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Different genes underlie adaptive melanism in different populations of rock pocket mice

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Abstract

Identifying the genes responsible for adaptation has been an elusive goal in evolutionary biology. Rock pocket mice (*Chaetodipus intermedius*) provide a useful system for studying the genetics of adaptation: most *C. intermedius* are light-coloured and live on light-coloured rocks, but in several different geographical regions, *C. intermedius* are melanic and live on dark-coloured basalt lava, presumably as an adaptation for crypsis. Previous work demonstrated that mutations at the melanocortin-1 receptor gene (*Mc1r*) are responsible for the dark/light difference in mice from one population in Arizona. Here, we investigate whether melanism has evolved independently in populations of dark *C. intermedius* from New Mexico, and whether the same or different genes underlie the dark phenotype in mice from these populations compared with the dark mice from Arizona. Seventy-six mice were collected from pairs of dark and light localities representing four different lava flows and adjacent light-coloured rocks; lava flows were separated by 70–750 km. Spectrophotometric analysis of mouse pelage and of rock samples revealed a strong positive association between coat colour and substrate colour. No significant differences were observed in the colour of rocks among the four lava flows, suggesting that mice in these separate populations have experienced similar selection for crypsis. Despite this similarity in environment, melanic mice from the three New Mexico populations were slightly, but significantly, darker than melanic mice from Arizona. The entire *Mc1r* gene was sequenced in all mice. The previously identified mutations responsible for the light/dark difference in mice from Arizona were absent in all melanic mice from three different populations in New Mexico. Five new *Mc1r* polymorphisms were observed among mice from New Mexico, but none showed any association with coat colour. These results indicate that adaptive melanism has arisen at least twice in *C. intermedius* and that these similar phenotypic changes have a different genetic basis.

Keywords: adaption, *Chaetodipus*, convergent evolution, crypsis, Mc1r, natural selection

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A classic problem in evolutionary biology is to connect genotype with phenotype for traits of ecological importance. Finding the 'genes that matter' in ecology has been difficult for a number of reasons. Many fitness-related traits are quantitative and unlikely to have a simple genetic basis. Even when the traits are relatively simple, phenotypic variation of ecological relevance has often been studied in species for which we have little genetic information. For example, one of the most famous cases of adaptation involves the colour morphs of the peppered moth, *Biston betularia*, yet the genes responsible for this variation remain unknown (Majerus 1998).

There are several situations in which links between genotype and ecologically relevant phenotypes have been made successfully in eukaryotes. One involves protein polymorphisms and is rooted in the allozyme studies of the 1970s. Among the best examples of this are studies of LDH in killifish (e.g. DiMichele & Powers 1982a,b; Crawford & Powers 1989), PGI in *Colias* butterflies (e.g. Watt 1977, 1983) and haemoglobin in many species, including deer mice (e.g. Chappell & Snyder 1984; Snyder 1988). In each of these examples, allozyme variation has been shown to affect phenotypic variation in fitness-related traits among

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individuals within a species. A second situation in which genotype–phenotype links have been successfully made involves response to artificial selection (Doebley *et al*. 1995, 1997; Wang *et al*. 1999) or selection induced by human changes to natural environments. A good example of the latter is the evolution of insecticide resistance in *Drosophila* and other species (e.g. ffrench-Constant *et al*. 1993; Newcomb *et al*. 1997; Daborn *et al*. 2002).

Finding the genes that underlie adaptation allows us to directly investigate genetic changes and genetic constraints in evolution. This, in turn, allows us to ask such questions as whether adaptation results from changes in coding regions or changes in regulatory regions, whether adaptation results from a few mutations of large effect or many mutations of small effect, and whether similar selection pressures result in similar genetic changes.

The rock pocket mouse, *Chaetodipus intermedius*, provides a useful system for studying the genetic basis of adaptation. This species is restricted to rocky habitats in the deserts of the southwestern US and adjacent areas in Mexico. Throughout most of its range, this species lives on light-coloured rocks, and individuals typically have light-coloured pelage. In several disjunct geographical regions, however, these mice live on dark lava flows and are correspondingly dark in colour, presumably as an adaptation against predation from avian predators (Dice & Blossom 1937). Many of these lava flows are separated from each other by hundreds of kilometres, and the intervening rocks are mostly light in colour. This raises the possibility that dark mice have evolved independently on different lava flows. First described by Sumner (1921), Benson (1933) and Dice & Blossom (1937), this classic system is amenable to genetic analysis because of the large number of mammalian pigmentation genes that have now been characterized at the molecular level (Jackson 1997).

In mammals, coat colour is determined by both the absolute and relative amounts of two pigments: eumelanin, which is responsible for brown to black colour, and phaeomelanin, which is responsible for yellow to red colour (Barsh 1996). The switch between the production of these two pigments is controlled by the interaction of the melanocortin-1 receptor (MC1R), which is a transmembrane receptor found on melanocytes, and the agouti signalling protein. When MC1R is activated, intracellular cAMP levels are elevated through a G-protein signalling pathway, and eumelanin is produced. Agouti is an antagonist of this signalling pathway, and in the presence of agouti, cAMP levels are reduced and phaeomelanin is produced. Thus, the interaction of these two proteins plays an important role in determining the pigmentation patterns on individual hairs.

