Adaptive Variation in Beach Mice Produced by Two Interacting Pigmentation Genes

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Adaptive Variation in Beach Mice Produced by Two Interacting Pigmentation Genes

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Little is known about the genetic basis of ecologically important morphological variation such as the diverse color patterns of mammals. Here we identify genetic changes contributing to an adaptive difference in color pattern between two subspecies of oldfield mice (Peromyscus polionotus). One mainland subspecies has a cryptic dark brown dorsal coat, while a younger beach-dwelling subspecies has a lighter coat produced by natural selection for camouflage on pale coastal sand dunes. Using genome-wide linkage mapping, we identified three chromosomal regions (two of major and one of minor effect) associated with differences in pigmentation traits. Two candidate genes, the melanocortin-1 receptor (Mc1r) and its antagonist, the Agouti signaling protein (Agouti), map to independent regions that together are responsible for most of the difference in pigmentation between subspecies. A derived mutation in the coding region of Mc1r, rather than change in its expression level, contributes to light pigmentation. Conversely, beach mice have a derived increase in Agouti mRNA expression but no changes in protein sequence. These two genes also interact epistatically: the phenotypic effects of Mc1r are visible only in genetic backgrounds containing the derived Agouti allele. These results demonstrate that cryptic coloration can be based largely on a few interacting genes of major effect.

Introduction

Animal pigmentation has attracted substantial evolutionary interest because changes in color, be they driven by natural or sexual selection, can have profound effects on fitness. Dissecting the genetic basis of morphological variation, such as adaptive pigmentation, allows us to answer several long-standing evolutionary questions: How many genes contribute to adaptive phenotypes? What are the relative sizes of their effects? Are adaptive alleles generally dominant, semidominant, or recessive? What types of genes are involved in adaptive change? Do adaptive mutations generally occur in coding or regulatory regions? What is the role of epistasis in evolutionary change?

To understand the genetic processes involved in generating adaptive color patterns, we revisited a series of classic natural history studies [1–3] that described geographic variation in coat-color pattern of the oldfield mouse (P. polionotus). The extreme coat-color variation within this species is driven by selection for camouflage [4], yielding a strong geographical correlation between coat color and reflectance of the substrate [5,6].

We focused on the two subspecies of P. polionotus showing the greatest difference in color pattern: P. p. subgriseus and P. p. leucoecephalus. The mainland subspecies (P. p. subgriseus) occupies oldfield habitats in the southeastern United States and has a coat that is dark brown on top and light gray on the belly, as well as a striped tail. In contrast, the light-colored Santa Rosa Island beach mouse (P. p. leucoecephalus), like other “beach mice” that have colonized Florida’s barrier islands and sandy coastal dunes, lacks visible pigmentation on its face, flank, and tail (Figure 1).

