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The genetic basis of adaptive melanism in pocket mice

Michael W. Nachman*, Hopi E. Hoekstra, and Susan L. D'Agostino

Department of Ecology and Evolutionary Biology, Biosciences West Building, University of Arizona, Tucson, AZ 85721

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Identifying the genes underlying adaptation is a major challenge in evolutionary biology. Here, we describe the molecular changes underlying adaptive coat color variation in a natural population of rock pocket mice, *Chaetodipus intermedius*. Rock pocket mice are generally light-colored and live on light-colored rocks. However, populations of dark (melanic) mice are found on dark lava, and this concealing coloration provides protection from avian and mammalian predators. We conducted association studies by using markers in candidate pigmentation genes and discovered four mutations in the melanocortin-1-receptor gene, *Mc1r*, that seem to be responsible for adaptive melanism in one population of lava-dwelling pocket mice. Interestingly, another melanic population of these mice on a different lava flow shows no association with *Mc1r* mutations, indicating that adaptive dark color has evolved independently in this species through changes at different genes.

natural selection | pigmentation | melanocortin receptor | adaptation | *agouti*

A key problem in evolutionary biology is to connect genotype with phenotype for fitness-related traits (1, 2). Finding the genes underlying adaptation has been difficult for a number of reasons. First, it requires that we identify traits that are ecologically important and that we have some understanding of how these traits affect fitness in different environments. Second, phenotypic variation of ecological relevance has often been studied in species for which we have little genetic information, making the genetic basis of the traits difficult to analyze. For example, one of the best known cases of adaptation involves color morphs of the peppered moth, *Biston betularia*. Yet, after more than a half-century of study, the genes responsible for these color differences remain unknown (3). Finally, many fitness-related traits are quantitative and are unlikely to have a simple genetic basis. Because of these difficulties, the molecular basis for adaptation is known in only a handful of cases. Most involve either biochemical polymorphisms (4–6) or response to human disturbance, such as heavy metal tolerance in plants, insecticide resistance, warfarin resistance in rats, or antibiotic resistance (7), and in many cases, the specific nucleotide changes have not been identified.

The rock pocket mouse, *Chaetodipus intermedius*, provides a useful system for studying the genetics of adaptation. This species is found in rocky habitats in southern Arizona, New Mexico, and in adjacent areas in northern Mexico. Classic studies (8, 9) in the 1930s revealed a strong correlation between the color of the dorsal pelage and the color of the substrate on which *C. intermedius* live. In most places, these mice have a sandy dorsal pelage and white underbelly, and they inhabit light-colored rocks. In several different regions, however, these mice are found on lava flows. The mice from these lava sites are typically melanic, with dark-colored dorsal hairs and white underbellies. Most of the lava flows are surrounded by light-colored substrate and are isolated from one another by hundreds of kilometers, raising the possibility that melanic mice have evolved independently on different lava flows. The close match between the color of the mice and the color of the substrate on which they live is thought to be an adaptation against predation (8, 9). Owls are common predators of these mice, and experi-

ments by Dice (10) on deer mice showed that owls can effectively discriminate between light and dark mice even at night. Thus, it is likely that owls exert strong selection on coat color in *C. intermedius*, and that differences in coat color are an adaptation for crypsis. This view is strengthened by the strong correlation between substrate color and coat color across 18 different populations in Arizona and New Mexico, ranging from very light to very dark (9).

