

The Genetics of Adaptive Coat Color in Gophers: Coding Variation at *Mclr* Is Not Responsible for Dorsal Color Differences

GABRIELA WLASIUK AND MICHAEL W. NACHMAN

From the Department of Ecology and Evolutionary Biology, Biosciences West Building, The University of Arizona, Tucson, AZ 85721 (Wlasiuk and Nachman).

Address correspondence to G. Wlasiuk at the address above, or e-mail: wlasiuk@email.arizona.edu.

Abstract

The genetics of adaptation is a key problem in evolutionary biology. Pocket gophers of the species *Thomomys bottae* provide one of the most striking examples of coat color variation in mammals. Dorsal pelage color is strongly correlated with soil color across the range of the species, presumably reflecting the selective pressure exerted by predation. To investigate the genetic basis of coat color variation in *T. bottae*, we cloned and sequenced the *melanocortin-1 receptor* locus (*Mclr*), a candidate pigmentation gene, in 5 dark and 5 light populations of the species. Our results show that, in contrast to many other species of mammals and other vertebrates, coding variation at *Mclr* is not the main determinant of coat color variation in *T. bottae*. These results demonstrate that similar phenotypic variation may have a different genetic basis among different mammalian species.

A basic goal in evolutionary genetics is to find the genes underlying adaptive traits. Finding the genes underlying adaptations might allow us to answer a number of questions. For example, do adaptive phenotypes derive mainly from changes in gene structure or gene regulation? Does adaptation usually involve one or a few mutations of major effect or many mutations of small effect? What kinds of genes are involved? Are similar phenotypes produced by similar genetic changes? Finding genes involved in adaptation has been difficult and many of the best examples come from response to human disturbance (e.g., antibacterial drug resistance: Walsh 2000; insecticide resistance: Daborn et al. 2002). One promising approach is to choose ecologically important phenotypes for which a clear set of candidate genes exists (Palopoli and Patel 1996).

Thomomys bottae is a species of pocket gopher in the southwestern United States and northwestern Mexico that exhibits a high degree of geographic variation, both morphologically and genetically (Patton and Smith 1990). In particular, these gophers exhibit dramatic variation in the color of the dorsal pelage, and this color variation is strongly correlated with soil color across the range of the species (e.g., Ingles 1950; Patton and Smith 1990), presumably due to selection from predation. Avian predators, including owls,

are known to prey upon *Thomomys* (Fassler and Leavitt 1975; Janes and Barss 1985; Cutler and Hays 1991; Young et al. 1997), and several species of owls discriminate between light and dark mice on light and dark backgrounds in experimental tests, even under very low light intensities (Dice 1947).

A large amount of information exists on the genetic, developmental, and biochemical details underlying pigmentation (reviewed in Bennett and Lamoreux 2003), making this an excellent phenotype to explore using a candidate gene approach. In mammals, pigment is produced in specialized cells known as melanocytes, and there are 2 basic kinds of pigment: eumelanin (dark brown or black) and pheomelanin (light cream, yellow, or red). A key switch between the production of these 2 kinds of pigment involves the interaction of 3 proteins: the agouti signalling protein (ASP), alpha melanocyte stimulating hormone (α -MSH), and the melanocortin-1 receptor (MC1R). When α -MSH binds to MC1R, the latter is activated and eumelanin is produced. ASP, on the other hand, is an antagonist of MC1R and when expressed pheomelanin is produced. In a surprisingly large number of vertebrate species, mutations in *Mclr* have been shown to cause changes from light to dark color over much of the body (e.g., cattle: Klungland et al. 1995; mouse: Barsh 1996; horse: Marklund et al. 1996;