Results/Discussion

To analyze the genetic basis of color-pattern difference, we made reciprocal genetic crosses between three mainland and three beach mice, yielding 28 F1 hybrids that were then intercrossed to produce 465 F2 progeny. A genome-wide linkage map was generated using both anonymous microsatellite markers and single nucleotide polymorphisms (SNPs) in candidate pigmentation genes (Figure 2). This represents the first genome-wide linkage map for Peromyscus, with the exception of an allozyme-based map with few markers [7]. We scored all F2 progeny for 113 microsatellite markers and single nucleotide polymorphisms (SNPs) in candidate pigmentation genes (Figure 2). This represents the first genome-wide linkage map for Peromyscus, with the exception of an allozyme-based map with few markers [7]. We scored all F2 progeny for 113 microsatellite markers and single nucleotide polymorphisms (SNPs) in candidate pigmentation genes (Figure 2). This represents the first genome-wide linkage map for Peromyscus, with the exception of an allozyme-based map with few markers [7]. We scored all F2 progeny for 113 microsatellite markers and single nucleotide polymorphisms (SNPs) in candidate pigmentation genes (Figure 2). This represents the first genome-wide linkage map for Peromyscus, with the exception of an allozyme-based map with few markers [7]. We scored all F2 progeny for 113 microsatellite markers and single nucleotide polymorphisms (SNPs) in candidate pigmentation genes (Figure 2). This represents the first genome-wide linkage map for Peromyscus, with the exception of an allozyme-based map with few markers [7]. We scored all F2 progeny for 113 microsatellite markers and single nucleotide polymorphisms (SNPs) in candidate pigmentation genes (Figure 2). This represents the first genome-wide linkage map for Peromyscus, with the exception of an allozyme-based map with few markers [7]. We scored all F2 progeny for 113 microsatellite markers and single nucleotide polymorphisms (SNPs) in candidate pigmentation genes (Figure 2). This represents the first genome-wide linkage map for Peromyscus, with the exception of an allozyme-based map with few markers [7]. We scored all F2 progeny for 113 microsatellite markers and single nucleotide polymorphisms (SNPs) in candidate pigmentation genes (Figure 2). This represents the first genome-wide linkage map for Peromyscus, with the exception of an allozyme-based map with few markers [7]. We scored all F2 progeny for 113 microsatellite markers and single nucleotide polymorphisms (SNPs) in candidate pigmentation genes (Figure 2). This represents the first genome-wide linkage map for Peromyscus, with the exception of an allozyme-based map with few markers [7]. We scored all F2 progeny for 113 microsatellite markers and single nucleotide polymorphisms (SNPs) in candidate pigmentation genes (Figure 2). This represents the first genome-wide linkage map for Peromyscus, with the exception of an allozyme-based map with few markers [7]. We scored all F2 progeny for 113 microsatellite markers and single nucleotide polymorphisms (SNPs) in candidate pigmentation genes (Figure 2).
The tremendous amount of variation in color patterns among organisms helps individuals survive and reproduce in the wild, yet we know surprisingly little about the genes that produce these adaptive patterns. Here we used a genomic analysis to uncover the molecular basis of a pale color pattern that camouflages beach mice inhabiting the sandy dunes of Florida’s coast from predators. We identified two pigmentation genes, the melanocortin-1 receptor (Mc1r) and its ligand, the agouti signaling protein (Agouti), which together produce a light color pattern. We show that this light pigmentation results partly from a single amino acid mutation in Mc1r, which reduces the activity of the receptor but does not affect the gene’s expression level, and partly from the derived Agouti allele, which shows no change in protein sequence but does exhibit an increase in mRNA expression. We also show that these two genes do not act additively to produce pale color; rather, the derived Agouti allele must be present to see any effect of Mc1r on pigmentation. Thus, the light color pattern of beach mice largely results from the physical interaction between a structural change in a receptor (reducing Mc1r activity) and a regulatory change in the receptor’s antagonist (increasing Agouti expression).

To identify which genomic regions were statistically associated with the pigmentation differences, we determined the phenotypes of F2 progeny in seven regions of the body. These regions show the most divergence in pigmentation between the subspecies and together accurately encapsulate the difference in color and pattern. We measured total pelage reflectance (brightness) and scored pigment pattern on individual hairs for four facial traits (rostrum, cheek, eyebrow, and earbase) and also calculated the extent of dorsal, rump, and tail pigmentation as three additional traits (Figure 3A). The phenotypic data show no evidence for sexual dimorphism or maternal effects. The phenotypic correlation (r) between traits ranged from 0.29 to 0.82 (the highest value between earbase and cheek), suggesting that while some genes cause similar pigmentation differences among different body parts, other genes have more localized effects (Figure S1A).

The distribution of phenotypic scores among F2 individuals was not consistent with simple Mendelian inheritance for any of the traits with the exception of tail stripe, which shows a bimodal distribution of scores (Figure S1B). We analyzed these phenotypic values, along with the molecular marker data, using MapQTL 5 [11]. Only three LGs harbored quantitative trait loci (QTL) that influence pigmentation differences between the subspecies (Figure 3B–3D). Because pigmentation has served as a model pathway for studies of gene action and interaction in a variety of biological processes, there are over 100 well-characterized genes known to affect pigmentation in laboratory mice.
Figure 2. A Genome-Wide Linkage Map of *P. polionotus* Comprising 27 LGs Ordered by Size

Microsatellite loci are indicated in black and candidate pigmentation genes in red. The cumulative genetic distance between markers is given in centimorgans (cM). All candidate genes were on separate LGs with the exception of *Pldn*, *Atm*, *Slc24a5*, and *Agouti*, which clustered on LG 7. A total of three microsatellite loci and one pigmentation gene *Tyr* failed to show linkage to any other markers.