This system is amenable to genetic analysis because the biochemistry, development, and genetics of the pigmentation process have been intensively studied in other mammals. Approximately 80 genes have been identified that affect coat color in the laboratory mouse, and more than one-quarter of these have been molecularly characterized (11). A key distinction in melanogenesis is between the production of eumelanin (brown or black pigment) and pheomelanin (yellow or red pigment). This difference is controlled in large part by the interaction of two proteins, the melanocortin-1-receptor (MC1R) and the agouti-signaling protein (12–14). MC1R is a G protein-coupled receptor that is highly expressed in melanocytes, the specialized cells that are the site of pigment production. A peptide hormone, α -melanocyte-stimulating hormone, typically activates MC1R, resulting in elevated levels of cAMP and increased production of eumelanin. Agouti is an antagonist of MC1R; local expression of agouti results in decreased synthesis of eumelanin and increased production of pheomelanin. Wild-type laboratory mice have banded hairs on their dorsum; these hairs have a black tip, a middle yellow band, and a black base (the agouti hair). This banding is caused by a pulse of agouti expression during the middle phase of the hair cycle, resulting in deposition of pheomelanin during the middle of hair growth and deposition of eumelanin at the beginning and end of hair growth. Mutations at both *agouti* (14, 15) and at *Mc1r* (16) have been identified that produce unbanded dorsal hairs in the laboratory mouse. We observed banded dorsal hairs in all light *C. intermedius*, but unbanded, uniformly melanic hairs in all dark *C. intermedius*, suggesting a possible role for either *agouti* or *Mc1r*.

We used association studies with these candidate genes to investigate the genetic basis of adaptive melanism in *C. intermedius*. Here, we report that mutations at *Mc1r* are perfectly associated with coat color differences in one population of these mice, but that another population of mice shows no association between *Mc1r* mutations and coat color phenotype.

Materials and Methods

Samples. We sampled mice from two of the darkest lava flows, the Pinacate lava flow in Arizona and the Pedro Armendaris lava flow in New Mexico, separated by ≈ 750 km. We also sampled mice from light rocks adjacent to each lava flow; the distance between light and dark rock was ≈ 3 km at each site. In addition,

Abbreviation: MC1R, melanocortin-1-receptor.

Data deposition: The sequences reported in this paper have been deposited in the Genbank database (accession nos. AY258909–AY259034, AY247584, AY247585, AY247575, AY247576, AY247578, AY247580, AY247617, AY247619, AY250801–AY250829, AY259035, and AY259036).

*To whom correspondence should be addressed. E-mail: nachman@u.arizona.edu.

