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Striking coat colour variation in tuco-tucos (Rodentia: Ctenomyidae): a role for the melanocortin-1 receptor?

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South American tuco-tucos (*Ctenomys*) are characterized by striking variation in coat colour. A range of phenotypes, from pale blonde to dark black, is observed across species, with some of them matching their local substrate colour. Moreover, phenotypic convergence is evident in some taxa that occupy similar habitats. The present study investigated a role for the *melanocortin-1 receptor* (*Mc1r*) in determining coat-colour variation in a wide range of *Ctenomys* species. We sequenced 1250 bp, including the entire *Mc1r* coding region and a portion of the adjacent 5′ and 3′ untranslated regions, in 21 species. In total, 20 amino acid replacements were identified in Mc1r. However, our findings suggest that these changes have not contributed to coat-colour differences among tuco-tucos because no amino acid replacement was associated with pigmentation phenotype in a simple way. Levels of *Mc1r* expression were measured in skin samples from dorsal, flank, and ventral body regions in pale, brown, and melanic individuals. We did not observe any significant difference in transcript abundance among phenotypes, although we identified a significant reduction of expression level from the dorsal to ventral region in both pale and brown morphs but not in the completely melanic form. Thus, a role for *Mc1r* regulation in tuco-tucos colour pattern cannot be completely ruled out, although further functional assays are needed. Finally, selection analysis suggests that *Mc1r*, in a majority of lineages, has evolved under purifying selection but with relaxation in functional constraint in some regions, especially in the fourth transmembrane domain. In summary, the results obtained in the present study suggest that this trait may have a complex basis, and that other pigmentation genes are involved in generating the dramatic diversity in coat-colour phenotypes observed among *Ctenomys* species. *Biological Journal of the Linnean Society*, 2012, **105**, 665–680.

ADDITIONAL KEYWORDS: adaptation – crypsis – *Mc1r* – pigmentation – purifying selection.

INTRODUCTION

Coat-colour variation is one of the most conspicuous phenotypic traits in mammals. The investigation of this trait specifically allows inferences to be made about the mechanisms that generate phenotypic diversity more generally. For example, we can determine the kind of genes involved in phenotypic change, whether trait variation is caused primarily by changes in gene structure or gene regulation, and, finally, whether similar phenotypes are produced by similar genetic changes (Hoekstra, 2006). Targeting genes that are known to control hair colour in laboratory populations may provide insights into the mechanisms driving coat-colour evolution in the wild (Hofreiter & Schöneberg, 2010; Hubbard *et al*., 2010).

One promising candidate gene is the *melanocortin-1 receptor* (*Mc1r*). Hair and skin colour in mammals is largely determined by the amount, type, and distribution of melanin packaged in the melanosomes of epidermal cells and hair follicles (Jackson, 1997). *Mc1r* acts as a pigmentary switch in the production of

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Figure 1. Convergent pelage coloration and local habitat of ctenomyids. 1 (*C. flamarioni*) and 2 (*C. australis*) are pale forms; A and B represent Atlantic coastal dunes habitat; 3 (*C. porteousi*) and 4 (*C. minutus*) are dark phenotypes; C and D represent Pampa grasslands habitat.

melanin: when activated by α-melanocyte stimulating hormone (α-MSH), it signals the production of eumelanin (black/brown pigment) and in the absence (or inhibition) of α-MSH, pheomelanin (red/yellow pigment) is synthesized (Jackson, 1997). In mice, *Mc1r* dominant mutations are often associated with a hyperactive or constitutively active receptor resulting in predominantly black coat colour (Jackson *et al*., 1994), whereas recessive loss-of-function mutations tend to trigger the production of pheomelanin, which leads to predominantly yellow or red coat colour (Robbins *et al*., 1993). In addition, several pigmentation polymorphisms in domestic and wild mammals have been attributed to variation in *Mc1r*; for example, beach mice (Hoekstra *et al*., 2006), black bears (Ritland, Newton & Marshall, 2001), cattle (Klungland *et al*., 1995), dogs (Newton *et al*., 2000), foxes (Våge *et al*., 1997), grey squirrels (McRobie, Thomas & Kelly, 2009), horses (Marklund *et al*., 1996), jaguars and jaguarundis (Eizirik *et al*., 2003), pigs (Kijas *et al*., 1998), golden lion tamarins (Mundy & Kelly, 2003), rock pocket mice (Nachman, Hoekstra & D'Agostino, 2003), and sheep (Våge *et al*., 1999). Intraspecific studies suggest that the *Mc1r* may be a target of positive selection, in that it underlies melanism in lava-dwelling rock pocket mice (Nachman *et al*., 2003) and adaptive pale coloration in beach mice (Hoekstra *et al*., 2006). Despite the many cases of intraspecific pigment polymorphism associated with *Mc1r* mutations, much less is known about its role in the evolutionary pattern of coloration among more diverged mammalian species (Eizirik *et al*., 2003; Mundy & Kelly, 2003; Ayoub *et al*., 2009).

South American tuco-tucos (Ctenomyidae) represent a remarkable system for investigating the genetic basis of ecologically important traits such as pigmentation. This family of subterranean rodents is characterized by conspicuous ecological and morphological diversity (Busch *et al*., 2000; Lacey, Patton & Cameron, 2000; Mora, Olivares & Vassallo, 2003). Ctenomyids occur in a wide variety of habitats and across extensive areas (Lacey *et al*., 2000). They can be found, for example, in the pampas of Puna (above 4000 m), the high mountain steppes, low valleys of the west, the dunes of the Atlantic coast of the east, mesic and humid plains, desert or semi-deserts, open areas among subtropical forests, and the steppes of Terra del Fuego (Reig *et al*., 1990). In addition, tuco-tucos have a wide range of pelage coloration that vary both inter- and intraspecifically, ranging from pale blonde to brown with white spots and even melanic phenotypes (Langguth & Abella, 1970; Freitas & Lessa, 1984; Gonçalves & Freitas, 2009). In most cases, their dorsal coloration matches the substrate in which they live (Langguth & Abella, 1970). In addition, some taxa represent clear examples of phenotype convergence (Fig. 1). For example, the closely-related species *Ctenomys australis* and *Ctenomys flamarioni* inhabit pale coastal dunes in Argentina and Brazil, respectively (Mora *et al*., 2006; Fernández-Stolz, Stolz & Freitas, 2007), and both are characterized by pale coats. The more distantly related species *Ctenomys talarum* and *Ctenomys minutus* occupy darker sand habitats adjacent to the costal dunes and are significantly darker than their coastal counterparts (Freitas, 1995; Busch *et al*., 2000). These two pairs of closely- and distantly-related cryptically coloured taxa may represent a case of adaptation to minimize predation. To date, the genetic basis of this convergence has not been

explored; it is unknown, for example, whether mutations in the same gene or different genes cause such similar coat colours.

The present study aimed to investigate the evolution of *Mc1r* and its possible role underlying coat-colour diversity in *Ctenomys*. Specifically, we aimed to: (1) identify amino acid replacements associated with coat-colour differences; (2) measure levels of gene expression between extreme pale and dark phenotypes; and (3) quantify patterns of selective constraint and positive selection acting on this protein.

MATERIAL AND METHODS

Experimental procedures

A total of 21 ctenomyid species were surveyed in the present study. Their phenotypes were classified by considering overall appearance (dorsal coloration) and grouped into three classes: (1) pale; (2) dark: brown, brown with white spots, and dark brown; and (3) melanic. Tissue samples were obtained either from live caught specimens or from scientific collections (Table 1).