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Each of the three QTL regions contains only a single pigmentation gene from the homologous regions (bounded by homologous microsatellite markers) of the closely related model organisms Mus musculus and Rattus norvegicus: these genes are the Agouti signaling protein (Agouti; LG 7), the melanocortin-1 receptor (Mc1r; LG 1), and the c-kit receptor (Kit; LG 14). When mapped in Peromyscus, markers in these three candidate genes showed the highest LOD values for all seven pigmentation traits compared to other markers in the same LG (Table 1). Application of Multiple QTL model (MQM) mapping methods shows that none of the other eight candidate genes or 113 microsatellites is significantly associated with pigmentation variation. Our results suggest that nearly all of this difference is likely due to the three pigmentation genes Agouti, Mc1r, and Kit, although it is formally possible that other closely linked loci affect the color difference between subspecies. Below, we refer to these three QTLs using the names of the candidate genes.

Each of the two regions of largest effect, Agouti and Mc1r, influence all seven pigmentation traits (LOD > 5.8). Agouti explains the greatest amount of pigment variation for three traits (cheek, eyebrow, and tail), while Mc1r explains the greatest amount of variation for two traits (rostrum and earbase). Both regions contribute equally to the extent of dorsal and rump pigmentation. The relative phenotypic effect of these two regions varies among traits (Table 1). For example, Agouti explains 78% of the variation in tail striping, but only 9% of the variation in dorsal pigmentation, while Mc1r explains 27% of the variation in rostrum pigmentation but only 1% of the variation in tail striping. Depending on the trait, the combination of these two loci explains between 19% and 80% of the variation for each of the pigmentation traits. The candidate gene Kit mapped to the only QTL of small effect, which explained less than 3.2% of the phenotypic variation among traits. This region is associated with only four traits (rostrum, cheek, earbase, and tail; LOD > 3.0), thus showing more spatial specificity than the two regions of major effect. The remaining phenotypic variance is likely attributable to other loci of small effect that are undetectable in a cross of this size and to environmental and/or epigenetic variation. Thus, a small number of chromosomal regions—and perhaps only a few genes—are responsible for most of the difference in color pattern between subspecies.

One of the classical ways to determine the effects of genetic variation on pigmentation is to analyze the allelic composition of extreme classes in an F2 or backcross (e.g., 14). An analysis of the most extreme phenotypes among our F2 progeny shows a striking association between phenotype and the allelic variation (“light” allele derived from the beach parents [L] and “dark” allele derived from the mainland parents [D]) at Agouti and Mc1r. Of the 50 F2 progeny with the lightest dorsal pigmentation, 42 had at least one light Mc1r allele (LL or LD Mc1r genotypes; $\chi^2$ test, $p < 0.0001$). Similarly, of the 113 F2 progeny lacking a tail stripe, 112 had at least one light Agouti allele (LL or LD Agouti genotypes; $\chi^2$ test, $p < 0.0001$).

The direction and magnitude of QTL effects were gauged by comparing phenotypic means among the F2 offspring. For the two major QTLs, the derived Agouti and Mc1r alleles increase the average coat reflectance (i.e., produce lighter color) and reduce the extent of dorsal, rump, and tail

Figure 3. Genetic Architecture of Pigmentation in a Cross between Beach and Mainland Subspecies

(A) Each of seven pigmentation traits for which we found significant QTLs is highlighted in a different color on a cartoon of a mouse pelt. MQM analyses showed that two LGs harbored major effect QTLs and one LG carried a minor effect QTL. For each LG, LOD scores are shown as a function of genetic distance in centimorgans (cM). Black triangles on x-axes show the position of marker loci. Each line indicates the LOD score at 5-cM intervals along the LG and are coded by colors corresponding to each of the seven traits.

(B) One region of major affect maps to LG 7 and the Agouti locus maps to the peak in LOD score.