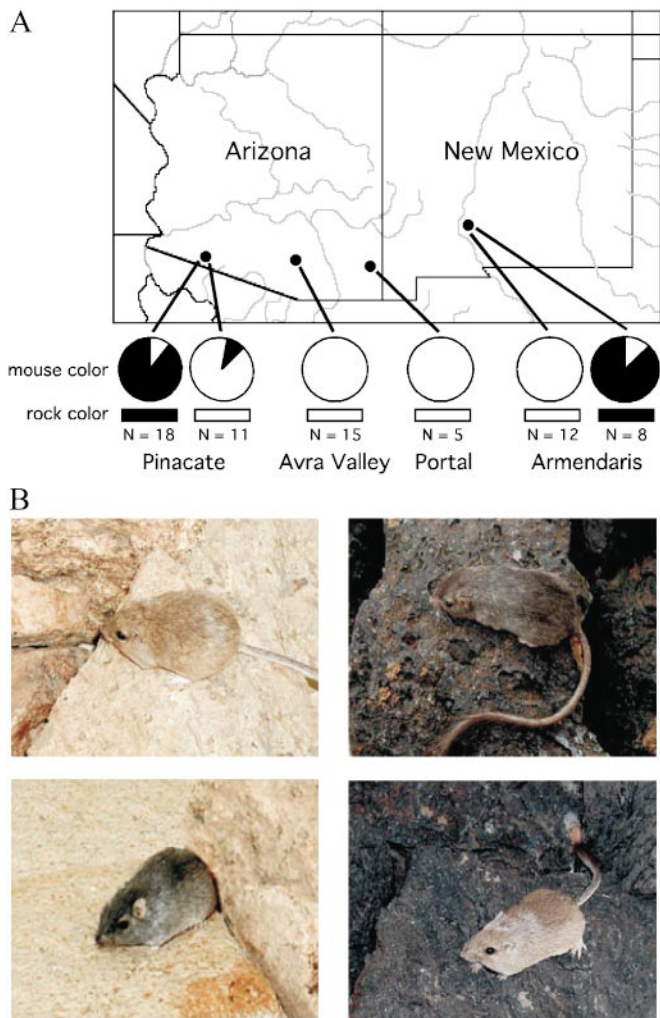


Fig. 1. (A) Collecting localities, substrate color, and mouse color. Sample sizes at each site are given. Pie charts indicate the proportion of light and dark mice at each site. Rectangles indicate the substrate color at each site. Mice from Pinacate and Armendaris were sampled on dark lava and also on light rock adjacent to the lava, whereas mice from Avra Valley and Portal were sampled only on light rock. (B) Light and dark *C. intermedius* from the Pinacate locality on light and dark rocks.

mice were sampled from light rocks at two other areas in Arizona (Avra Valley and Portal), both situated approximately midway between the Pinacate site and the Armendaris site (Fig. 1A). Mice were live trapped from each site; exact locations, sample sizes, and specimen numbers were as follows. (i) Pedro Armendaris dark rocks: Pedro Armendaris lava bed, Hard Luck Crossing, Socorro County, New Mexico, 33°30.8'N, 107°0.2'W ($n = 9$, HEH 521, 561–568); Pedro Armendaris light rocks: Fra Cristobal Mountains, Walnut Springs, Socorro County, New Mexico, 33°19.9'N, 107°7.1'W ($n = 12$, HEH 571, 573–582); (ii) 4.0 mi. E of Portal, Cochise County, Arizona, 31°54.9'N, 109°3.7'W ($n = 5$, MWN 1394, 1395, 1399–1401); (iii) Avra Valley Road, 1.0 mi. W of I-10, 15 mi. NNW of Tucson, Pima County, Arizona, 32°23.8'N, 111°8.5'W ($n = 15$, MWN 1327, 1328, 1331, 1333–1335, 1359, 1361–1367, 1370); (iv) Pinacate dark rocks: Cabeza Prieta National Wildlife Refuge, Pinacate Lava Bed, Yuma County, Arizona, 32°6.6'N, 113°30.4'W ($n = 18$, MWN 1371–1379, 1381–1387, 1414, 1416); Pinacate light rocks: Cabeza Prieta National Wildlife Refuge, western edge of O'Neill Hills, Yuma County, Arizona, 32°6.5'N, 113°22.5'W ($n = 11$, MWN

1402–1412). Museum specimens were prepared and have been deposited in the vertebrate collections of the Department of Ecology and Evolutionary Biology at the University of Arizona. DNA was extracted from frozen spleen or liver by following standard protocols.

Agouti. Approximately 1.5 kb of intronic DNA from the *agouti* locus was sequenced in a subset of mice ($n = 36$), including representatives from all six collecting sites. Conserved regions in the first two translated exons of *agouti* were identified from alignment of human and mouse *agouti* sequences, and the intervening intron was PCR amplified by using primers F1 (5'-GAT GTC ACC CGC CTA CTC CT-3') and R1 (5'-TGA TCT TTT TGG ATT TCT TGT TCA-3') in 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. This reaction produced a 1,500-bp fragment from which we sequenced 1,447 bp. Diploid PCR products were sequenced directly on an ABI 377 automated sequencer (Applied Biosystems).