In a previous study, we demonstrated that variation at the melanocortin-1 receptor locus (*Mc1r*) is responsible for phenotypic variation in coat colour among mice inhabiting adjacent light and dark rocks in the Pinacate region of south–central Arizona (Nachman *et al*. 2003). Specifically, four charge-changing amino acid polymorphisms in MC1R are perfectly associated with coat colour. These four amino acids are in complete linkage disequilibrium with each other and define two classes of *Mc1r* alleles: the light and dark alleles. In these mice, the dark *Mc1r* allele is dominant over the light *Mc1r* allele, consistent with dominance patterns observed in the laboratory mouse.

Here, we survey *Mc1r* in dark pocket mice from three different lava flows in New Mexico to ask whether adaptive dark phenotypes in different populations have the same underlying genetic basis. First, we document phenotypic variation in mice from paired light and dark populations. Second, we show that, despite having similar, although not identical, phenotypic changes in colour compared with the Pinacate mice, the dark mice from populations in New Mexico show no associations with *Mc1r* genotype. This indicates that dark colour in pocket mice from Arizona and New Mexico has evolved independently through changes at different genes.

Methods

Sampling

A total of 76 pocket mice were collected from four large lava flows in the southwestern US: Pinacate, Kenzin, Pedro Armendaris and Carrizozo (Tables 1 and 2; Fig. 1). These lava flows vary in both age (1000–2000 000 years) and size (150–1500 km^2) (Table 1). The Pinacate flow is located in northern Sonora, Mexico and extends slightly into south–central Arizona. The Pinacate region is separated from the three New Mexico flows by over 750 km. Within New Mexico, the Kenzin lava bed is separated from the Armendaris and Carrizozo flows by 220 km and the Rio

Table 1 Lava flow characteristics

Lava flow	Area (km^2)	Age (Myr)	Reference
Pinacates, Yuma Co., AZ	1500	1.7–0.12	(Lynch 1989)
Kenzin, Doña Ana Co., NM	142	0.530 ± 0.04	(Hoffer & Corbitt 1991)
Armendaris, Socorro Co., NM	435	0.760 ± 0.10	(Bachman & Mehner 1978)
Carrizozo, Lincoln Co., NM	329	< 0.001	(Renault 1970)

Table 2 Sampling of *Chaetodipus intermedius* showing substrate colour, number of samples (*N*), phenotype description, and geographical location

Collecting site	Substrate*	*N*	Phenotype†	Lat (N)/Lon (W)
Pinacates	D	20	melanic	32°05.9′/113°28.3′
	L	10	wild-type	32°06.5′/113°22.5′
Kenzin	D	4	melanic	32°03.4′/106°57.9′
	D	7	dark agouti	32°03.4′/106°57.9′
	L	6	wild-type	32°09.6′/107°10.4′
Armendaris	D	8	melanic	33°31.0′/106°60.0′
	L	12	wild-type	33°19.9′/107°07.1′
Carrizozo	D	9	melanic	33°41.5′/105°50.9′

*L = light-coloured granitic rock and D = dark-coloured volcanic basalt. †Melanic = eumelanic unbanded hairs, and Dark agouti = banded hairs with a reduced agouti band.

Grande River. Armendaris is located in the Jornada del Muerto valley and is separated by the San Andres and Oscura Mountains from the Carrizozo flow (70 km to the east) in the Tularosa valley. All four lava flows are surrounded by sandy habitat, which severely limits contemporary migration to and from the lava, as the mice are restricted to rocky substrates.

Pocket mice were caught using Sherman traps. Museum specimens (skins, skulls and skeletons) were prepared and have been deposited in the zoological collections of the Department of Ecology and Evolutionary Biology at the University of Arizona. Mice included in this study are as follows: Pinacates dark ($n = 20$, MWN 1385, 1412, 1434, 1459, 1472, 1506, 1509–10 and HEH 512–3, 534–41, 598, 608); Pinacates light ($n = 10$, HEH 529, 532–3, 546, 548, 552, 589, 592, 594, 603); Kenzin dark melanic ($n = 4$, HEH 522–3, 687, 691*); Kenzin dark agouti ($n = 7$, HEH 504–7, 557, 559, 690*); Kenzin light ($n = 6$, HEH 515–6, 517*,