(C) A second major-effect region is located on LG 1, and the Mc1r locus maps to the peak in LOD score. Both major-effect loci are statistically associated with all seven pigmentation traits studied.

(D) A QTL of minor effect is located in LG 14, and the Kit locus maps near the peak. This minor effect locus is associated with four of the seven pigmentation traits.

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pigmentation (Table 1), changes consistent with the idea that these alleles were fixed by natural selection in beach mice. In addition, population-specific alleles of \( Mc1r \) and \( Agouti \) show differences in dominance for all traits. For example, 30 F2 cheek, eyebrow and earbase, scores represent relative brightness (values range from 0 to 1). For dorsum and tail, scores are a percent of area that is pigmented (values range from 0 to 100). For rump, categorical scores represent the extent of pigmentation (values range from 0 to 4). See Methods and Materials for details. *Homzygous genotypes that are significantly different in phenotypic mean from the heterozygote genotype (Student's t-test, \( p < 0.001 \)).

Table 1. Location and Magnitude of QTLs Affecting Pigmentation Pattern

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<th>LG</th>
<th>LOD</th>
<th>p.v.e (%)</th>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td>22.9</td>
<td>13.7</td>
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<td>7.3</td>
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<td>9.3</td>
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<tr>
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<td>14</td>
<td>7.4</td>
<td>3.2</td>
<td>0.43 ± 0.12*</td>
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<td>9.5</td>
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<tr>
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<td>9.4</td>
<td>27.59 ± 4.48</td>
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<td>23.2</td>
<td>2.03 ± 1.42*</td>
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<td>44.43 ± 32.19*</td>
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For each significant QTL detected (LOD > 4.5), the name of the locus, the corresponding LG, the maximum LOD score, and the percentage of phenotypic variance explained (p.v.e) are provided. Phenotypic means (± standard deviation) are given by genotypic class (L, light allele from the beach mouse parent and D, dark allele from the mainland parent). For rostrum, cheek, eyebrow and earbase, scores represent relative brightness (values range from 0 to 1). For dorsum and tail, scores are a percent of area that is pigmented (values range from 0 to 100). For rump, categorical scores represent the extent of pigmentation (values range from 0 to 4). See Methods and Materials for details. *Homzygous genotypes that are significantly different in phenotypic mean from the heterozygote genotype (Student's t-test, \( p < 0.001 \)).

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and ear base) that show large differences in pigmentation between beach and mainland mice. There was no difference in Mc1r expression level among taxa or among body regions (analysis of variance, \(p = 0.05\)) and no correlation between Mc1r expression level and reflectance among taxa across body regions when all taxa were included (Figure 4; \(r = 0.45, R^2 = 0.20, \) and \(p = 0.10\)). Thus, taken together with earlier functional analyses [18], these data suggest that a single amino acid mutation in the coding region of Mc1r— and not mutations in neighboring cis-regulatory regions—produces light pigmentation in beach mice. Finally, in the same four body regions, Agouti expression was always significantly higher in tissues from beach mice than in tissues from mainland mice (Student’s t-test, \(p < 0.05\), two-tailed test).

Comparing Agouti expression in \(P.\) polionotus to its sister species \(P.\) maniculatus, we find that the increased expression in beach mice is a derived trait because both \(P.\) p. subgriseus and \(P.\) maniculatus have similarly low levels of Agouti expression. In addition, Agouti expression is significantly correlated with pelage reflectance when all three taxa are compared (Figure 4C; \(R^2 = 0.65, \) and \(p < 0.001\)). Agouti also explains spatial variation in light coloration within a subspecies; there is a significant positive correlation between pelage reflectance and Agouti expression across body regions in beach mice \((R^2 = 0.91, \) and \(p < 0.05)\). Together, these results suggest that increased expression of Agouti, caused by either mutation(s) in its cis-regulatory region or in closely

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**Figure 4.** Gene Expression Assays for Mc1r and Agouti in Five Pigmentation Regions in Three Peromyscus Taxa

(A) Phylogenetic relationship and approximate divergence times are shown for the \(P.\) polionotus subspecies and the sister species \(P.\) maniculatus. Photos show typical pigmentation pattern for each taxon. The phylogeny and pictures together highlight the similarity in pigmentation patterns between the mainland \(P.\) polionotus and \(P.\) maniculatus, the close evolutionary relationship between \(P.\) polionotus subspecies, and the derived nature of the beach mouse phenotype.