Genomic DNA (gDNA) was isolated using DNeasy Blood & Tissue Kit (Qiagen) in accordance with the manufacturer's instructions. Comparative analysis was performed using both nucleotide and amino acid sequences of *Mc1r* from additional rodents obtained from the GenBank (*Chaetodipus intermedius*, AY247634; *Eothenomys melanogaster*, GU001573; *Meriones unguiculatus*, AY800269; *Mus musculus*, AB306322; *Onychomys torridus*, DQ482899; *Peromyscus maniculatus*, GQ337977; *Sciurus carolinensis*, EU604831; *Thomomys bottae*, EF488834; *Rattus norvegicus*, AB306978).

Specific primers were designed for *Ctenomys*, targeting the *Mc1r* coding region, based on alignment of sequences across all rodents available in GenBank. Initially, six sets of primers were tested in all possible combinations with different annealing temperature and $MgCl_2$ concentration. Primers GLF1 (5'-CCAAGAACCGAAACCTGCAC-3') and GLR5 (5'-AAAGCATAGATGAGGGGGTC-3') amplified a 710-bp expected product using a touchdown polymerase chain reaction (PCR) profile (decreasing annealing temperatures from 60 °C to 50 °C) with AmpliTaq Gold (Perkin Elmer) and 1.8 mM $MgCl_2$. PCR products were purified using Exonuclease I (GE Heathcare) and Shrimp Alkaline Phosphatase, sequenced with a BigDye chemistry and analyzed on a ABI3730XL. Resulting sequences were checked using BLAST (Altschul *et al*., 1990) and used to design primers that would capture the complete *Mc1r* coding region (954 bp), as well as a small portion of the 5′ and 3′ untranslated regions (5' UTR and 3' UTR, respectively). The genome-walking technique was performed with a Universal Genome Walking kit (Clonetech) and a sample of high molecular weight gDNA with from *C. flamarioni*. First, we built a pool of gDNA fragments bound to adapters, defined as 'libraries', using restriction enzymes *Dra*I, *Eco*RI, *Pvu*II, and *Stu*I. Then, two amplifications were performed by PCR for each library. A single positive band (i.e. specific PCR product) was identified in three of four libraries and selected for further characterization. Each fragment was cut from a 1.5% agarose gel, purified by columns (Invitrogen) and directly sequenced. Finally, a new set of six pairs of primers was designed to amplify the entire *Mc1r* coding region and neighbouring UTRs in *Ctenomys* (1250 bp). Nucleotide sequences of primers are available upon request. This segment was amplified using recombinant Taq polymerase (Invitrogen), an annealing temperature of 67 °C, and 1.2 mM $MgCl_2$, and sequenced as previously described.

Gene structure and expression analysis

Mc1r nucleotide sequences from all 21 species were aligned using CODON CODE ALIGNER (CodonCode Corp.), and all chromatograms were manually visualized and checked. Analyses were performed in MEGA, version 5.0 (Tamura *et al*., 2011) and PAUP* 4.0 (Swofford, 2002), including analysis of nucleotide composition, codon usage bias, and phylogenetic relationships. To reconstruct *Mc1r* gene genealogies, we used the 1250 bp that includes the entire *Mc1r* coding region. A tree was constructed using maximum likelihood (ML) method with heuristic search option, tree bisection–reconnection, and an initial neighbour joining clustering. Branch support was estimated by 1000 bootstrap replications using a heuristic search of nearest-neighbour interchange. The nucleotide substitution model was estimated using JMODELTEST (Posada, 2008), following the Akaike information criterion. All the amino acid changes were tracked along branches of a phylogenetic tree, which was reconstructed based on cytochrome *b* (cyt *b*) data using a ML method, with MACCLADE, version 4.0 (Maddison & Maddison, 2000). Several species already had cyt *b* sequences available in GenBank; for all others, we sequenced a fragment of the gene (approximately 500 bp) using primers MVZ05 and TUCO06 (Wlasiuk, Garza & Lessa, 2003) (Table 1).

Because *Mc1r* plays a key role in the type and distribution of melanin throughout the hair, we hypothesized that transcript levels of mRNA may be higher in darker species compared to paler ones. Similarly, transcript abundance may vary across body regions within individuals: high on the dark-coloured dorsal region, intermediate on flank, and lowest on

Table 1. List of *Ctenomys* used in the present study

Species	*N*	Phylogenetic group	Coat colour	Habitat	Genbank acession number	
					Mc1r	*Cyt-b*
Ctenomys australis	4	*mendocinus*	Pale	Costal sand dunes	JF910108	AF370697
Ctenomys boliviensis	1	*boliviensis*	Brown	Forest meadows	JF910109	AF155869
Ctenomys azarae	3	*mendocinus*	Brown	Pampa grassland	JF910110	JN791406
Ctenomys dorbygni	1	*torquatus*	Brown	Pampa grassland	JF910111	AF144279
Ctenomys flamarioni	5	*mendocinus*	Pale	Costal sand dunes	JF910112	AF119107
Ctenomys haigi	1	*magellanicus*	Brown	Steppe grassland	JF910113	GU433046
Ctenomys lami	5	*torquatus*	Brown	Sand fields	JF910114	HM777477
Ctenomys leucodon	1	No group	Brown	Altiplano grassland	JF910115	AF007056
Ctenomys maulinus	1	No group	Brown	Steppe grassland	JF910116	AF370703
Ctenomys mendocinus	1	*mendocinus*	Brown	Pampa grassland /Forest meadows	JF910117	AF007062
Ctenomys minutus	5	*torquatus*	Brown	Sand fields/Costal sand dunes	JF910118	HM777482
Ctenomys nattereri	1	*boliviensis*	Brown	Forest meadows	JF910119	AF144298
Ctenomys pearsoni	3	*torquatus*	Brown	Pampa grassland	JF910120	HM777486
Ctenomys perrensi	1	*torquatus*	Brown	Pampa grassland	JF910121	AF500067
Ctenomys porteousi	3	*mendocinus*	Brown	Pampa grassland	JF910122	AF370681
Ctenomys rionegrensis	4	*mendocinus*	Brown; melanic	Inland sand dunes	JF910123	AF119114
Ctenomys roigi	1	*torquatus*	Brown	Pampa grassland	JF910124	AF144278
Ctenomys sociabilis	1	No group	Brown	Steppe grassland	JF910125	EU035177
Ctenomys steinbachi	1	*boliviensis*	Brown	Forest meadows	JF910126	JN791407
Ctenomys talarum	3	*talarum*	Brown	Sand fields	JF910127	HM777498
Ctenomys torquatus	31	*torquatus*	Brown; brown with white spots; dark-brown; melanic	Pampa grassland	JF910128	EF372287

pale ventrum, in accordance with the colour gradient generally observed in tuco-tucos. Thus, we quantified levels of *Mc1r*-mRNA in the three classes of phenotypes: pale (*C. australis* and *C. flamarioni*; $N = 4$), dark (*C. minutus* and *Ctenomys torquatus*; $N = 4$), and melanic (*C. torquatus*; $N = 2$) using quantitative PCR. Skin tissue samples of 5 mm^3 were collected on the dorsal, flank, and ventral from field-trapped adults, preserved in RNAlater (Ambion), and stored at –80 °C. RNA extraction was performed using RNeasy Fibrous Tissue kit (Qiagen). This RNA then served as a template for complementary DNA (cDNA) synthesis, using random hexamer oligonucleotides primers (poly-dT 20) and SuperScript III reverse transcriptase (Invitrogen). We purified the cDNA using columns (Invitrogen). We next designed a custom TaqMan assay (Invitrogen) to measure *Mc1r* transcript abundance in a Realplex2 Mastercycler (Eppendorf). The 'housekeeping' gene (β-actin, *act*) was used as a control for starting RNA amounts. These experiments were carried out in duplicate. PCR was initiated with a 'hot start' of 15 min at 95 °C followed by 50 cycles of denaturation (94 °C for 15 s), annealing (50 °C for 30 s), and extension (72 °C for 30 s). We calculated the relative *Mc1r*-mRNA levels using the ratio of *Mc1r*-mRNA to *act*-mRNA. We compared relative expression levels of each gene with Student's *t*-test (two-tailed, unequal variance).