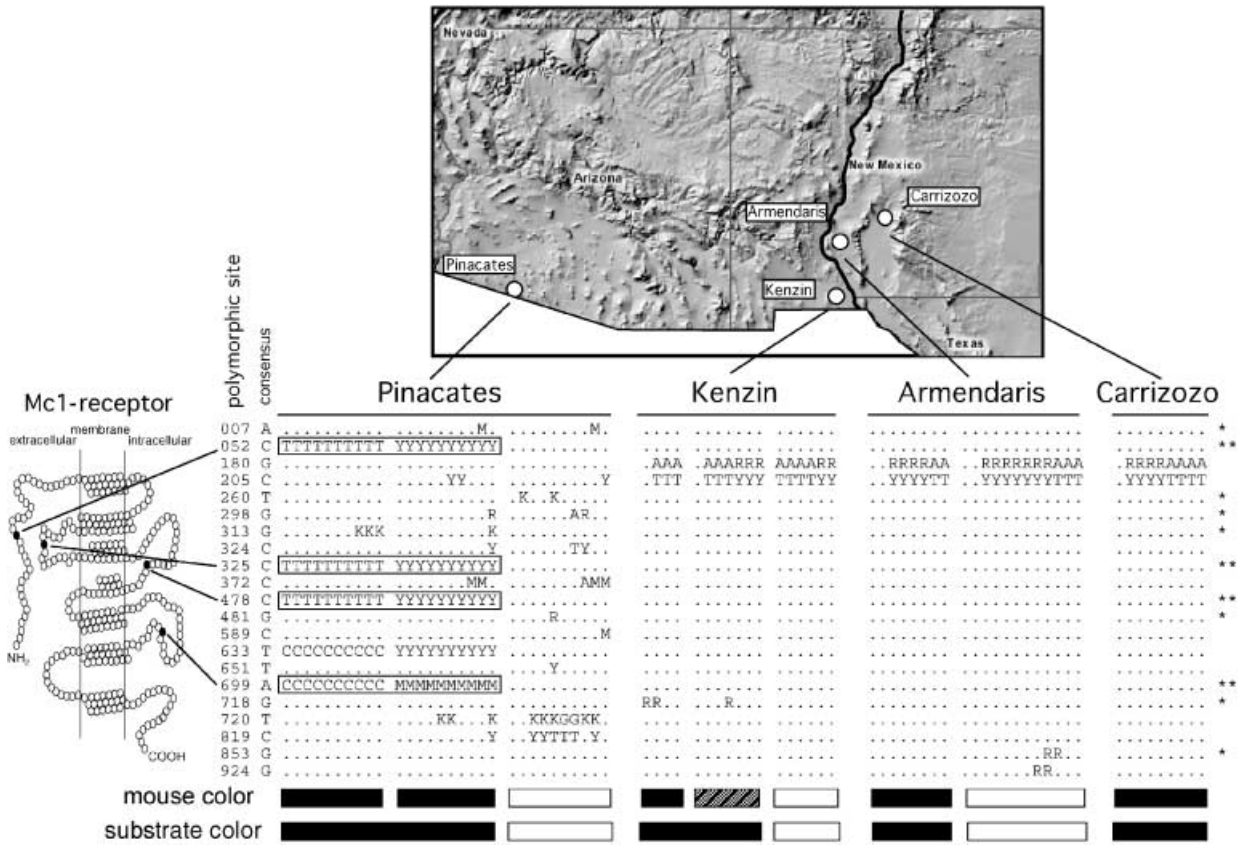

Fig. 1 Collecting localities and *Mc1r* polymorphisms for 76 mice representing 152 alleles. The MC1R protein is shown schematically on the left, and mouse and substrate colour are shown at the bottom. Dark and light mice were caught on adjacent dark and light substrates at each of the three localities (Pinacates, Kenzin and Armendaris), whereas only dark mice were caught at Carrizozo. Each column in the table represents the diploid *Mc1r* genotype for one mouse. Heterozygous sites are indicated with standard one-letter nucleotide codes: M (A/C), Y (C/T), R (A/G), K (T/G). Each row in the table depicts nucleotide variation at the 21 polymorphic sites relative to the consensus shown on the left. Pinacate dark mice are arranged by *Mc1r* genotype: homozygotes followed by heterozygotes. Melanic Kenzin mice are listed before the dark agouti mice. Four nonsynonymous polymorphisms that are perfectly correlated with coat colour in the Pinacate mice are highlighted, and the corresponding positions of the amino acid sites in the MC1R protein are shown. Asterisks (*) on the right of the figure indicate nonsynonymous nucleotide changes. Two asterisks (**) indicate charge-changing amino acid polymorphisms.

518–20); Armendaris dark ($n = 8$, HEH 521, 561–7); Armendaris light ($n = 12$, HEH 568, 571, 573–9*, 580–2); Carrizozo dark ($n = 9$, MWN 1337–45). Asterisks (*) indicate subadult individuals. The location of each collecting site is given in Table 2. To quantify habitat colour, representative rocks were collected from the trap-lines at each site.

Spectrophotometry measurements and analysis

The reflectance spectra of mouse pelage and of rocks were measured using a USB2000 spectrophotometer (Ocean Optics) with dual deuterium and halogen light source (Analytical Instruments Systems, Inc.). The program OOIBASE32 (Ocean Optics) was used to capture reflectance readings over the visible (400–850 nm) and UV (200–400 nm) spectra, using an integration time of 1460 ms. UV measurements (long-, medium- and short-range) were included because several bird species are known to see in the UV (Bennett & Cuthill 1994), although it is not known if nocturnal predators such as owls have UV-sensitive visual pigments. A standard reflection probe (400μ) with 25μ receptor fibre was held perpendicular to the surface, using a specialized probe holder, to capture both diffuse and spectral reflection.