(B) Qualitative (RT-PCR) and qPCR expression levels of Mc1r and Agouti mRNA relative to beta-Actin (control gene) for five distinct tissue samples in \(P.\) polionotus subspecies (\(P.\) p. leucocephalus [Ppl; white] and \(P.\) p. subgriseus [Pps; brown]) and \(P.\) maniculatus (Pm; black) are shown. A 100-bp ladder (L) flanks both sides of the RT-PCR gels. For the qPCR assays, since low Ct values indicate high expression level, we transformed the raw expression data to be more intuitive: relative expression values represent the averaged Ct values for each species subtracted from the sum of expression values across all species. Significant differences in relative expression levels between \(P.\) polionotus subspecies are indicated by asterisks (Student’s t-test, \(p < 0.05\)). Bars indicate the standard error for each assay.

(C) Association between level of Mc1r and Agouti relative expression and pigmentation (measured by reflectance) is shown among the three taxa. Correlation (r) values are shown. doi:10.1371/journal.pbio.0050219.g004
linked trans-factors, also contributes to the light phenotype of beach mice.

Because the Mc1r and Agouti proteins interact physically, we tested for epistasis by performing gene interaction analyses (MapManager QTxb [20]). We found evidence of epistasis in several pigmentation traits (e.g., eyebrow: LOD score = 11.28; \( \chi^2 \) test, \( p = 0.001 \) and rostrum: LOD score = 10.32; \( \chi^2 \) test, \( p = 0.001 \)). We also examined the effect of Mc1r genotypes on different Agouti backgrounds (and vice versa) using a categorical measurement of pigmentation. We detected epistasis for all seven of the traits but most strikingly for cheek pigmentation (Figure 5): F2 mice with the Agouti DD genotype always have fully pigmented hairs regardless of Mc1r genotype. In fact, for all seven traits there is no difference in phenotype between individuals who have either the DD or DL Mc1r genotypes on an Agouti DD genetic background (Student’s t-test, \( p > 0.05 \)), and for only a few traits (e.g., tail) is the Mc1r LL genotype visible on the Agouti DD background. In contrast, in F2 mice having the Agouti LL genotype, the Mc1r genotype explains all of the variation in cheek pigmentation (\( r = 1.0 \) and \( p < 0.0001 \)); and double mutants (Agouti LL and Mc1r LL) always lack visible pigment in their cheek hairs (Figure 5). Clearly, Mc1r genotype has a significant effect on pigmentation only on the light Agouti (LL and LD) background, a pattern mirrored in all seven traits. Thus, the effect of each of the two genes on phenotype clearly depends on the genotype at the other locus.

It is striking that the interaction between Agouti and Mc1r in these mice is just the reverse of what we would predict from the classical genetics of the laboratory mouse. In the pigmentation pathway, Mc1r is downstream of Agouti action, and in the laboratory variation in Mc1r has been shown to mask the action of Agouti alleles [21,22]. In contrast, we show here that Agouti can mask Mc1r, even though the dominance hierarchy of alleles remains identical to that seen in laboratory mice. We can explain this pattern of “reverse epistasis” mechanistically. The single mutation in Mc1r significantly decreases agonist (αMSH) binding, and hence cAMP signaling, but does not eliminate the receptor functionality of its protein product [18]. However, it is only with increased expression of Mc1r’s antagonist Agouti that the phenotypic effects of this weakened Mc1r-receptor are revealed, a result consistent with Agouti’s ability to decrease cAMP production independent of αMSH. Thus, it is clear that epistasis is a property of particular alleles rather than of loci themselves, and thus epistatic interactions observed in the laboratory may differ from those seen in natural populations [see 23].