Test for *Mc1r* selection

Variation in selective pressure (e.g. positive selection) acting on *Mc1r* was characterized by estimating the ratios of nonsynonymous to synonymous substitution rates (dN/dS or ω) using the codeml routine of PAML, version 4.2 (Yang, 1997; Yang, 2007). When the ω ratio is significantly less than 1, this is consistent with the action of purifying selection. Neutral evolution is considered likely if the ratio is approximately equal to 1. Positive selection is inferred ω is significantly greater than 1. The dataset comprised 21 sequences of ctenomyids and other nine rodent species. The latter were included to compare the evolution of *Mc1r* in ctenomyids to other families and, specifically, to test whether there is acceleration of nonsynonymous substitution rate in tuco-tucos. The phylogeny used for this analysis was a consensus of the published data (Blanga-Kanfi *et al*., 2009).

The data set was adjusted to six models of codon substitution (Yang *et al*., 2000: M0 (one ratio), fitting a single value of ω across all sites: M1, which allows for two site classes ($\omega = 1$, $0 < \omega < 1$); M2 (positive selection) with three site classes ($\omega = 1$, $0 < \omega < 1$ and $\omega > 1$); M3 (discrete), which has three discrete site classes with different ω values; M7 (β), which assumes a β distribution of class sites that does not allow for selection ($0 < \omega < 1$); and M8 (continuous), which is similar to M7 but has an additional class with $\omega > 1$. We calculated Bayesian posterior probabilities for positively selected sites using either naïve empirical Bayes (NEB) for model M3 or Bayes empirical Bayes (BEB) in case of models M2 and M8. We also conducted a likelihood ratio test (LRT) to compare the corresponding models with and without selection (i.e. M2 versus M1, M3 versus M0 and M8 versus M7). Finally, we performed tests to evaluate variation in selective pressures in each domain of the Mc1r protein defined as: extracellular (EL1–EL4), intracellular (IL1–IL4), and transmembrane (TM1–TM7), using all *Ctenomys* and other 35 rodent sequences. In addition, we performed branch-site models types A and D. These analyses allow the ω ratio to vary among branches in the phylogeny and are useful for detecting selection acting on particular lineages (Yang, 1998; Yang & Nielsen, 1998). All statistical significance was determined by comparing twice the log-likelihood scores (2ΔLnL) to a chi-squared distribution with degrees of freedom equal to the difference in the number of parameters between the models to be compared (Yang, 1997).

RESULTS

Mc1r variability and coat colour

In ctenomyids, the *Mc1r* gene consisted of a single exon of 948 bp (315 amino acids). The complete coding region and small 5' and 3' UTR fragments (approximately 150 bp on each side) were successfully aligned for all species but *Ctenomys leucodon*, for which only a partial sequence was obtained. High levels of *Mc1r* variability were observed among species, including 66 variable nucleotide sites and 20 amino acid changes (Fig. 2, Table 2). UTR segments were almost entirely conserved across species with only three substitutions of C/T: site 90 at 5' UTR and sites 1114 and 1119 at 3' UTR (Table 2). Two indels were identified in the *Ctenomys* coding region compared to other rodents, corresponding to a 6-bp deletion and a 3-bp insertion (Fig. 2). Intraspecific variability was not observed, except for *C. torquatus*, in which a T/C mutation was identified at nucleotide site 612 (Table 2). The existence of heterogeneity in evolutionary rates was apparent among both lineages and *Mc1r* domains (Fig. 2, Table 2). Fourteen replacements were located in transmembrane domains (mainly TM3 and TM4), four in the extracellular regions (EL1 and EL2), and only one in the intracellular loops (IL3) (Fig. 2). Most of the variation was identified in one species, *C. leucodon*, including 15 unique nonsynonymous substitutions (Fig. 2, Table 2). Assessment of nucleotide composition in *Ctenomys* sequences revealed high GC

Table 2. Variable sites ($N = 66$) in the *melanocortin-1 receptor* (*Mc1r*) nucleotide and amino acid sequences from 21 species of tuco-tucos

Variable sites

Nucleotides

Species	90	148	201	213	228	249	256	276	277	284	285	288	292	318	325	355	358	371	372	396	408	414	435	465	471	477	487	496	512	517	523	530	552	557	568	575	589	592	598	605	620	632	633
Ctenomys australis	C	C	A	G	T	C	T	A	T	C	G	C	G	A	G	G	A	C	C	T	C	T	T	T	G	C	C	A	C	G	T	C	C	C	C	C	T	T	T	C	G	T	G
Ctenomys boliviensis	.	.	T	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	C	.	.	.	T	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.
Ctenomys azarae	.	.	T	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Ctenomys dorbigny	.	.	T	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	C	.	.	.	T	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.
Ctenomys flamarioni	.	.	T	A	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Ctenomys haigi	.	.	T	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Ctenomys lami	.	.	T	A	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	C	.	.	.	T	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.
Ctenomys leucodon	.	.	T	.	.	T	C	G	C	.	C	T	A	G	A	A	T	T	T	C	T	C	C	C	A	A	.	G	T	A	G	.	G	G	T	G	G	C	C	A	A	A	T
Ctenomys maulinus	.	.	T	.	C	.	.	.	.	T	.	.	A	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Ctenomys mendocinus	.	.	T	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Ctenomys minutus	.	.	T	.	.	T	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	C	.	.	.	T	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.
Ctenomys nattereri	.	.	T	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Ctenomys pearsoni	T	.	T	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	C	.	.	.	T	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.
Ctenomys perrensi	.	.	T	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	C	.	.	.	T	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.
Ctenomys porteousi	.	.	T	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Ctenomys rionegrensis	.	.	T	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Ctenomys roigi	.	.	T	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	C	.	.	.	T	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.
Ctenomys sociabilis	.	.	T	.	C	.	.	.	.	T	.	.	A	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Ctenomys steinbachi	.	.	T	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	A	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Ctenomys talarum	.	T	T	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Ctenomys torquatus	.	.	T	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	C	.	.	.	T	.	.	.	.	T	.	.	.	.	.	.	.	.	.	Y	.