Light and dark individuals (Fig. 2) were collected from the Pinacate, Kenzin and Armendaris lava flows, whereas dark mice only were collected from Carrizozo (Table 2). Spectrophotometric measurements were taken only on nonmolting adults ($N = 66$), whereas *Mc1r* sequences were generated for all individuals ($N = 76$), as described below. To characterize overall dorsal coloration, 10 reflectance measurements were taken across the dorsal surface; each measurement is taken over a circular area with a diameter of 2 mm. The measurements were restricted to areas behind the head. The 10 spectra for each individual were combined into an average spectrum for that individual by calculating the mean reflectance intensity from each of the 10 measurements at each of 2019 increments from wavelengths of 200–865 nm (Fig. 3). In addition, three rocks from each site were chosen to represent the habitat at both light and dark sites, and a single average spectrum for each rock was calculated, as above.

Differences in pelage colour were quantified by calibrating all measurements to white and black standards and measuring the reflectance intensity at all wavelengths. We concentrated on two primary measurements: peak reflectance intensity and total reflectance intensity (or brightness), which was calculated by summing the total area (visible and UV) under the reflectance curve. We present the raw data (intensity counts) here, but total reflectance intensity can be converted to per cent reflectance (relative to a white standard) by multiplying by a factor of 0.038,

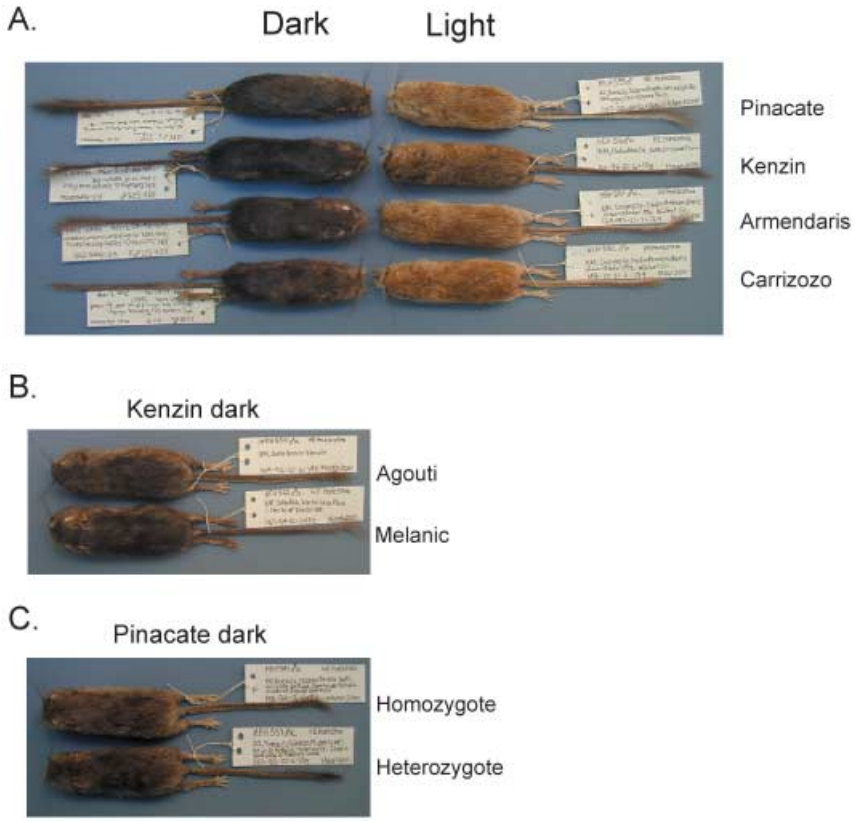

Fig. 2 (A) Representative light and dark *Chaetodipus intermedius* from each of the four collecting sites in this study. Carrizozo dark individual is paired with a light mouse from Armendaris. (B) Two dark Kenzin mice are shown: one with melanic hairs and one with agouti-banded hairs. (C) Two dark Pinacate mice are shown: one that is homozygous for the *Mc1r* dark allele and one that is heterozygous for this allele.

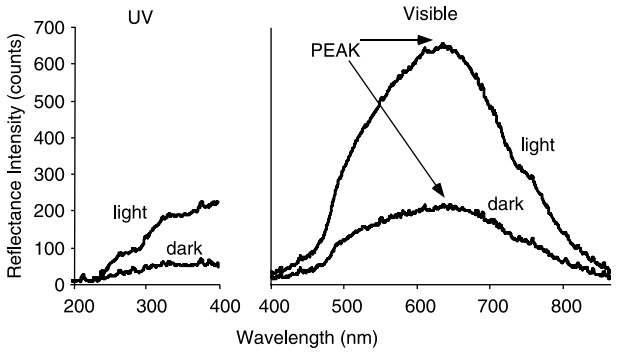

Fig. 3 Reflectance curves (not standardized) for a typical dark and light mouse over visible (400–850 nm) and UV (200–400 nm) wavelengths. The curves shown are from two representative individuals from Armendaris (HEH 521 = dark, HEH 581 = light). Total intensity (area under the curve) includes both the UV and visible spectra. Because the reflectance intensity is a function of both the light source and the illuminated object, the UV and visible spectra are not directly comparable as they are measured using different light sources.

and peak intensity can be converted to the peak per cent reflectance by multiplying by 0.047. Differences in reflectance were compared with ANOVA and *t*-tests using JMP (SAS Institute).