chicken: Takeuchi et al. 1996; fox: Vage et al. 1997; pig: Kijas et al. 1998; sheep: Vage et al. 1999; dog: Newton et al. 2000; black bear: Ritland et al. 2001; bananaquit: Theron et al. 2001; jaguar and jaguarundi: Eizirik et al. 2003; pocket mouse: Nachman et al. 2003; and lesser snow geese and arctic skuas: Mundy et al. 2004). The common finding of a role for *Mc1r* in color variation is probably due in part to publication bias. *Mc1r* is a single-exon gene that is easily studied and has been investigated far more than other pigmentation genes. There are some cases, however, in which *Mc1r* does not seem to be involved in color differences (pocket mice: Hoekstra and Nachman 2003; old-world leaf warblers: MacDougall-Shackleton et al. 2003; and some primates: Mundy and Kelly 2003), although at least in the case of warblers the phenotypic differences investigated were the presence or absence of unmelanized pattern elements rather than overall body coloration. In spite of these negative results, the widespread role of *Mc1r* in coat color evolution in many species, and the fact that some of the phenotypic changes described in these species resemble *T. bottae* color phenotypes, make *Mc1r* a reasonable candidate gene for the variation in pelage color in *T. bottae*. We note that other genes, particularly *Agouti*, may also be good candidates.

Here, we have cloned and sequenced the entire *Mc1r* gene in pocket gophers from 10 populations, representing 5 paired light and dark localities across the range of the species. We found no association between *Mc1r* variation and coat color, in contrast to many other vertebrates, suggesting that similar phenotypes may evolve through changes at different genes in different species.

Materials and Methods

Sampling

Fifty-two individuals were studied (Appendix 1). Specimens were obtained from the Museum of Vertebrate Zoology at the University of California Berkeley and the Mammal Collection of the Department of Ecology and Evolutionary Biology at the University of Arizona, Tucson. The sampling was designed to simultaneously maximize dorsal color differences and minimize genetic distances between populations for each comparison. Five pairs of populations were chosen (Figure 1). Four of these pairs belong to different major intraspecific genetic units, defined previously by Patton and Smith (1990) based on allozyme allele frequencies. The fifth comparison (*T. bottae. connectens* and *T. b. ruidosidae*) involves populations from different genetic units (Patton and Smith 1990) that are very similar with respect to their mtDNA (Smith 1998). The maximum geographic distance between populations within pairs (*T. b. awanbee* and *T. b. perpallidus*) is 517 km. The rest of the populations (within pairs) are 85–424 km apart.

Isolation of *Mc1r* in Gophers

Genomic DNA was extracted from tissue samples preserved in ethanol using Qiagen (Valencia, CA) extraction

kits. The entire *Mc1r* gene consists of a single exon and was isolated in gophers by polymerase chain reaction (PCR) amplifying and cloning a conserved central region and then using genome walking to capture the 5' and 3' ends. First, primers GWMc1rF: 5'-CTCTTYCTCDGCTYGGGGCT-3' and GWMc1rR: 5'-ACCABRAGMAYDKYAGCACCT-3' were designed in conserved regions of *Mc1r* across mammals to PCR amplify and sequence the middle portion of the coding sequence in *T. bottae*. This sequence was then used to design 2 pairs of species-specific nested primers (MC1RTB1R: 5'-AGTAGTACATGGGCGAGTGCAGGTTT-3', MC1RTB2R: 5'-AATCACTACCAGCACATTCTCCACCA-3', MC1RTB1F: 5'-CTCCTGGGCATTTTCTTCTTATGCTG-3', MC1RTB2F: 5'-TAACTCCATTGTTGACCCCTCATCT-3') that were used to capture the 3' and 5' ends of *Mc1r* by genome walking (Universal Genome Walking Kit; Clontech, Palo Alto, CA). A third set of primers (described below) flanking the coding region were then developed from the sequences in the previous step to amplify a fragment of 1123 bp.

DNA Sequencing

The entire coding sequence of *Mc1r* was PCR amplified using the following primers: Mc1r3F: 5'-TGACACCATGAAATGAGCAG-3' and Mc1r8R: 5'-CATAGGGATCAGGACACTGG-3', under the following conditions: 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 65 °C for 1 min, preceded by a denaturing step of 2 min at 94 °C, and followed by an extension step of 8 min at 65 °C. The diploid PCR products were sequenced with the same primers. Potential heterozygous sites were identified by visual inspection of the chromatograms and in most cases confirmed by sequencing the complementary strand.