Columns in grey represent the 5' and 3' untranslated regions.

content: 59% in the 5' noncoding fragment, 61% in the 3' fragment, and 63% in the coding region. When the three different codon positions were analyzed separately, the GC content was 58% in the first, 40% in the second, and 83% in the third position. Finally, phylogenetic analysis using *Mc1r* nucleotide sequences recovered major groups of ctenomyids previously determined by published phylogenies (Parada *et al*., 2011) but with limited internal resolution of relationships (Fig. 3); only a few clades of the tree were resolved with high bootstrap support. Basal positions of *C. leucodon* and *Ctenomys sociabilis* identified in previous studies (e.g. Lessa & Cook, 1998; Parada *et al*., 2011) were also recovered. However, *Ctenomys haigi* also showed a basal position, not consistent with previous results (Castillo, Cortinas & Lessa, 2005; Parada *et al*., 2011).

There was no simple association between the 20 amino acid replacements and coat-colour phenotypes among species (Fig. 4). *Ctenomys australis* showed a unique, but conservative, amino acid change, Ile^{98}Val, although this was not present in *C. flamarioni*, the other member of this clade with similar coat colour. In addition, both melanic forms of *C. rionegrensis* and *C. torquatus* had *Mc1r* sequences identical to brown morphs of the same species (Fig. 4). *Ctenomys boliviensis*, *C. nattereri* and some members of the Mendocinus group showed the same replacements, including Try^{163}Arg and Iso^{177}Thr (Fig. 4). In addition, we identified a unique Thr^{95}Met mutation in *C. maulinus* and *C. sociabilis*. The most unexpected result was the 15 amino acid replacements observed only in *C. leucodon* (Fig. 2, Table 2). Finally, none of the 20 amino acid changes observed in *Ctenomys* overlapped with those previously shown to be associated with coat-colour variation in other species.

LEVELS OF *MC1R* EXPRESSION

Mc1r transcript levels were not significantly different between body regions when we compared pale and brown morphs: the dorsal (mean ± SE, unit $2^{\Delta CT} \times 10^{-5} = 6.21 \pm 0.15$ and 6.26 ± 0.28) and flank regions (4.47 ± 0.31 and 4.54 ± 0.17) showed similar expression levels (Fig. 5). However, comparison of the ventral regions indicated a significant increase in *Mc1r* transcript abundance ($P < 0.01$) in the melanic morph (3.92 ± 0.14) compared to pale (2.97 ± 0.43) and brown (2.97 ± 0.19) morphs. Moreover, when transcript abundance was compared within each phenotype but across body regions, there was a

Amino acids

634	660	663	690	691	692	696	698	717	768	780	783	786	804	816	825	837	846	848	858	876	1149	1149	959	985	1090	1191	1202	1243	1663	1667	1713	1735	1757	1774	1844	1866	1899	1912	2133	2333	2383
A	G	T	A	G	T	T	G	T	C	T	C	T	T	G	T	C	T	C	T	C	T	T	T	V	A	V	I	T	R	T	A	V	S	T	N	T	L	L	T	V	R	S
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G	C	C	C	A	C	C	A	C	.	C	G	G	C	C	C	T	C	T	C	.	C	.	.	I	T	I	F	I	.	A	V	I	A	.	K	S	V	M	A	T	Q	F
.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	C	.	M	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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significant reduction ($P < 0.01$) in Mc1r transcript in both the pale and brown forms (from high in dorsal to low in ventral) but not in the uniformly melanic form (Fig. 5).

Purifying selection and relaxation of functional constrains in *Mc1r*

Site-specific models identified variation in selection pressure among rodent lineages (Fig. 6, Table 3). Model M0 (one ratio) assumes the same ratio for all sites and fits the data more poorly than any of the other models, which is probably a result of variable ω ratios across sites. The M3 (discrete) model involves four more parameters than M0, and the LRT statistic $2\Delta\text{LnL} = -4508.53$ is greater than the critical value with d.f. = 4 (Table 3). M0 was rejected compared to M3 ($P < 0.01$), which is consistent with variation in selection pressure among sites. All the three models that allow for the presence of positive selection sites [i.e. M2a (positive selection), M3 (discrete), and M8 (β and ω)] failed to detect the presence of such sites in *Ctenomys* (Table 3). Only one site was identified as positively selected (199 T) in the *Chaetodipus intermedius* sequence, although the posterior probability (BEB and NEB) for this case was lower than 95%.

Allowing for the presence of positively selected sites (with $\omega > 1$) significantly improves the fit of some models. The comparison of models M1a and M2a can be considered as a test of the null hypothesis of neutral evolution versus the alternate hypothesis that some sites are also under negative selection ($\omega < 1$) and some are under positive selection ($\omega > 1$). The neutral model (M1a) does not allow sites with $\omega > 1$, whereas the positive selection model (M2a) adds an additional site class, with the ω ratio estimated to be 3.866. The log-likelihood improvement was low ($2\Delta\text{LnL} = 4547.98$) and hence not a better fit than M1a ($P > 0.01$). Comparison between M7 (β) and M8 (β and ω) produced similar results. Most of *Mc1r* domains showed variable ω ratio among sites (Table 4). We observed a signal of positive selection in transmembrane 4 (TM4) and extracellular loop 3 (EL3), where model M2 (selection) was a significantly better fit ($P < 0.01$) for the data than M1 (neutral). Comparison between M7 (β) and M8 (β and ω) in TM4 produced a similar result.

We next used the branch-site models A and D to detect possible heterogeneity of selective constraint between two rodent clades (Clade 1: ctenomyids, Clade 2: other rodents). Model A was not significantly better model than M1a ($P < 0.01$) in either clade, consistent with purifying selection. In addition, the