Mc1r. Both alleles of the entire *Mc1r* gene (954 bp) were sequenced in all 69 mice. The *Mc1r* locus consists of a single exon encoding a 317-aa protein. Human and mouse *Mc1r* sequences were aligned, and PCR amplification primers were designed in conserved regions at the beginning and end of the coding region: F1 5'-TCT GGG TTC TCT CAA CTC CA-3' and R2 5'-AAA GCA TAG ATG AGG GGG TC-3'. These primers were used to amplify an 869-bp fragment in 35 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min. This fragment was sequenced, and nested primers were designed for genome walking to capture the 5' and 3' ends of *Mc1r*. Genomic DNA was digested with *PvuII* and *StuI*, and a 48-bp adapter containing two nested primer sequences was ligated to digested DNAs (Genome Walker Kit, K1807, CLONTECH). The 5' end of *Mc1r* was recovered in a 1-kb *StuI* fragment, and the 3' end of *Mc1r* was recovered in a 3-kb *PvuII* fragment. These fragments were sequenced, and new PCR primers were designed to amplify the entire 951-bp coding region (F11 5'-ATG CCT GGG CTG ACC TGT-3'; R9 5'-GGG CTC TTG TTC CTG ATG-3'). For all individuals, diploid PCR products were sequenced directly. From these sequences, we were able to identify putative heterozygous sites. Individual alleles from these PCR products were then cloned (Topo TA Cloning Kit, Invitrogen), and clones were sequenced to resolve haplotypes and confirm heterozygous sites. Two to five clones were sequenced per individual, until both alleles were captured. PCR error can be common in sequences derived from a single clone but is typically uncommon in direct amplification and sequencing of genomic DNA, which begins with many template sequences. Thus, by the combination of direct sequencing and sequencing of multiple clones for each individual, we were able to identify true heterozygous sites, resolve phase, and eliminate PCR errors.

Mitochondrial Genes. We assessed population structure among light and dark mice in the Pinacate population from mitochondrial DNA sequences. The mitochondrial *ND3* gene (345 bp) and *COIII* gene (783 bp) were PCR amplified by using conserved primer sequences designed from the published mouse sequence (GenBank accession no. V00711). PCR products were sequenced directly as described above.

Data Analysis. Sequences were aligned by using the SEQUENCHER program (Gene Codes, Ann Arbor, MI) and verified through visual inspection of all chromatograms. Polymorphic sites were identified, and these sites were used to test for associations with pigmentation phenotypes. The strength of genotype–phenotype associations was measured by using Fisher's exact test. Nucleotide heterozygosity, π , was calculated from the number of polymorphic sites and the frequencies of polymorphic nucle-

Table 1. *Mclr* polymorphisms in light and dark *C. intermedius* from the Pinacate population

Phenotype	Specimen no.	52	126	205	243	298	313	324	325	478	481	495	633	651	699	720	787	819	915	927
	Consensus	C	T	C	T	G	G	C	C	C	G	C	T	T	A	T	C	C	C	C
Dark	1371	T	T	T	.	.	C	.	C
Dark	1373	T	T	T	.	.	C	.	C
Dark	1374	T	T	T	.	.	C	.	C
Dark	1375	T	T	T	.	.	C	.	C
Dark	1376	T	T	T	.	.	C	.	C
Dark	1377	T	T	T	.	.	C	.	C
Dark	1378	T	T	T	.	.	C	.	C
Dark	1381	T	T	T	.	.	C	.	C
Dark	1383	T	T	.	T	T	.	.	C	.	C
Dark	1385	T	T	T	.	.	C	.	C
Dark	1386	T	T	.	T	T	.	.	C	.	C
Dark	1372	T	T	T	.	.	C	.	C
Dark	1379	T	T	.	T	T	.	.	C	.	C
Dark	1382	T	T	T	.	.	C	.	C
Dark	1384	T	T	.	T	T	.	.	C	.	C
Dark	1412	T	T	T	.	.	C	.	C
Dark	1416	T	.	T	.	.	T	.	T	T	.	.	C	.	C
Light	1387	.	.	T	T	.	.	.	G	.	T	.	.
Light	1402	.	.	T	G	T	.	.	.
Light	1403	A	.	T	G	.	T	.	.
Light	1404
Light	1405	.	.	.	C	A	G	.	T	T	.
Light	1406	.	C	.	.	A	.	T	G	.	T	.	.
Light	1407	.	.	.	C	A	G	.	T	T	.
Light	1408	G	.	T	.	.
Light	1409	.	.	T	A	.	C
Light	1410	A	.	T	G	.	T	.	.
Light	1411	A	.	T	G	.	T	.	T
Light	1414	A	.	T	G	.	T	.	.
<i>C. penicillatus</i>	1396	.	C	C
<i>C. baileyi</i>	1357	.	C	T	C
		R				V	A		R	R	A				Q		L			
		C				M	S		W	W	T				H		F			
		18				100	105		109	160	161				233		263			