Molecular methods

DNA was extracted from fresh liver tissue using DNeasy tissue kits (Qiagen). Primers (mcr.F11-5′-ATGCCTGGGCTGACCTGT-3′ and mcr.R9-5′-GGGCTCTTGTTCCTGATG-3′) designed to amplify the entire coding region of *Mc1r* in *Chaetodipus intermedius* were used to generate a 1200-bp fragment (Nachman *et al*. 2003). Polymerase chain reaction (PCR) products were purified using QiaPrep spin columns. Diploid PCR products were completely sequenced on both strands using an ABI 3700 automated sequencer, with four internal sequencing primers: mcr.F1-5′-ACTGGGTCCTTTCAACTCCAC-3′, mcr.F5-5′-CCGAAGCACCCTACC-3′, mcr.R2-5′-AAGGCATAGATGAGGGGGTC-3′ and mcr.R6-5′-ACCACCAGCACATTTTCCACCAAGC-3′. Nucleotide sequences were analysed in SEQUENCHER (GeneCodes) and corrected by eye. These sequences have been deposited in GenBank (Accession nos AY247560–AY247635).

Results

Phenotypic and environmental variation

A total of 28 light and 48 dark individuals from four volcanic regions were included: Pinacates ($N = 30$), Kenzin ($N = 17$), Armendaris ($N = 20$) and Carrizozo ($N = 9$) (Table 2). All light individuals were characterized by dorsal hairs with a banded pattern. On these hairs, the tip and base are dark, as a result of the deposition of eumelanin, and the subterminal or central band is light, as a result of the deposition of phaeomelanin. This light phenotype results in an overall sandy colour and is referred to as agouti. All dark individuals had completely melanic hairs (i.e. no band of phaeomelanin) with the exception of a subset of dark mice from the Kenzin lava, which had an overall dark appearance but had a greatly reduced phaeomelanin band. Thus, there are three groups of mice from Kenzin: dark melanic mice, dark mice with a reduced agouti band (referred to as dark agouti) and light mice. The Pinacate sample included 10 light individuals and 20 dark individuals, 10 of which are heterozygous for the *Mc1r* dark allele and 10 of which are homozygous for the *Mc1r* dark allele.

Spectrophotometric readings were taken from 66 adult mice and 27 rocks. Peak intensity, total intensity and peak wavelength were recorded for 10 measurements taken on each specimen. There was substantial variation among measurements on each specimen (both for mice and for rocks); individual measurements taken on two representative mice and two representative rocks are given in Table 3. Variation in measurements was primarily due to variation in colour across the sample surface (dorsal pelage or rock face). However, variation in the distance of the probe from the sample surface and curvature of the sample surface can introduce measurement error. In general, standard deviations among measurements were ≈ 30% of the mean value. The mean for each specimen (mouse or rock) was used in all analyses below.

Phenotypic and environmental variation in colour within and among populations is shown in Table 4 and Fig. 4. Several patterns emerge from these data. First, there were significant differences in wavelength at the peak

Table 3 Measurements of total reflection intensity (counts) at 10 sites across the surface of a representative dark (HEH 521) and light mouse (HEH 581) and representative dark and light rock. All samples are taken from the Armendaris region

Measure	Dark mouse	Light mouse	Dark rock	Light rock
1	78 188	226 843	68 292	447 036
2	103 170	252 372	157 075	512 978
3	77 286	193 691	67 864	692 864
4	52 289	186 544	86 326	472 421
5	24 411	82 548	98 194	486 124
6	82 965	216 783	62 272	457 566
7	101 005	242 741	85 545	511 810
8	76 311	137 442	130 197	497 732
9	44 286	259 671	70 840	581 640
10	46 014	168 711	49 053	622 524
Mean	68 592	196 734	87 566	528 270
SD	25 816	55 712	33 216	79 411

Table 4 Summary of spectrophotometric analysis across populations

			MOUSE COAT				ROCK SUBSTRATE		
Site	Phenotype	*N**	λ† (SD)	Peak intensity‡ (SD)	Total intensity§ (SD)	*N**	λ† (SD)	Peak intensity‡ (SD)	Total intensity§ (SD)
Pinacates	D (homo)	10	624.3 (12.9)	374.2 (96.1)	131 643 (35 829)				
	D (hetero)	10	629.2 (11.9)	431.1 (87.5)	152 298 (28 285)	9	620.0 (12.3)	555.1 (185.3)	163 609 (47 971)
	L	10	634.1 (7.7)	668.9 (141.6)	207 940 (46 059)	3	612.0 (0.4)	1153.2 (181.1)	354 887 (60 648)
Kenzin	D (melanic)	3	612.0 (0.0)	164.4 (60.4)	62 907 (27 896)				
	D (agouti)	6	612.0 (0.0)	179.7 (59.7)	59 746 (20 163)	3	612.0 (0.0)	524.9 (215.6)	159 042 (61 815)
	L	5	631.6 (10.9)	337.0 (50.2)	111 663 (17 233)	3	612.0 (0.0)	1655.4 (144.1)	449 513 (31 909)
Armendaris	D	8	621.2 (12.7)	207.5 (96.3)	68 848 (32 385)	6	612.0 (0.0)	477.8 (154.8)	141 809 (39 659)
	L	5	630.4 (12.3)	571.7 (146.0)	181 803 (43 824)	2	612.0 (0.0)	1891.3 (48.1)	521 468 (19 994)
Carrizozo	D	9	623.5 (25.2)	110.7 (47.7)	36 435 (13 595)	1	612.0	466.5	141 619

*Adults. †Wavelength at peak intensity. ‡Peak reflectance intensity (counts). §Total area under the spectral curve (UV and visible).

