Gulf Coast that have similar pigmentation. PBMs are very light, similar in color to the PKBM and SABM Gulf Coast subspecies (and only slightly darker than the SRIBM); this similarity in color pattern is even noted in the first description of the PBM subspecies (Howell 1939). The $Arg^{65}Cys$ was not present in the PBM subspecies, even though this allele is at high frequency (>95%) in the two lightest Gulf Coast subspecies. Moreover, we show here that there are no amino acid differences between PBMs and the mainland *Mc1r* allele—that is, there are no new mutations in PBM *Mc1r* alleles that contribute to their light coloration. Thus, the convergence in pigment pattern between the PBMs and the phenotypically similar Gulf Coast subspecies clearly rests on different molecular changes.

Although the light *Mc1r* allele is not found in any of the Atlantic coast subspecies, this does not preclude the possibility that other mutations in this gene could contribute to light coloration on the Atlantic coast. Complete sequences of the *Mc1r*-coding region reveal two patterns relevant to this possibility. First, compared with Gulf Coast populations, Atlantic coast populations show higher levels of nucleotide (and amino acid) variability in *Mc1r*. Although demographic processes may explain this pattern (e.g., larger, more stable, older populations on the Atlantic coast or more immigration from the mainland), it is not consistent

with a recent selective sweep of a *Mc1r* allele, which may be expected if this gene contributed to adaptive pigmentation on the Atlantic coast. Second, and more convincingly, we found no new *Mc1r* mutations in these populations that are correlated with pigmentation. The entire sample of beach mice yielded six new amino acid polymorphisms, none of which are known to affect pigmentation in other species (Hoekstra 2006). Two other mutations were polymorphic only in the CBM subspecies (amino acid sites 120 and 294) and are thus not relevant to phenotypic differences between subspecies. Only one mutation, at amino acid site 230, produced a change in amino acid charge ($Arg^{230}Gly$), and this site was polymorphic in the two Atlantic coast populations as well as the mainland (PPSu) population. Thus, these sequence results did not identify any new candidate mutations in *Mc1r* that were specific to the light-colored Gulf or Atlantic coast subspecies. However, the phenotypic effect of *Mc1r* alleles depends critically on its genetic background (e.g., genotype at the *Agouti* locus; Steiner et al. 2007), which probably differs between mainland and beach forms. This raises the possibility that the same *Mc1r* mutations found in mainland and beach populations (e.g., $Arg^{230}Gly$) could affect pigmentation very differently in each.

The most direct evidence then for a lack of *Mc1r* involvement in these convergent phenotypes stems from functional assays of the amino acid mutations. Cell-based assays show that none of these six amino acid mutations (even a charge-changing mutation at a conserved amino acid position; $Arg^{230}Gly$) has a measurable effect on receptor activity. These results allow us to unambiguously rule out any additional mutations in the *Mc1r*-coding sequence as individual contributors to light pigmentation in beach mice.

Thus, although many recent studies have reported that the same genes are involved in the repeated evolution of traits across taxa (even *Mc1r* in several melanic vertebrates; Majerus and Mundy 2003), this work shows that the convergence of pigmentation among populations of a single species rests, at least in part, on different genetic changes. Whereas different genes undoubtedly also contribute to phenotypic convergence among distantly related taxa, these disparate genetic bases might be due to differences in developmental constraint or differences in the available genetic variation. By studying convergence within species, in which developmental constraints are presumably similar, we can largely eliminate one of these possibilities. In beach mice, then, light color pattern probably evolved through different genes because Gulf and Atlantic coast beach mice differed in their genetic starting materials (i.e., either different standing genetic variation in the founding populations or the independent appearance of different novel mutations).

This question remains: which genes (if not *Mc1r*-coding mutations) are responsible for light coloration in the Atlantic coast beach mice (and also some Gulf Coast subspecies)? Although levels of *Mc1r* messenger RNA do not differ between mainland and Gulf Coast (SRIBM) mice (Steiner et al. 2007), it is possible that *Mc1r* expression levels differ between Atlantic coast and mainland mice. However, a more promising place to look may be the *Agouti signaling protein* (*Agouti*), a locus known to have a large effect on pigmentation in genetic crosses between Gulf Coast (SRIBM) and mainland mice, and whose expression level is positively correlated with coat color reflectance in *Peromyscus* (Steiner et al. 2007). It is possible that although Atlantic coast beach mice do not share *Mc1r* alleles with Gulf Coast mice, they may share alleles at other pigmentation loci like *Agouti*. Association studies using candidate genes (e.g., *Agouti*) and genome-wide quantitative trait locus analysis in Atlantic coast beach mice will help us answer this remaining question.

Supplementary Material

Supplementary tables S1–S4 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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