Numbers in the top row correspond to nucleotide positions, and numbers in the bottom row correspond to amino acid positions in the *Mclr* sequence. Dots indicate identity to the consensus sequence; only differences are shown. For each individual, the two cloned alleles are shown. For nonsynonymous substitutions, alternative polymorphic residues are indicated at the bottom using the single-letter amino acid code.

tides (17). Phylogenetic relationships of mitochondrial alleles were estimated by using parsimony and neighbor-joining algorithms in PAUP* (18).

Results and Discussion

Mice were collected from six sites in four geographic regions, including both light and dark substrates (Fig. 1A). In all cases, we observed a close match between substrate color and the color of the dorsal pelage (Fig. 1B). Mice from Portal and Avra Valley were uniformly light. Of the 29 mice from the Pinacate site, 16 of 18 mice (89%) caught on the dark lava were dark, whereas 10 of 11 mice (91%) caught on the light-colored rocks were light (Fig. 1B). Similarly, of the 20 mice from Armendaris, 7 of 8 mice (88%) caught on the dark lava were dark, whereas all 12 mice (100%) caught on the light-colored rocks were light. In these populations, phenotypic variation in color was largely discrete rather than quantitative; all mice were easily classified as light or melanic based on the presence or absence of a subterminal band of pheomelanin on individual hairs on the dorsum.

In principle, several approaches are possible for identifying the genes underlying a phenotype of interest. However, *C. intermedius* are difficult to breed in captivity, and therefore any approach that relies on crosses is not practical. Instead, we used association studies with candidate genes to identify mutations responsible for the observed phenotypic differences. Approximately 1.5 kb of intronic DNA from the *agouti* locus were sequenced in 36 mice, including representatives from all sites. The black-and-tan mutation in the laboratory mouse produces a dark dorsum with unbanded hairs and is caused by an insertion disrupting the dorsal promoter, ≈ 15 kb 5' of the start codon (15). We reasoned that a similar mutation in *C. intermedius* might be detected through linkage to polymorphisms at neutral sites over moderate genomic distances (0–50 kb, depending on the age of the mutation). We observed 16 single-nucleotide polymorphisms and 5 insertion/deletion polymorphisms, including several at intermediate frequencies; none showed an association with coat color. There are two possible explanations for this lack of association: Either *agouti* is not a principal determinant of the observed phenotypic differences, or *agouti* is involved but there is little or no linkage disequilibrium between the sites we surveyed and the functional site(s).

Both alleles of the entire *Mclr* gene (954 bp) were sequenced in the 69 mice in Fig. 1. Twenty-four single-nucleotide polymorphisms were observed: 15 were synonymous and 9 were non-synonymous. Four of the nine amino acid polymorphisms were observed only in the dark mice from the Pinacate locality (Arg-18 \rightarrow Cys, Arg-109 \rightarrow Trp, Arg-160 \rightarrow Trp, and Gln-233 \rightarrow His). These four amino acid variants were present at high frequency (82%) among the Pinacate dark mice and were in complete linkage disequilibrium with one another. All other *Mclr* amino acid polymorphisms were present at low frequencies and showed no association with mouse color. The distribution of *Mclr* nucleotide variation among light and melanic mice from the Pinacate site is shown in Table 1.

Several observations suggest that one or more of these four amino acid mutations (sites 18, 109, 160, and 233) are responsible for the light/dark phenotypic differences seen in the Pinacate population. First, there is a perfect association between genotype and phenotype (Table 2). In a single panmictic population with no assortative mating, an association between genotypic and phenotypic variation is unexpected unless the gene or a tightly linked gene is responsible for the phenotype. However, population structure can produce a spurious association even when a gene is not involved in the phenotypic differences (19). We tested this hypothesis by sequencing the mitochondrial *COIII* and *ND3* genes in all mice from the Pinacate locality ($n = 29$). A phylogeny of these mitochondrial genes shows that haplotypes from light and dark mice are intermingled, providing no evi-