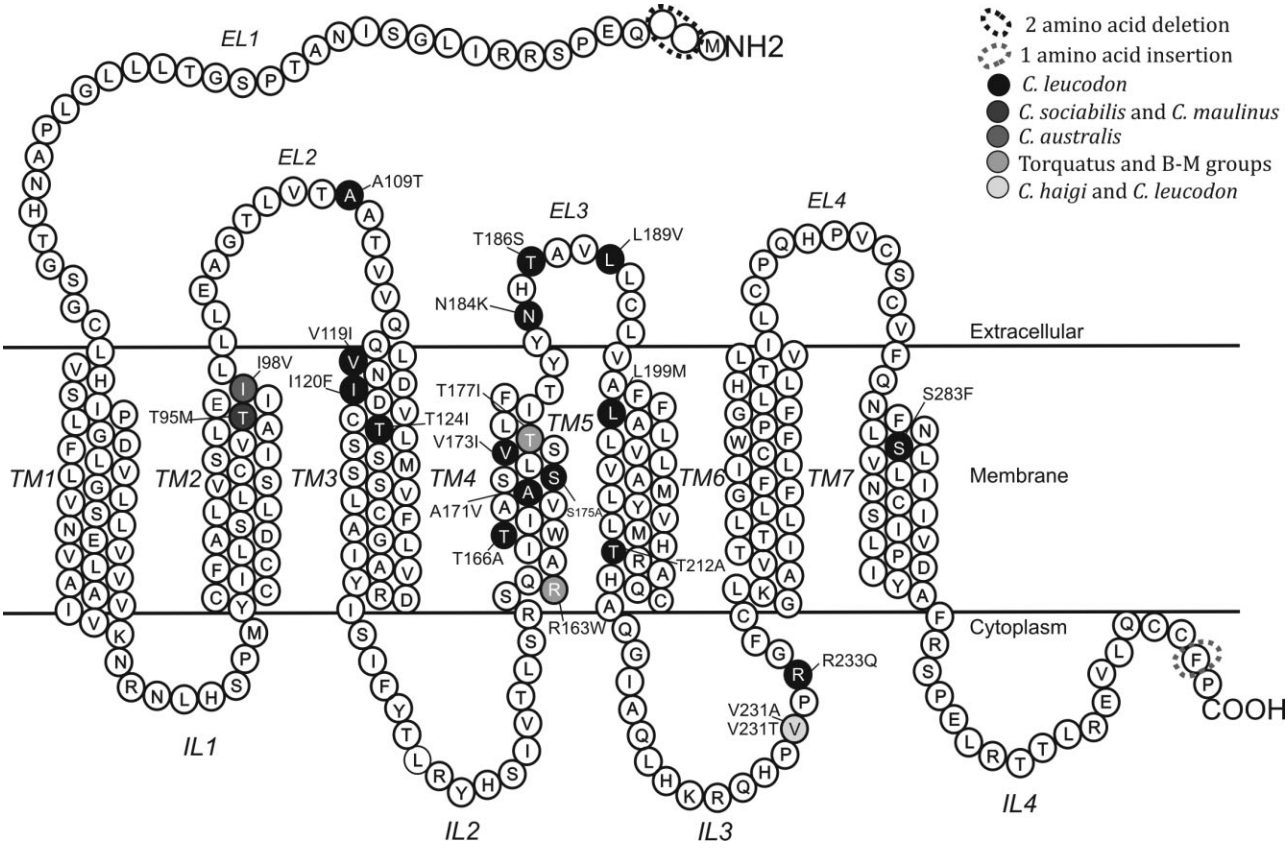

Figure 2. Two-dimensional structure of the deduced *Ctenomys* melanocortin-1 receptor (Mc1r) protein sequence. The 20 amino acid changes observed are represented by colors and site position. Domains: EL, extracellular regions; TM, transmembrane domains; IL, intracellular loops.

low estimated ω value (ω = 0.1956) and no significant difference between models A and A0 ($P > 0.01$) suggests that relaxed selection is a likely explanation for high ω values.

DISCUSSION

Coat-colour variation and *Mc1r*

Comparisons among both closely- and distantly-related taxa suggest *Mc1r* has not played a major role in producing the dramatic pigmentation differences observed across species of tuco-tucos. In several interspecific comparisons, taxa with differing coat colours had identical *Mc1r* sequences. In addition, a preliminary analysis indicated intraspecific variability is also not associated with coat-colour variation found by Gonçalves & Freitas (2009) for southern Brazilian populations of *C. torquatus*. Thus, in these cases, a role for mutations in the *Mc1r* coding region in pigment diversity clearly can be ruled out.

In other comparisons, however, conclusions are more difficult to make. For example, some species (e.g. *C. australis*) have unique amino acid mutations, although most are conservative changes (e.g. similar amino acid size, polarity and/or charge) and none overlap with those previously shown to affect coat colour. However, any conclusions linking those mutations to colour differences require additional data, such as functional assays to measure Mc1r signalling potential and/or ligand binding. Even these cell-based functional assays may be difficult to interpret because of possible *in vivo* epistatic interactions with other pigmentation loci (e.g. *Agouti*) (Mundy & Kelly, 2003) and Mc1r may have different effects on pigment-switching in different regions of the body.

In the present study, we also had focused on the convergent evolution of cryptic phenotypes that probably confer the same ecological function (Nevo, 1979). We first hypothesized a genetic convergence in *Mc1r* in *C. australis* and *C. flamarioni* because other studies had shown that similar blanched phenotypes can be produced by different mutations in the same gene (e.g. sand lizard species and beach mice: Rosenblum *et al.*, 2010; see also Hoekstra *et al.*, 2006). Unexpectedly, our data indicated that there was no convergence at mutational level in the *Mc1r* coding

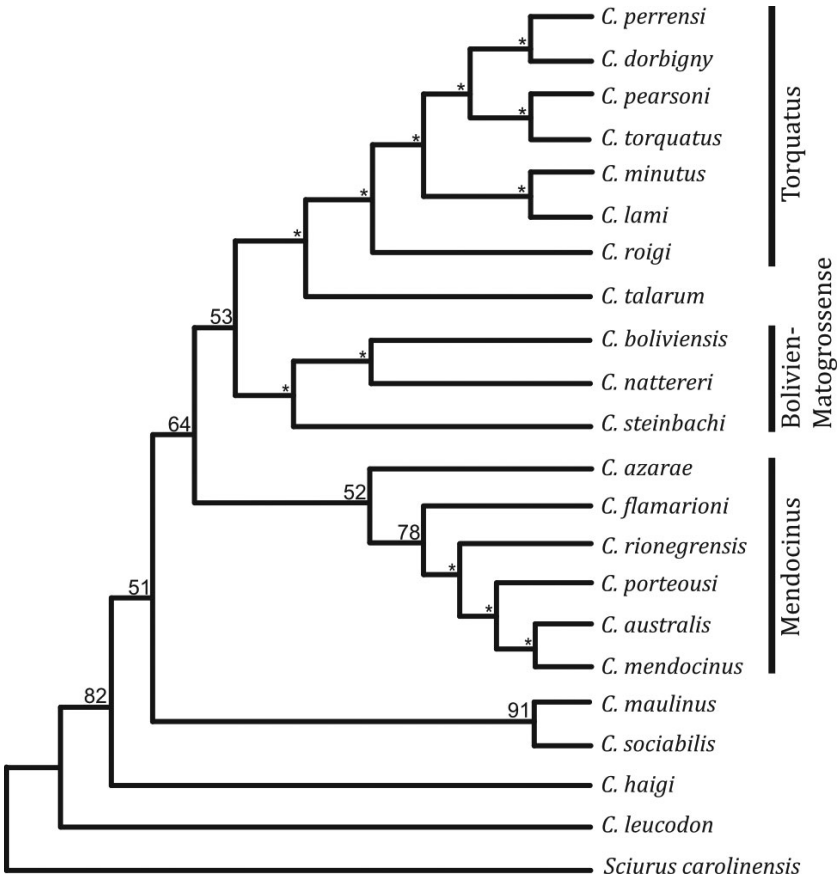

Figure 3. Maximum likelihood tree constructed for 21 species of *Ctenomys* based on 1250 bp of the *melanocortin-1 receptor* (*Mc1r*) gene using the HKY + G substitution model. Numbers above branches represent bootstrap support. Asterisks indicate values below 50%. Major phylogenetic groups recovered (Mendocinus, Bolivien-matogrossense, Torquatus) are consistent with those reported by Parada *et al*. (2011).

region associated with such cryptic pattern identified in these species. This result contrasts with previous work, which suggests that *Mc1r* is a 'hotspot' for evolutionary change, and hence an emerging model for understanding convergence at the mechanistic level (Manceau *et al*., 2010).

The majority of *Mc1r* variants previously described in mammals are either loss-of-function mutants associated with only yellow/red hair (e.g. mice, horses, black bears, dogs) or gain-of-function mutants with completely melanic hair (e.g. fox, jaguar and jaguarundi, grey squirrel, cattle, pig, sheep). Among the tuco-tuco species investigated in the present study, we hypothesized the pale *C. australis* may have a loss-of-function mutation(s), and melanic forms of *C. torquatus* and *C. rionegrensis* have a gain-of-function mutation(s), although in neither case are the unique mutations in these lineages associated with changes in coat colour. Importantly, however, such gross changes in *Mc1r* function caused by complete loss- or gain-of-function mutation do not easily allow for the simple control of the spatial distribution of hair colour as observed in some species of tuco-tucos (see below).