In sum, our genome-wide linkage map for Peromyscus allowed us to identify three genetic regions, two of which have major phenotypic effects on the adaptive difference in color pattern between subspecies of P. polionotus. Moreover, these regions contain the well-studied Agouti and Mc1r pigmentation genes [13]. While mutations in Mc1r are correlated with pigmentation in a number of wild vertebrates [see 13], our results are, to our knowledge, the first example of variation at the well-studied Agouti locus being associated with adaptive variation of animal coloration in nature.

Our results also have several implications for understanding the genetic basis of adaptation. First, this subspecific difference in color pattern is produced by a few interacting genes of large effect, supporting the idea that adaptations can involve relatively few genes rather than, as is often believed, many genes of small effect [24]. When an animal suddenly invades a novel habitat, their ancestral phenotype is often very different from the new optimal phenotype (as was almost certainly true for beach mice). Indeed, population genetic theory predicts that mutations of large effect will often be involved in adaptation in these circumstances [25], a prediction consistent with several studies on the genetic basis
of mimicry and cryptis [26–28]. Second, both structural mutations (a single amino acid change reducing McIr signaling potential) and regulatory mutations (a derived increase in Agouti expression) contribute to adaptive change, and this change involves both recessive (McIr L) and dominant (Agouti L) alleles. These results support the idea that adaptation is not necessarily driven largely by cis-regulatory changes [29,30] or by (semi) dominant alleles [31,32]. Third, we show that the nature of epistasis between McIr and Agouti in wild populations does not mirror that seen in the laboratory, suggesting that one should be cautious not only about extrapolating the genetics of laboratory strains to evolution in nature, but also about inferring the directionality of biochemical pathways from patterns of gene interactions. Finally, most genetic studies of morphological change have concentrated on the loss of phenotypic traits through loss-of-function mutations (e.g., reduced armor in stickleback fish [33,34], absence of wing spots in Drosophila [35], and lack of pigment in cavefish [36]). This study provides a novel example of how adaptation can result from mutations involving a gain of function.

**Materials and Methods**

**Genetic crosses.** Parental stocks were maintained at the Peromyscus Genetic Stock Center (University of South Carolina, United States). Maintenance of stocks and the crossing design have been described previously [19].

**Phenotyping.** A total of seven pigmentation traits (rostrum, cheek, eyebrow, earbase, and the extent of pigmentation on the dorsum, rump, and tail) were scored in all 465 F2 individuals. A spectrophotometer (Ocean Optics, http://www.oceanoptics.com) was used to capture reflectance spectra from the four facial traits (rostrum, cheek, eyebrow, and earbase). A reflectance probe was held at a 45° angle to the surface, and the program OOBase32 (Ocean Optics) was used to capture reflectance measurements from 300–700 nm. Brightness was calculated by summing the area under the reflectance curve and converting to a normalized reflectance [37]. The extent of dorsal and tail pigmentation was measured as the proportion of the area that was pigmented. Rump pattern was scored using five categories from minimally (0) to fully (4) pigmented (following [1–3]). In addition, categorical pigmentation values (0–2) were scored for all seven pigmentation traits in the F2 progeny (following [18]). All statistical correlation analyses for the color traits were performed using JMP version 5.1.2 statistical software package (SAS Institute, http://www.sas.com).

**Genotyping assays.** All F2 individuals were genotyped for a total of 113 anonymous microsatellite markers and 11 SNPs in pigmentation genes.

Microsatellites were cloned from enriched partial genomic libraries developed for *P. maniculatus bairdii* and *P. polionotus subgigresens* [38]. Cloned sequences were edited in Sequencher 3.1.1 (GeneCodes, http://www.genecodes.com), and microsatellite motifs were identified by eye. PCR primers designed to amplify the repeat motifs were used to genotype the six parental mice (three beach and three mainland parents). Out of 400 microsatellite loci tested, 113 showed diagnostic differences between individuals from the two subspecies, and these were scored in the 465 F2 progeny. All microsatellite loci were inherited in a codominant manner and were anonymous (with the exception of one microsatellite identified in a pigmentation gene, t-box protein 15 (Tbx 15)). Microsatellite markers used to construct the linkage map are listed in Table S1.