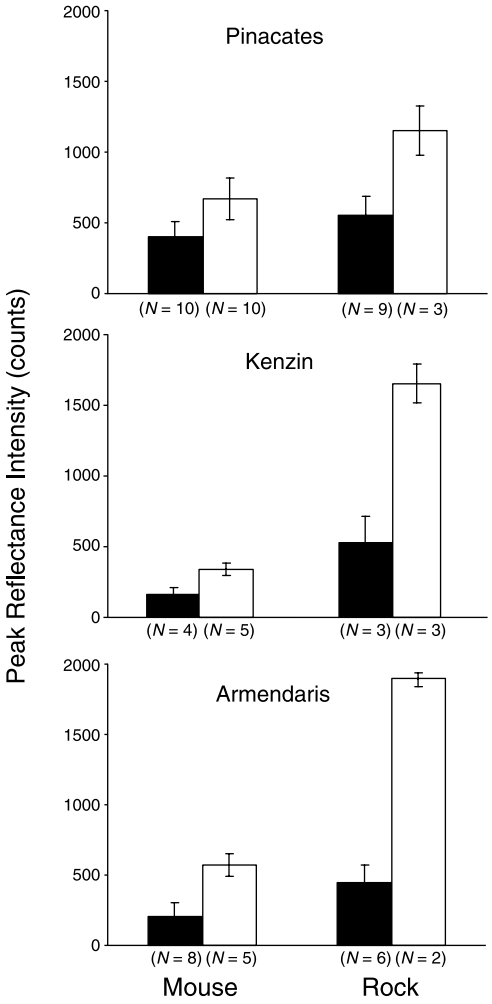

Fig. 4 Comparison of peak reflectance intensity for dark and light mice and dark and light rock from the Pinacate, Kenzin and Armendaris lava flows. Pinacate dark mice include only the 10 individuals homozygous at the *Mc1r* locus. Kenzin dark mice include only the four melanic individuals. Error bars represent one standard deviation.

intensity between light and dark mice ($t_{47} = 2.49$, $P = 0.017$); dark mice reflected light at shorter wavelengths than did light mice. However, no significant differences in wavelength were observed between light and dark rock ($t_{25} = 1.20$, $P = 0.24$). Peak reflectance for both mice and rocks occurred between 610 and 640 nm. Spectral reflectance intensities from both light and dark mice and light and dark rock have a unimodal distribution (Fig. 3). Second, both peak and total intensity differed significantly between dark and light mice at each site (peak intensity: Pinacates: $t_{28} = 5.93$, $P < 0.0001$; Kenzin: $t_6 = 3.09$, $P < 0.02$; Armendaris: $t_{11} = 5.47$, $P < 0.0002$ and total intensity: Pinacates: $t_{28} = 4.51$, $P < 0.0001$; Kenzin: $t_6 = 3.12$, $P < 0.02$; Armendaris: $t_{11} = 5.36$, $P < 0.0002$). Similarly, both peak intensity and total intensity differed significantly between light and dark rocks at each site (peak intensity: Pinacates: $t_{10} = 4.86$, $P < 0.0007$; Kenzin: $t_4 = 7.55$, $P < 0.0016$; Armendaris: $t_6 = 12.14$, $P < 0.0001$ and total intensity: Pinacates: $t_{10} = 5.65$, $P < 0.0002$; Kenzin: $t_4 = 7.23$, $P < 0.0019$; Armendaris: $t_6 = 12.53$, $P < 0.0001$). Third, there was a significant positive correlation between the total reflectance intensity of mouse pelage and of the substrate on which the mice live (Fig. 5; Spearman rank correlation, rho = 0.786, $P = 0.05$). In all three paired populations, dark mice and dark rocks reflect significantly less light (lower reflectance intensity) than their light counterparts (Fig. 4).

Three additional general patterns emerged in these comparisons (Fig. 4): (1) the magnitude of the difference in reflectance between light and dark rocks was always greater than the magnitude of the difference between light and dark mice, (2) there was a larger difference between light mice and light rocks than between dark mice and dark rocks, and (3) the reflectance intensity from mouse pelage was always lower than the reflectance intensity of the substrate on which the mice were found.