In addition to the present study, several studies have also demonstrated that *Mc1r* was not responsible for pigmentation phenotypes. For example, in pocket gophers (*Thomomys bottae*), there was no consistent association between replacement polymorphisms and coat colour, suggesting that *Mc1r* is not a major determinant of variation within this species (Wlasiuk & Nachman, 2007). In the same way, association between *Mc1r* coding region and differences in eumelanin and pheomelanin in some species of primate (Mundy & Kelly, 2003) and cetartiodactyls (Ayoub *et al*., 2009) and reptiles (Rosenblum, Hoekstra & Nachman, 2004) was ruled out. We also hypothesized an amino acid change and/or an in frame indel in *Mc1r* sequence of melanic forms of tuco-tucos (*C. torquatus* and *C. rionegrensis*). Yet, similar to other studies (Kingsley *et al*., 2009), we observed that *Mc1r* amino acid changes are not

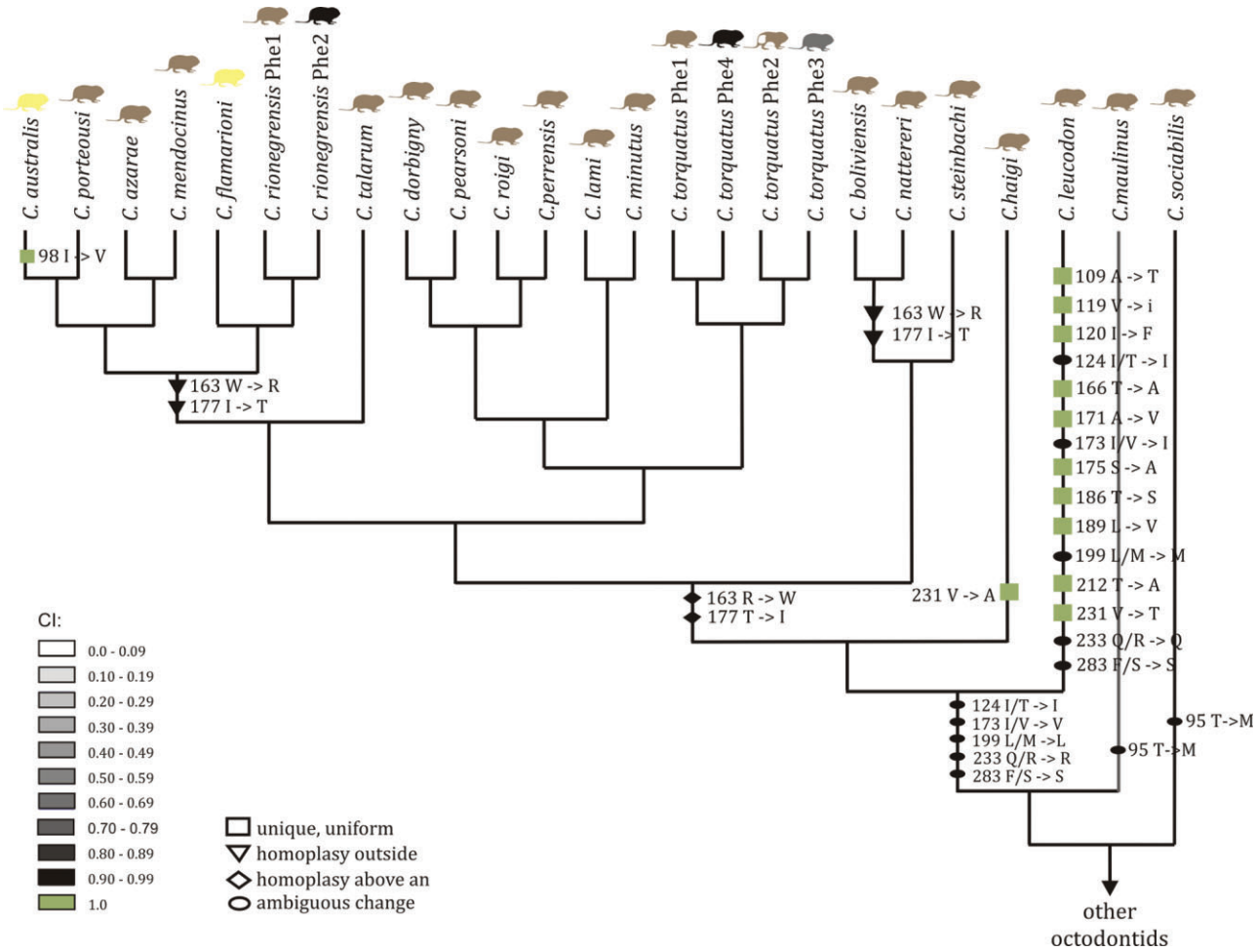

Figure 4. Evolution of the *melanocortin-1 receptor* (*Mc1r*) gene in 21 ctenomyid species. Amino acid substitutions were mapped on a phylogenetic tree constructed with the maximum likelihood method from cytochrome *b* gene data. Colours refer to the confidence index of each replacement. Representative coat colors for each species are depicted above taxa.

responsible for melanism in tuco-tucos. However, changes in the regulatory regions of *Mc1r* may be involved or, alternatively, other loci may contribute to this conspicuous pattern of coloration, such as the *Agouti* gene (Kingsley *et al*., 2009).

Thus, a central question remains: why is structural variation in *Mc1r* not involved in colour variation in tuco-tucos? One possible explanation is that in many of the species in which *Mc1r* has been implicated in colour variation differences are relatively discrete rather than continuous. For example, in black bears, *Mc1r* mutations are associated with a blonde or 'ghost' morph, and intermediates have not been observed. Similarly, of 200 pocket mice captured on and adjacent to lava flows in Arizona, all were easily categorized as light or dark, although some minor variation within the classes is also evident (Hoekstra, Drumm & Nachman, 2004). In such cases, *Mc1r* alleles appear to have a large effect, and therefore phenotypic variants segregate approximately as Mendelian traits. In other situations, however, *Mc1r* mutations explain a smaller amount of phenotypic variation, and the observed colour variation is more quantitative. For example, in beach mice, *Mc1r* mutations account for 10–36% (depending on trait) of the variation in pigmentation (Hoekstra *et al*., 2006). Studies on coat colour in tuco-tucos suggest that they can be easily classified into discrete groups in most cases; however, there is more colour variation than can be easily explained by a single locus, perhaps as a result of more subtle differences in environmental variation (Freitas & Lessa, 1984; Reig *et al*., 1990; Wlasiuk *et al*., 2003; Gonçalves & Freitas, 2009). Thus, *Mc1r* mutations of large effect may not be tolerated in environments where selection favours incremental differences.