All PCRs were performed in a 15 μl volume using Eppendorf Mastercycler Gradient thermal cyclers (http://www.eppendorf.com). Each reaction included 30 ng of template DNA, 10× Taq Buffer with 1.5 mM MgCl2 (Eppendorf), 0.3 μL of 10 mM dNTPs, 0.6 μM each of a fluorescently labeled forward primer, unlabeled reverse primer, and 0.15 units Taq DNA polymerase (Eppendorf). The majority of microsatellite primers were synthesized with a known CAG (5’-CATGAGGCTGAGCTCA-3’), or M13R sequence (5’-GGAACAGCTATGACCAC-3’), or M13R sequence (5’-GGAACAGC-TATGACCAC-3’) attached to the 5’ end. The PCR master mixes used in this system included 0.06 μM of the sequence-tagged primer, 0.6 μM of the unlabeled primer, and 0.54 μM of the fluorescently labeled probe.

The cycling conditions for all primer pairs followed a touchdown protocol (successively lower annealing temperatures). PCR parameters were: 94°C for 90 s, followed by 21 cycles of denaturation at 94°C for 30 s, 55°C annealing for 30 s, and 72°C for 1 min. The initial annealing temperature decreased by 0.5°C for each of 20 cycles. An additional 15 cycles were performed as follows: 94°C for 30 s, followed by 30 s at the last temperature, and 72°C for 1 min. The final extension occurred at 72°C for 5 min.

Amplification products were scored on an ABI 3100 (http://www.appliedbiosystems.com) in a 96-well format and genotyping was multiplexed by labeling loci with different 5’ fluorescent dyes: FAM (blue), VIC (green), and NEL (yellow). Rox Genescan 400HD (Applied Biosystems) was used as internal size standard, and PCR products were analyzed with Genemapper version 3.5 software (Applied Biosystems).

In addition to microsatellite markers, 11 candidate pigmentation genotypes were screened for SNPs that were diagnostic between the two *P. polionotus* subspecies (Table S2). Candidate genes were chosen based on their known phenotypic effects, both on pigmentation and pleiotropic effects on other traits, in laboratory mice. For each candidate gene, PCR primers were designed in conserved exonic regions based on alignments of mouse, rat, and human sequences. Amplification primers were designed to span introns to maximize variation between subspecies. Following PCR optimization, introns were amplified in all six parents to identify diagnostic polymorphism. Sequences were edited using Sequencher, and diagnostic markers were identified by eye. PCR primers and amplification conditions are listed in Table S3.

Genotyping of three candidate loci, Kit, Kitl, and Hps4, was performed using a restriction enzyme digest assay. One microgram of Kit amplification product was digested at 60°C for 60 min with 1 unit of Bgl II × NEB (New England Biolabs), and the total reaction volume of 50 μL. Kitl and Hps4 amplitcons were digested in a total reaction volume of 15 μL at 37°C for 4 h using HpaI188 III and PspOM I enzymes, respectively. Digestion products were visualized on a 1.5% agarose gel stained with ethidium bromide.

A polymorphic microsatellite was identified in the first intron of *Tbx 15*. Genotyping of *Tbx 15* was scored on an ABI 3100 in a 96-well format. Genotyping of seven candidate pigmentation genes (McIr, Agouti, Tyr, Attn, Sl24a5, Pdhl, and Mgenl) was performed on an ABI 37000 using a TaqMan assay. A total of 60 ng of genomic DNA was used in each reaction, and cycling parameters were as follows: 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. The TaqMan primer sequences are listed in Table S4.

**Linkage map construction.** A genetic linkage map was generated using JoinMap version 3.0 [8] on a locus file containing genotypes of a total of 124 molecular markers in 465 F2 progeny, with the population type set for segregation of two alleles per locus (F2 population). JoinMap was used with an LOD score threshold of 6.0 to assign 120 of 124 loci to 27 LGs. For each LG, a map was created connecting Kosambi mapping function, dominant alleles were added, and recombination (0.4) thresholds, jump threshold of 5.0, and not fixed order. A ripple analysis was performed after all markers on the LG were added to the map. This analysis attempts to improve the order of the loci in a chromosome by testing alternative orders created by local permutations of the locus order.