Sequence data for the mitochondrial *cytochrome b* (*Cyt b*) gene were obtained for the 10 specimens of *T. b. awanbee* and *T. b. perpallidus* because in that comparison a statistically significant association was found between a replacement polymorphism in *Mc1r* and coat color. Primers MVZ69 and MVZ14 (Smith 1998) were used to PCR amplify the entire coding region (1140 bp), according to the conditions described by Smith and Patton (1991). MVZ69 and TBcytb14b (5'-GGTCTTCATCTYHGGYTTAC-3') were used as sequencing primers.

In all cases, PCR products were sequenced on an ABI377 or ABI3700 automated sequencer (Applied Biosystems, Foster City, CA). Sequences were edited and aligned using SEQUENCHER (Gene Codes, Ann Harbor, MI). Sequences were deposited in GenBank under the following accession numbers: *Mc1r* (EF488832–488875) and *Cyt b* (EF488876–488885).

Spectrophotometric Measurements

Reflectance spectrophotometry was used to characterize dorsal coloration. Reflectance, calibrated to a white standard, was measured over the UV (200–400 nm) and visible (400–850 nm) spectra with a USB2000 spectrophotometer

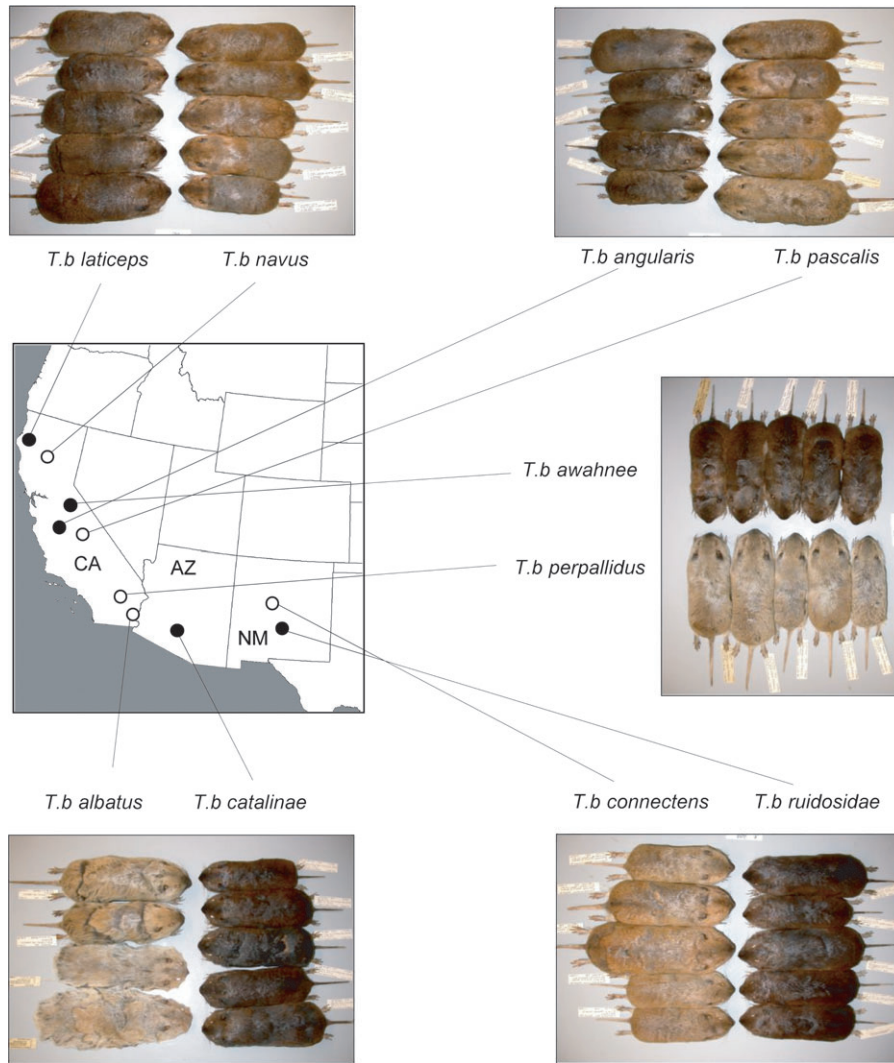


Figure 1. Map of collecting localities in California, Arizona, and New Mexico and phenotypic differences between populations for each of the 5 comparisons. Black and white circles represent dark and light coat colors, respectively.