In addition to testing for associations with *Mc1r* structure, we also tested for an association between

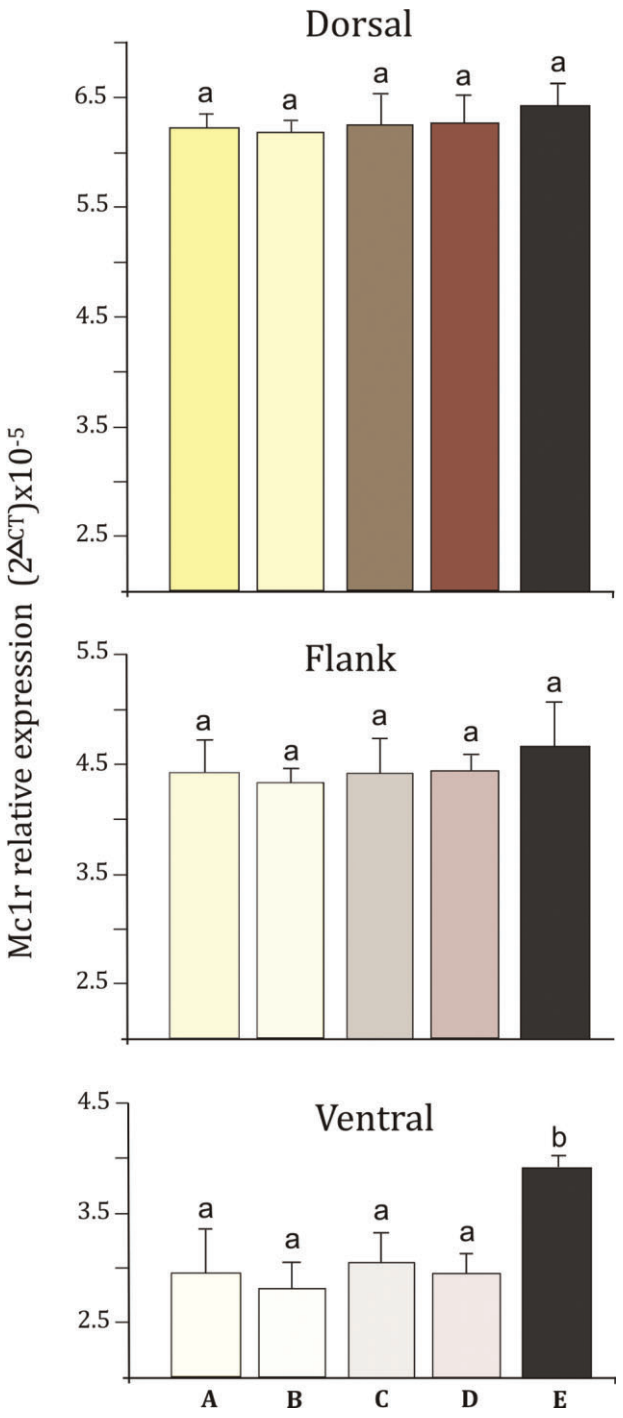

Figure 5. Gene expression of *melanocortin-1 receptor* (*Mc1r*) in three classes of phenotypes in *Ctenomys*: pale (A, *Ctenomys australis*/B, *Ctenomys flamarioni*), dark (C, *Ctenomys minutus*/D, *Ctenomys torquatus*) and melanic (E, *C. torquatus*). Relative expression of *Mc1r* transcripts in dorsal, flank, and ventral skin was measured by quantitative polymerase chain reaction and standardized with β-actin. Distinct lowercase letters above the bars indicate statistical significance among species, within a given body region.

Mc1r expression level and coat-colour phenotype. First, we did not find significant variation in *Mc1r* expression across different colour phenotypes. Although a few studies have investigated *Mc1r* expression level in mammalian populations (Steiner, Weber & Hoekstra, 2007; Kingsley *et al*., 2009), they report similar results: none have found a difference in transcript abundance between pale and dark morphs. We did, however, find a marked difference among the dorsal, flank, and ventral regions within both pale and brown morphs. The coat colour along the body of tuco-tucos typically has a continuous pattern: dorsal is darker, flank is paler, and ventral is almost white (in all phenotypes, except the melanic form). Thus, a reduction in the abundance of transcripts from dorsal to ventral may be explained by (or associated with) a gradient of *Mc1r* expression. This result is also consistent with the finding of the present study that, in the melanic form of *C. torquatus* (including a melanic ventrum), we found no significant difference in *Mc1r* expression among body regions. However, such association does not necessarily mean that *Mc1r* itself was modified to produce the genetic change. Changes in a gene expression pattern may also be the result of changes in upstream regulators of *Mc1r* (Protas & Patel, 2008) or explained by differences in the spatial distribution of melanocytes. Thus, further studies are required to determine what role, if any, *Mc1r* plays in producing variation across body regions.

A longstanding question in evolutionary biology is whether genes identified originally from laboratory mutations (such as *Mc1r*), most of which are of large effect, will also contribute to adaptive evolution in nature (Palopoli & Patel, 1996). The results reported in the presented study suggest that simple *Mc1r* mutations of large effect have not contributed to adaptive differences among species of tuco-tucos. However, the variation in coat colour among tuco-tucos suggests that this trait might have a more complex or even polygenic basis. Finding the genes underlying this variation will probably be a more daunting task, requiring mapping and association studies involving more markers and defined populations.

Evolution of the *Mc1r* coding region

The *Mc1r* coding region exhibited high GC content at third-codon positions, which is consistent with codon-usage bias. Similar findings have been reported for other genomic regions of high GC content, and interpreted to derive either from biased mutation pressure or from selection favouring a high GC proportion or particular codons (Li, 1997). The pattern observed in *Mc1r* in *Ctenomys* is difficult to reconcile with a strictly mutationalist scenario because the GC content at third codon position is approximately 40%

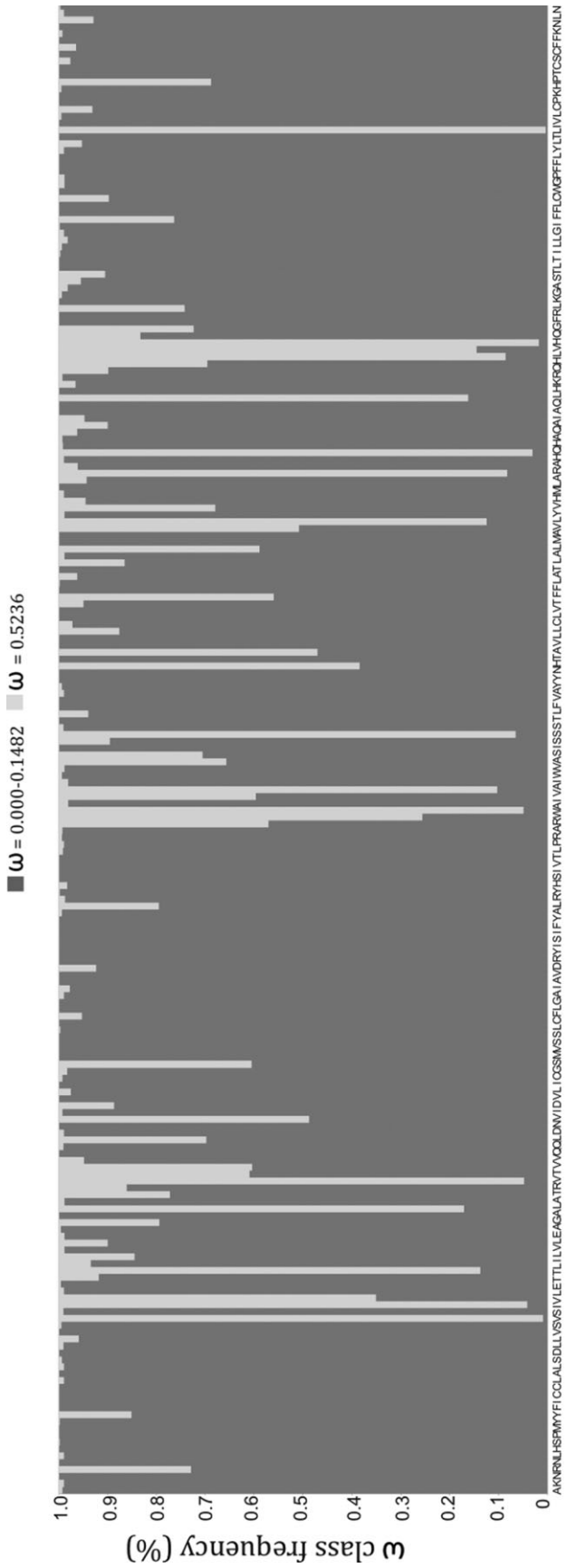

Figure 6. Analysis of the naïve empirical Bayes (NEB) posterior probability under the model M3, from each codon of the *melanocortin-1 receptor* (*Mc1r*) in ctenomyids and other rodent families.