Table 2. Genotype–phenotype associations between *Mclr* alleles and coat color in *C. intermedius* from the Pinacate site

Genotype	Mouse phenotype	
	Light	Dark
DD	0	11
Dd	0	6
dd	12	0

The dark *Mclr* allele, D, has the following amino acids: Arg-18, Arg-109, Arg-160, and Gln-233. The light allele, d, has the following amino acids: Cys-18, Trp-109, Trp-160, and His-233. A Fisher's exact test comparing light homozygotes with dark homozygotes and heterozygotes is highly significant (i.e., 2×2 table with row entries 12 and 0, 0 and 17; $P < 0.0001$). Observed numbers of heterozygotes are slightly lower than expected under Hardy–Weinberg equilibrium ($\chi^2 = 4.89$, $P = 0.087$), consistent with disruptive selection across the two habitats.

dence for hidden population structure (Fig. 2). Because most gene flow in *Chaetodipus* is likely mediated by males (20) and because the mitochondrial genome has an effective population size one-quarter the effective size of autosomes, mitochondrial DNA provides a sensitive marker for detecting population structure. These mtDNA data also provide further evidence of selection on the dark/light phenotypic differences. In the Pinacate population, the frequency of dark mice on light substrate (9%) and the frequency of dark mice on dark substrate (89%) can be used to estimate the degree of population differentiation

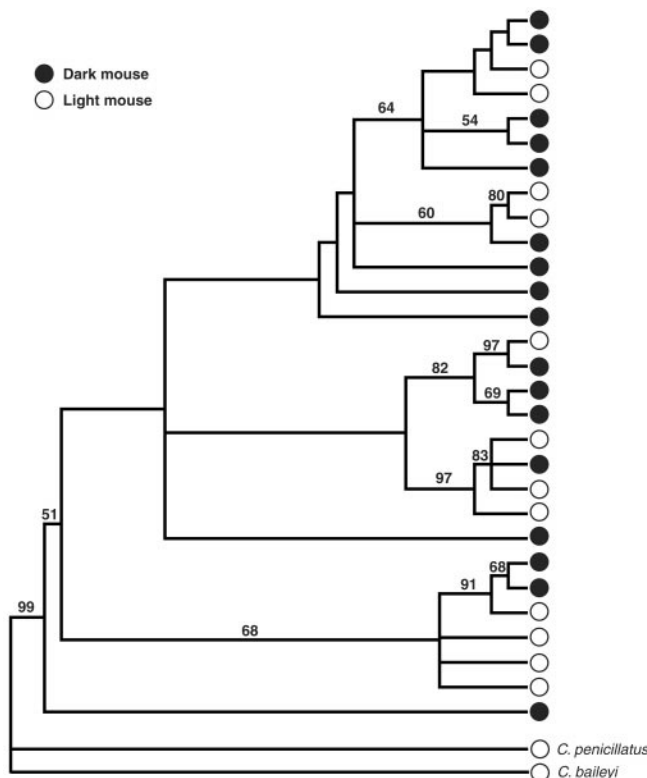


Fig. 2. Phylogeny of combined mitochondrial *COIII* and *ND3* sequences of the 29 *C. intermedius* from the Pinacate site. *Chaetodipus penicillatus* and *Chaetodipus baileyi* were used as outgroups; all individuals in these species are light. Light and dark mice are indicated with open and filled circles, respectively. Unweighted parsimony analysis using PAUP* resulted in a single shortest tree (length 132; consistency index 0.765). Numbers on branches indicate bootstrap values. The same topology was obtained when transversions were weighted 2 or 10 times more than transitions using parsimony. The same topology was also obtained by using the Neighbor-Joining algorithm.

substitutions present in all dark animals; this pattern is consistent with genetic hitchhiking of this silent site on the selected haplotype. However, two standard tests of neutrality (29, 30) fail to detect selection on *Mclr* in the Pinacate population, either in the total sample or in the subpopulations on light or dark substrate. The McDonald–Kreitman test (29) compares the ratio of synonymous to nonsynonymous changes within and between species; here, this ratio is changed only slightly by the four amino acid substitutions that distinguish the light and dark *Mclr* alleles. The HKA test (30) compares variation within and between species for two different genes (in this instance, *Mclr* and mtDNA); however, the level of nucleotide variability at *Mclr* is not significantly reduced even in the subpopulation on dark substrate, due to the presence of *Mclr* heterozygotes.