(Ocean Optics) coupled to a MiniDT1000 light source (Analytical Instrument Systems, Inc.). Five measurements were taken per individual on the dorsum and then combined into an average individual spectrum, as in Hoekstra et al. (2005). Total reflectance intensity (brightness) was obtained summing the total area (UV + visible) under the average reflectance curve.

Data Analysis

Associations between *Mc1r* genotypes at each polymorphic site and phenotype (dark versus light) were tested in 3 × 2 contingency tables using Monte Carlo simulations. The 3 rows thus correspond to the 3 possible genotypes, and the 2 columns correspond to light versus dark animals.

To assess population structure, the statistic F_{ST} (Hudson et al. 1992) was calculated from the mitochondrial sequences using the software DNAsp (Rozas et al. 2003). This was

done with the pair of populations showing strong association between variation at *Mc1r* and coat color.

Results and Discussion

The amino acid alignment of a consensus sequence of *Mc1r* and several other "wild-type" *Mc1r* mammalian sequences is shown in Appendix 2. The consensus length of *Mc1r* in vertebrates is 954 bp. In *T. bottae*, we observed a deletion of 21 bp (in frame) in the first extracellular region, resulting in a gene length of 933 bp (including the stop codon). The first extracellular region constitutes the most variable portion of the protein among vertebrates, probably indicating low functional constraints.

The distribution of nucleotide variation within and among different pairs of populations is presented in Figure 2. We observed 18 single-nucleotide polymorphisms, of which

		Pol. Site #	33	138	235	288	304	426	438	464	498	499	501	591	597	622	664	702	735	744	
		Pol. Site # consensus	54	159	256	309	325	447	459	485	519	520	522	612	618	643	685	723	756	765	
Population	Specimen		C	G	C	A	C	A	T	G	C	T	G	A	A	C	C	C	A	C	
albatu (light)	MVZ15 4176	
	MVZ15 4177	
	MVZ15 4178	
	MVZ15 6116	
	MVZ15 6117	
cata linae (dark)	UA25203	
	MVZ14 6822		C
	MVZ14 6824		C/T	A/C
	MVZ14 6825		A/C
	MVZ14 6826		C
connectens (light)	MVZ15 0251		A
	MVZ15 0260		C/T	A
	MVZ15 0262		A
	MVZ15 0263		A
	MVZ14 7023		C/T
ruidosidae (dark)	MVZ14 7026		C/T	C/T
	MVZ14 7027		C/T
	MVZ14 7055		T
	MVZ14 7056	
navus (light)	MVZ16 2073		.	A/G
	MVZ16 2075	
	MVZ16 3174		G	.
	MVZ16 3194	
laticeps (dark)	MVZ16 0608		A/C
	MVZ16 0614	
	MVZ16 0618	
	MVZ16 0674	
perpallidus (light)	MVZ16 6252		C/T	C/T	.	.	.	G/T	.	.	C/T
	MVZ16 6253		T	C	.	.	.	T	.	.	T
	MVZ16 6256		T	C	.	.	.	T	.	.	T
	MVZ16 6257		C/T	C/T	.	.	.	G/T	.	.	C/T
	UA26596		T	C	.	.	.	T	.	.	T
awabnee (dark)	MVZ15 8712		T
	MVZ15 8713		T
	MVZ15 8718		T
	MVZ15 8722		T
	MVZ15 8726		T
pascalis (light)	MVZ15 6161		.	.	.	C	.	A/G	.	A/G	A/G	.	C/T
	MVZ15 6179		.	.	.	C
	MVZ15 6180		.	.	.	C	.	A/G	.	A/G	G
angularis (dark)	MVZ15 6221		T	C	.	.	.	C	T	T	.
	MVZ16 2159		.	.	A	C	A/G
	MVZ16 4595		T	C	.	.	.	C	T	T	.
	MVZ16 4601		T	C	.	.	.	C	T	T	.
Codon position			3	3	1	3	1	3	3	2	3	1	3	3	3	1	1	3	3	3	3
Amino acid					Leu Ile		Arg Trp			Arg Gln			Leu Phe*			Arg Cys	His Tyr				
Amino acid position			11	46	79	96	102	142	146	155	166	167	167	197	199	208	222	234	245	248	

* Note: both 1st and 3rd codon positions of codon 167 are variable and result in the same amino acid change.