Table 3. Estimated parameters on diverse models of codon substitution considering the the *melanocortin-1 receptor* (*Mc1r*) gene in ctenomyid and other rodent families

Model	*dN/dS**	Estimates of parameters†	*l*	*P*	Positively selected sites‡
M0: one-ratio	0.1170	$\omega = 0.1170$	–4592.69	< 0.01	None
M3: discrete	0.1346	$p_0 = 0.4385$, $p_1 = 0.4246$ ($p_2 = 0.1368$) $\omega_0 = 0$, $\omega_1 = 0.1482$, $\omega_2 = 0.5236$	–4508.53		None
M1a: neutral	0.1956	$p_0 = 0.8730$ ($p_1 = 0.1269$) ($\omega_0 = 0.0786$) ($\omega_1 = 1$)	–4547.98	> 0.01	Not allowed
M2a: selection	0.1956	$p_0 = 0.8730$, $p_1 = 0.1269$ ($p_2 = 0.0000$) ($\omega_0 = 0.078$) ($\omega_1 = 0$), $\omega_2 = 3.8663$	–4547.98		None
M7: β	0.1350	$p = 0.3477$, $q = 2.1587$	–4510.89	> 0.01	Not allowed
M8: β and ω	0.1361	$p_0 = 0.9974$ ($p_1 = 0.00257$) $p = 0.3564$, $q = 0.2785$, $\omega = 1.9134$	–4510.26		199T

ω, dN/dS ratio; *p*, proportion of codons in each ω class; M0–M3: chi-squared, d.f. = 4; M1a–M2a: chi-squared, d.f. = 2; M7–M8: chi-squared, d.f. = 2.
**dN/dS* ratio is the average across all codons.
†Parentheses indicate fixed parameters.
‡Posterior probability of the positively selected site is < 95%.

Table 4. Likelihood ratio test between models of codon substitution without selection (M0, M1, and M7) and with selection or relaxation of functional constrains (M3, M2, and M8) for each of the 15 domains of the melanocortin-1 receptor (Mc1r) protein in 30 rodent species

	Model of substitution comparison		
Domain	M0 versus M3	M1 versus M2	M7 versus M8
EL1	M3**	M1	M7
EL2	M3**	M1	M7
EL3	M3**	M2*	M7
EL4	M3**	M1	M7
TM1	M3**	M1	M7
TM2	M3**	M1	M7
TM3	M3**	M1	M7
TM4	M3**	M2**	M8**
TM5	M3**	M1	M7
TM6	M3**	M1	M7
TM7	M0	M1	M7
IL1	M3**	M1	M7
IL2	M3**	M1	M7
IL3	M3**	M1	M7
IL4	M3**	M1	M7

Asterisks indicate the level of significance: $*P < 0.01$, $**P < 0.001$; M0 versus M3: chi-squared, d.f. = 4; M1a versus M2a: chi-squared, d.f. = 2; M7 versus M8: chi-squared, d.f. = 2.

higher than that observed in surrounding small non-coding genomic areas sequenced.

The high number of variable sites and the low rate of homoplasy resulted in a well-resolved phylogeny reconstructed for the 21 species of tuco-tucos using *Mc1r* sequences, recovering major groups previously identified, specifically Mendocinus, Torquatus, and Bolivien-matogrossense (Cook & Lessa, 1998; Lessa & Cook, 1998; D'Elia, Lessa & Cook, 1999; Slamovits *et al*., 2001; Castillo *et al*., 2005; Parada *et al*., 2011). Although some internal relationships and conflicted position of species such as *C. haigi* and *C. leucodon* were not resolved, the results of the present study indicated that this nuclear marker has potential to be useful in phylogenetic studies.

Selection on *Mc1r*

Our analysis of *Ctenomys* suggests that purifying selection has been the predominant mode of selection acting in *Mc1r* evolution, although there is evidence for relaxation in functional constrain at some sites/regions. The high divergence observed in *C. leucodon* (15 amino acid replacements) could indicate a potential acceleration in the rate of substitution along this lineage, although there is no conspicuous difference in its coat colour (which is a typical brown). Unexpectedly, the strength of selection has been constant through evolutionary time in *Ctenomys*. There was, however, an increase in the rate of *dN*/*dS* in *C. leucodon* in relation to all other species of tuco-tucos (Gonçalves, 2011). We considered that a phylogenetic signal effect might be involved in the higher ratio of

dN/*dS* because the position of *C. leucodon* in Ctenomyidae is not well resolved and such species have even been considered as a member of a different genus (*Haptomys*; Osgood, 1946), although never recognized as such.

When we examined domain-specific rates of *Mc1r* evolution, we found a pattern of variation in ω among sites. We first considered possible substitution-rate variation, specifically in transmembrane domains, because more replacements were observed in such regions than other domains in *Ctenomys*. Our findings indicated that the fourth transmembrane domain (TM4) and the third extracellular loop (EL3) have a more variable rate of ω (i.e. potential selection acting on some sites could be involved), especially in TM4 compared to other domains. Alternatively, such variation might be a result of the relaxation of function constraints in these regions. We did not find evidence of positive selection on the *Mc1r*, using an analysis in which *dN*/*dS* ratios varied either among lineages or among sites. This finding is consistent with several other studies on mammals that report no evidence of global selection in *Mc1r* (e.g. cetartiodactyls: Ayoub *et al*., 2009; primates: Mundy & Kelly, 2003; mustelids: Hosoda *et al*., 2005).

As discussed above, the power to detect positive selection on *Mc1r* as a whole is probably low because there are many examples in which a single amino acid substitution in *Mc1r* leads to a large change in coat colour. These selection tests require many repeated substitutions among lineages. Alternatively, directional selection on *Ctenomys* coat colour may have occurred in relatively short bursts followed by long periods of purifying selection, leading to a low signal in the gene(s) involved. However, the lack of positive selection acting on *Mc1r* is also consistent with the lack of evidence for *Mc1r* contributing to adaptive coloration in tuco-tucos.

CONCLUSIONS

In conclusion, the present study serves as an evaluation of the potential genetic underpinnings responsible for the tremendous coat-colour diversity observed among tuco-tucos. Although *Mc1r* is an excellent candidate locus, given its role in generating colour diversity among a wide range of vertebrates, we found no simple associations between *Mc1r* amino acid variation and overall coat-colour phenotype. This is consistent with our lack of evidence for positive selection acting on *Mc1r* in ctenyomids. Future studies will focus on a more careful analysis of additional candidate genes, such as the *Agouti* locus, amongst others, and test for associations with respect to both protein structure and gene expression.

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