**QTLM mapping.** All quantitative measures of pigmentation traits were analyzed with MapQTL 5 [11] using the interval mapping (IM) method, which fits a single QTL model (additive versus dominant model). Using likelihood ratio tests in MapManager QTb [20], we determined the additive versus dominance model was the best model of allelic effects. Similar mapping results were observed for the quantitative and categorical datasets. The MapQTL 5 parameters used were: mapping step size of 2.0 cM, maximum of 200 interactions, functional tolerance value of 1.0e−06, and a minimum of five flanking markers for resolved incomplete genotypes. MQM mapping was performed in LGs where several QTLs were detected. Cofactors for MQM analyses were automatically selected with a p-value of 0.02. Results from MQM analyses improved initial IM outputs by identifying from multiple to a single QTL per LG. Significance thresholds for determining QTLs were chosen based on criteria for genome-wide linkage mapping in noninbred individuals: significant linkage of LOD ≥ 4.5 [39]. Significance of LOD values for each trait was confirmed by permutation tests in MapQTL 5, with a genome-wide significance level of p = 0.05 and 1,000 iterations. Calculation of the percentage of phenotypic variance explained (p2/e) by a QTL was performed in MapQTL 5 on the basis of the population variance found within the progeny of the cross.

Gene interaction analyses to identify epistasis between QTLs were
performed using MapManager QT Xb. Probability of association was set at \( p = 0.0001 \), and the LOD thresholds for each quantitative trait were estimated by permutation tests.

RT-PCR and qPCR. Total RNA was isolated for four facial regions and the dorsum from shaved adult skin tissue of *P. polionotus* *leucocephalus*, *P. p. subgriseus*, and *P. maniculatus* using TRIzol reagent (Invitrogen, http://www.invitrogen.com) following the manufacturer’s protocol. Total RNA was treated using DNase I (New England BioLabs, http://www.neb.com). Subsequent reverse transcriptase reactions were performed using the Titan One tube RT-PCR kit (Roche, http://www.roche.com) with specific *Peromyscus* primers of *Agouti* (forward 5'-TTCICGGTGTTGCACTCT-3' and reverse 5'-TGTGATTTTAGCCTCCATTAGGTTTCC-3'), *Mc1r* (forward 5'-TGGACATACAGAATTGCCATGAG-3' and reverse 5'-CAACCCA-CACACGCGCTCTA-3'; exon 1), and beta-Actin (forward 5'-TCCAAGGAGGATTCAGAAG-3' and reverse 5'-TCCTGACTGAGCGTGGCTATAG-3'; exon 4) genes. Although *Agouti* has two differentially expressed transcripts, these primers were designed to measure expression of both isoforms simultaneously. For all experiments, both no-RT and no-Template controls were included.

PCR products were visualized on a 1.5% agarose gel. RT-PCR were also performed using the SuperScript III Reverse Transcriptase (Invitrogen), RNaseOUT (Invitrogen), and the oligo(dT) 20. qPCR also performed using the SuperScript III Reverse Transcriptase designed to measure expression of both isoforms simultaneously. For PCR Master Mix, and 1

The mean phenotypic value of the parental subspecies (LS and PO) differs among all seven traits are shown. Comparisons within facial and body regions are boxed. The exon structure of the *Agouti* gene; black squares are coding regions of the gene. The exon

The authors thank the *Peromyscus* Genetic Stock Center for maintaining crosses, T. Glenn for generating microsatellite libraries, and A. Bree and T. Linder for help measuring phenotypes. R. Hirschmann and L. Mullen contributed to the molecular work. Comments from J. Coyne, J. Jaenike, E. Kingsley, T. Price, M. Shapiro, J. Stinchcombe, and three anonymous reviewers greatly improved this manuscript.

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**Author contributions.** HEH conceived and designed the study (with input from CCS) and helped conduct the genetic crosses, prepare specimens, and measure phenotypes. CCS carried out all the molecular experiments, including genotyping, sequencing and expression studies, as well as the data analysis. JNW designed and optimized all the microsatellites and SNP markers in pigmentation genes. CCS and HEH wrote the paper with comments from JNW.

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