Figure 2. Nucleotide polymorphism at *Mc1r*. Nucleotide positions are indicated above each column. Dots represent identity with respect to the first sequence. For heterozygous sites, the genotype is indicated. Codon positions, amino acid positions, and nonsynonymous changes are indicated at the bottom of the corresponding column.

6 resulted in nonsynonymous changes. The overall level of nucleotide variability ($\pi = 0.25\%$) was slightly higher than seen in other mammals at *Mc1r* (e.g., humans, $\pi = 0.1\%$; Harding et al. 2000; pocket mice, $\pi = 0.21\%$; Nachman et al. 2003).

Despite this variability, there was no consistent association between replacement polymorphisms (Figure 2) and coat color (Figure 1 and Table 1), suggesting that *Mc1r* is not a major determinant of coat color variation in this species.

In one pair of populations (*T.b. perpallidus*-*T.b. awabnee*), however, we did observe an association between Arg208Cys and dorsal color (Monte Carlo simulation $P < 0.004$). In spite of this significant association between Arg208Cys and dorsal color among these 10 animals, several observations suggest that this mutation is not causing the observed color differences. First, the amino acid associated with the light phenotype in *T.b. perpallidus* (e.g., specimen MVZ166253) is associated with the dark phenotype in another population

(*T.b. ruidosidae*; e.g., specimen MVZ147056). Second, there are no obvious color differences between Arg/Cys and Cys/Cys among *T.b. perpallidus*. Individuals of both genotypes exhibit similar total reflectance intensities and reflectance peaks at the same wavelengths in the UV and visible spectra. This pattern would, in principle, be compatible with a dominant light mutation. However, all known dominance relationships among *Mc1r* alleles in other species show the reverse: dark alleles are dominant over light ones (e.g., Klungland et al. 1995; Vage et al. 1997, 1999; Kijas et al. 1998; Newton et al. 2000; Nachman et al. 2003). Third, *T.b. perpallidus* and *T.b. awabnee* represent the most distant comparison in our sample, in terms of both genetic and geographic distances. A spurious correlation between genotype and phenotype can be generated due to population structure. To test for population structure, we used mitochondrial *cyt b* sequences to calculate F_{ST} . The observed F_{ST} (0.988) confirms an extreme level of genetic differentiation

Table 1. Total reflectance from dorsum of gophers from 5 light and 5 dark populations

Population	Sample size	Phenotype	Total intensity (range)
Albatus	4	Light	10806.7 (9689.2–12739.6)
Catalinae	5	Dark	4689.8 (776.8–7230.4)
Connectens	5	Light	6571.8 (4232.7–10788.6)
Ruidosidae	5	Dark	3864.5 (1313.6–7009.9)
Navus	5	Light	4581.7 (1861.8–10218.3)
Laticeps	4	Dark	2438.9 (1311.1–3651.3)
Perpallidus	5	Light	8752.6 (6918.2–10756.8)
Awahnee	5	Dark	2211.4 (1008.9–4505.6)
Pascalis	5	Light	4052.8 (3121.2–5432.6)
Angularis	5	Dark	4283.6 (1805.4–8589.3)

between these populations. Therefore, the observed differences at *Mc1r* might simply reflect population divergence. Fourth, the Arg208Cys change lies at a site that is variable across mammals (Appendix 2) and is not associated with coat color polymorphisms in other species (Mundy 2005), suggesting that it may not be functionally important. Together these observations suggest that population structure may account for the association between the Arg208Cys genotype and color phenotype.

Thus, coding sequence variation in *Mc1r* does not appear to be the principal determinant of coat color differences in *T. bottae*. Our reduced sample sizes, however, only allow the identification of strong associations between phenotypic and genotypic variation. The possibility that weak associations might exist remains open. Similarly, despite the fact that so far associations between regulatory variation at *Mc1r* and coat color differences have not been described in other species, a role for variation in the regulatory region of *Mc1r* cannot be ruled out with this data.