In comparisons among populations, there were no statistical differences in the peak reflectance measurements of

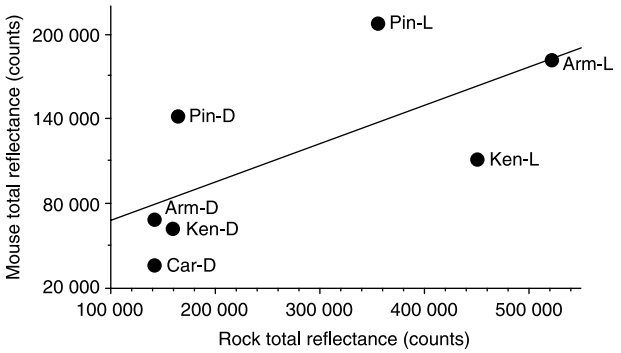

Fig. 5 Correlation between total reflectance from rock substrate and mouse pelage for seven sites (Spearmen rank correlation, rho = 0.786, $P = 0.05$). Abbreviations for the collecting locales are as follows: Pinacates = Pin, Armendaris = Arm, Kenzin = Ken and Carrizozo = Car. L = light and D = dark.

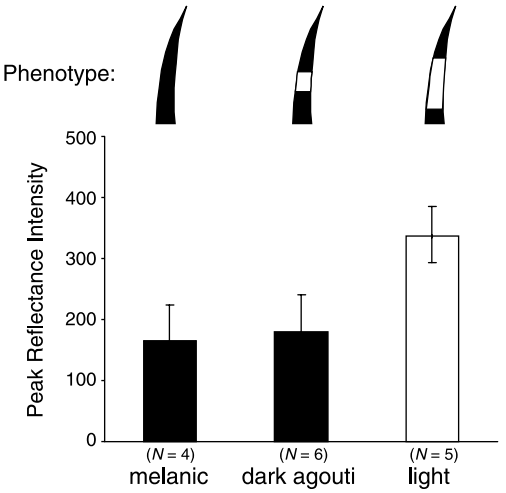

Fig. 6 Peak reflectance intensity of three phenotypic classes of mice from the Kenzin lava flow. Schematic pigmentation pattern of typical hairs is shown above. Error bars represent one standard deviation.

rocks among the four lava flows ($F_{3,15} = 0.25$, $P > 0.86$). Rocks from the Pinacates showed the largest range in peak reflectance measurements (but also had the largest sample size), and had the highest mean peak reflectance. However, there were differences in reflectance from mouse pelage on the different lava flows. Interestingly, the melanic mice from the three New Mexico populations were significantly darker than those from the Pinacates (peak reflectance: $F_{3,36} = 27.80$, $P < 0.0001$). In fact, the light Kenzin mice with banded hairs had lower peak reflectance and lower total reflectance than the melanic Pinacate mice (Table 4). Measurements from the melanic Pinacate mice have the largest range in peak and total reflectance (but also the largest sample size). Excluding the melanic Pinacate mice, there are minor differences in peak reflectance between the dark mice from the three New Mexico populations ($F_{2,17} = 3.74$, $P < 0.045$).

In the Kenzin population, we observed two types of dark mice: one with completely melanic hairs, and a second with banded hairs, in which the width of the phaeomelanic band was reduced (Fig. 6). Despite these morphological differences in individual hairs, overall peak reflectance intensity was not statistically different between these two dark phenotypes (Table 4; $t_7 = 0.36$, $P > 0.73$): mean peak reflectance for melanic mice was 164.4 (SD = 60.4) and for dark agouti mice was 179.7 (SD = 59.7).

Genetic analysis

The entire coding region of the melanocortin-1 receptor (954 bp) was sequenced for all 76 individuals (Fig. 1). In the total sample, there were 21 polymorphic sites, 4 of which were singletons. Of these 21 polymorphic sites, 10 were synonymous changes and 11 were nonsynonymous. The only nonsynonymous mutations that resulted in a change in amino acid charge are those four sites in the dark Pinacate mice that co-segregate with the dark phenotype in this population (Fig. 1). Most of the nucleotide variation occurred within the Pinacate population: 15 of the 21 segregating sites were exclusive to the Pinacate mice. In the Kenzin, Armendaris and Carrizozo populations there were only five segregating sites; of these, two rare variants resulted in an amino acid change (Ala240Thr and Ala285Thr) in the functionally less constrained 3′ region of the receptor.

Nachman *et al.* (2003) identified four amino acid polymorphisms (at nucleotide sites 52, 325, 478 and 699) in the *Mc1r* coding region that appear to be responsible for the phenotypic differences in colour seen in the Pinacate population (Fig. 1). Sequence data from both dark and light individuals from three additional dark populations showed no nucleotide variation at these four amino acid sites; these three populations were fixed for the ancestral 'light' allele.

In the three New Mexico populations, there were two high-frequency polymorphisms at nucleotide positions 180 and 205 that appear to be in linkage disequilibrium (Fig. 1). Both of these polymorphisms were silent and were found in both light and dark individuals. Thus, there are no *Mc1r* polymorphisms associated with coat colour variation in any of the three New Mexico populations, suggesting that dark colour in the New Mexico and Arizona populations evolved through changes at different genes.