Because of complete linkage disequilibrium among amino acid sites 18, 109, 160, and 233 (Table 1), our data do not allow us to determine the relative contributions of each of these sites to the observed differences in color, nor do they rule out the possibility that a linked gene is responsible for observed phenotypic differences. The latter possibility seems unlikely for the reasons described above and also because no candidate pigmentation genes are closely linked to *Mclr* in either humans or *Mus*. In the laboratory mouse, single amino acid changes at *Mclr* are sufficient to produce a dark color (16). Functional studies using *Mclr* constructs in an *in vitro* expression system can be used to measure the relative effect of the four mutations on the activation of the receptor. Preliminary results from cAMP assays indicate that the dark allele encodes a hyperactive receptor relative to the light allele, providing strong support for the role of *Mclr* in the observed phenotypic differences (H.E.H., H. Fujino, J. Regan, and M.W.N., unpublished data). Using this approach with constructs that contain each of the four mutations individually and in combination, we hope to distinguish between two different models for *Mclr* evolution: (i) a single amino acid difference is responsible for the observed phenotypic differences, and the other amino acid variants have hitchhiked with the selected site, or (ii) two or more amino acid variants (acting additively or epistatically) are required to produce the observed phenotypic differences.

Strikingly, the data presented here implicate amino acid changes at *Mclr* in the dark phenotype in the Pinacate population but not in the Armendaris population. Only one *Mclr* amino acid polymorphism (Ala-285 → Thr) was observed among the 40 alleles from Armendaris; this variant was present in 2 of 24 alleles in light mice and 0 of 16 alleles in dark mice. Two silent polymorphisms were

present at intermediate frequencies (48%) among the 40 Armendaris alleles, but neither showed any association with mouse color. In fact, the frequencies of these polymorphisms were very similar among dark (50%) and light (45%) mice. The absence of an association between nucleotide polymorphisms in the *Mclr* coding region and mouse color at Armendaris also makes it unlikely that unsurveyed sites in the promoter region are responsible for the phenotypic differences, unless linkage disequilibrium decays extremely rapidly. The promoter region of *Mclr* has been well characterized in both mice (31) and humans (32), and it lies ≈500 bp upstream of the start codon. In general, patterns of nucleotide variation in the coding region of *Mclr* in the samples from Armendaris (both light and dark), Portal, and Avra Valley were unexceptional. Levels of nucleotide diversity ranged from 0.11% to 0.19%, similar to the value seen for the light animals from the Pinacate site.

The fact that the four mutations seen in the melanic Pinacate mice (Arg-18 → Cys, Arg-109 → Trp, Arg-160 → Trp, and Gln-233 → His) are absent in the melanic mice from Armendaris indicates that a similar dark phenotype has evolved independently on these different lava flows and has done so through different genetic changes, although the gene(s) involved in the Armendaris population have not yet been identified. The distinct molecular basis for the same phenotype in two different populations provides strong evidence for convergent phenotypic evolution on a relatively short timescale; both lava flows are less than one million years old.

Whereas *Mclr* darkening mutations have been identified in other species (16, 21–25), the ecological context for the changes either is not understood (24, 25) or is due to artificial selection (22, 23). Owls are important predators of pocket mice (8, 9) and are known to discriminate between light and dark mice on light and dark substrates even when foraging at night under low light intensity (10). Thus, it is likely that owls play an important role in selecting for concealing coloration. The data reported here present a rare example of the molecular changes underlying adaptation in a simple and natural ecological setting.

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