Discussion

The results presented here indicate that adaptive melanism has evolved independently in different populations of *Chaetodipus intermedius* and has done so through changes at different genes. Spectrophotometric analyses of rocks

from different lava flows reveal that these rocks are similar in colour and therefore suggest that similar selection pressures may have existed in these different populations. Despite the similarity of the environments, spectrophotometric analyses of mouse pelage reveal subtle differences among populations. The melanic Pinacate mice have higher total reflectance than melanic mice from any of the New Mexico populations. Mice from the different New Mexico populations have similar reflectance intensities. These minor phenotypic differences between Arizona and New Mexico mice are consistent with an independent origin for melanism in these two geographical regions, a hypothesis that is corroborated by genetic data. Whereas four amino acid variants in the *Mc1r* coding region are involved in dark pigmentation in the Pinacate population, none of these four polymorphisms are present in the New Mexico mice. This observation points unambiguously to an independent origin of melanism in New Mexico and Arizona, but does not allow us to address whether melanic forms evolved once or multiple times within New Mexico.

Mutations in *Mc1r* have previously been shown to result in melanic phenotypes in other species of mammals as well as in birds (e.g. Robbins *et al*. 1993; Vage *et al*. 1997; Newton *et al*. 2000; Theron *et al*. 2001). These studies reveal that melanic phenotypes can result from a number of different amino acid mutations and can occur in several regions of the MCIR protein: transmembrane domains, intracellular regions and extracellular regions. These mutations can result in constitutive or hyperactivation of MC1R, and often only a single amino acid change is responsible for a change in receptor function (Robbins *et al*. 1993). In addition, MC1R is specific to the pigmentation pathway, and is thus unlikely to have antagonistic pleiotropic effects, making for a less constrained target of selection (Price & Bontrager 2001). These observations led us to expect the involvement of MC1R in other populations of *C. intermedius*. Our results, however, reveal no new polymorphisms in the *Mc1r* coding region associated with coat colour, suggesting that neither this gene nor linked regions are involved in the adaptive dark colour in Kenzin, Armendaris or Carrizozo mice.

Cryptic coloration is commonly thought to be maximized if spectral profiles are identical between an organism and its habitat. Despite a strong correlation between the colour of the mice and their respective habitats in this study (Fig. 5), reflectance intensities are not identical, or even overlapping in some cases, between mouse pelage and rock (Fig. 4). Instead, our results show consistent disparity between the reflectance of substrate and the mice that inhabit these areas. In all cases (in both light and dark mice and across lava flows), the mice are darker than their respective environments. This pattern is also observed in other nocturnal rodent species thought to be cryptically coloured. For example, this pattern is seen in oldfield mice, *Peromyscus polionotus* (Belk & Smith 1996), but not in diurnal gophers (Krupa & Gelusa 2000). It is possible that the match between reflectance intensity of mice and substrate may not be an accurate measure of crypsis, and that for antipredator crypsis, it may be advantageous to be darker than the surroundings.

However, it is also important to note that the reflectance measurements of rock do not convey the true complexity of the environment. For example, volcanic basalt becomes lighter over time as the environment wears away the dark outer layers, and as sand and dirt accumulate in the pores of the rocks. In addition, lava flows become vegetated over time; density of vegetation increases with the age of the lava flow. Kenzin, one of the older volcanic flows, is covered with several grass species that shade the rock substrate. In the Kenzin population, we observe the greatest disparity between brightness of the dark mouse and the dark rock. Similarly, Carrizozo is the youngest of the lava flows (< 1000 years; Table 1) and has the least amount of vegetation, and the pocket mice from Carrizozo are among the darkest.

In light of the recent origin of the Carrizozo lava flow, it is striking to find dark mice in this area. It is noteworthy that the three New Mexico populations are geographically close together and that the phenotypes of mice from these populations are statistically indistinguishable (Table 4). It is certainly possible that melanism evolved just once in these populations and spread via migration. Phylogeographical analyses at unlinked neutral loci may reveal historical migration patterns between these populations. A second approach to determine if the dark phenotype has evolved independently in the different New Mexico populations is to identify the genes responsible for melanism in each case.

Several loci involved in pigmentation are particularly likely candidates for the coat colour differences seen among populations of *C. intermedius*. Agouti, the antagonist to *Mc1r*, is a particularly appropriate candidate locus because multiple *agouti* alleles have been identified in the laboratory mouse which produce a range of colour phenotypes. Recessive, loss-of-function *agouti* mutations result in nonagouti, all black phenotypes. For example, a mouse mutation known as black-and-tan results from a large insertion near the dorsal promoter of the *agouti* gene (Bultman *et al*. 1992, 1994). This insertion eliminates dorsal expression of *agouti* but has no effect on ventral expression. The phenotype of these mice includes a light belly and all-black, unbanded dorsal hairs similar to the phenotype observed in lava-dwelling *C. intermedius* populations.

On the Kenzin lava we observed two classes of dark mice with similar reflectance intensities: dark mice with banded hairs, in which the light-coloured agouti band is greatly reduced, and dark mice with completely melanic hairs. Because the duration of *agouti* expression is thought to

control the width of the phaeomelanin bands on individual hairs, phenotypic variation among the Kenzin mice may be due to genotypic variation at the *agouti* locus. Association studies at *agouti* as well as at other pigmentation genes will help reveal the nature and number of mutations involved in adaptive melanism in multiple populations of *C. intermedius*.

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