Although there are a few documented cases in which *Mc1r* is not implicated in pigmentation differences (e.g., some dog breeds: Kerns et al. 2003; mustelids: Hosoda et al. 2005; and some populations of pocket mice: Hoekstra and Nachman 2003), for a wide range of taxa, *Mc1r* has been shown to be responsible for coat color differences both in domestic and wild species.

Why is *Mc1r* not involved in color variation in gophers? In many of the species in which *Mc1r* has been implicated in color variation, the color differences are relatively discrete rather than continuous. For example, in black bears, *Mc1r* mutations are associated with a light race, and intermediates have not been observed. Similarly, of 200 pocket mice captured on and adjacent to a lava flow in Arizona, all were easily categorized as light or dark, although some minor variation within the classes is also evident (Hoekstra et al. 2004). In cases such as these, *Mc1r* alleles seem to have large effects, and phenotypic variants presumably segregate roughly as Mendelian traits. In several situations, however, *Mc1r* mutations explain a smaller amount of phenotypic variation, and color variation is more quantitative. For example, in beach mice, *Mc1r* mutations account for

10–36% of the variation in pigmentation phenotypes (Hoekstra et al. 2006). Pocket gophers show nearly continuous variation from very dark to very light (Patton and Smith 1990). *Mc1r* mutations of large effect may not be tolerated in environments where selection is favoring incremental differences. A long-standing 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 (e.g., Palopoli and Patel 1996). The results presented here suggest that *Mc1r* mutations of large effect have not contributed to adaptive differences among gopher populations. However, the continuous variation in coat color in pocket gophers suggests that this trait might have a polygenic basis. Finding the genes underlying this variation will likely be a more daunting task, requiring mapping and association studies involving many more markers and individuals.

Appendix 1

Individuals are listed by population. Collection number is followed by sex: m, male; f, female.

T.b. albatus: MVZ 154176 (m), MVZ 154177 (f), MVZ 154178 (m), MVZ 156116 (f), MVZ 156117 (f), UA25203.

T.b. angularis: MVZ 156221 (f), MVZ 162159 (m), MVZ 162162 (f), MVZ 164595 (m), MVZ 164601 (f).

T.b. awahnee: MVZ 158712 (f), MVZ 158713 (f), MVZ 158718 (f), MVZ 158722 (f), MVZ 158726 (f).

T.b. catalinae: MVZ 146822 (m), MVZ 146823 (f), MVZ 146824 (m), MVZ 146825 (m), MVZ 146826 (f).

T.b. connectens: MVZ 150251 (m), MVZ 150260 (f), MVZ 150261 (m), MVZ 150262 (m), MVZ 150263 (m).

T.b. laticeps: MVZ 160608 (f), MVZ 160614 (f), MVZ 160618 (m), MVZ 160674 (m), MVZ 160682 (f).

T.b. navus: MVZ 162073 (m), MVZ 162075 (f), MVZ 163174 (f), MVZ 163176 (m), MVZ 163194 (f).

T.b. pascalis: MVZ 156161 (f), MVZ 156179 (f), MVZ 156180 (m), MVZ 162166 (f), MVZ 162863 (m).

T.b. perpallidus: MVZ 166250 (f), MVZ 166252 (m), MVZ 166253 (m), MVZ 166256 (m), MVZ 166257 (f), UA26596.

T.b. ruidosidae: MVZ 147023 (f), MVZ 147026 (f), MVZ 147027 (f), MVZ 147055 (f), MVZ 147056 (f).

Appendix 2

Protein alignment of the consensus *T. bottae* *Mc1r* with several species of mammals (pig, AF326520; cow, U39469; dog, AF064455; horse, AF288357; human, AF326275; and pocket mouse, AY258992). Putative transmembrane portions are shaded. Amino acid position corresponding to the Arg208Cys replacement change is indicated with an arrow

	1	60
T_bottae_cons	MPTQ-----GSLNSTPTATPHLRLSA---NQTGPWCLQVSI	PDGLFSLGLVSLVENV
S_scrofa	..VLGPERRLLA.LS.A.P.A.R.G.A.NQT.....Q.E.....	
B_taurus	..ALGSQRLL..L.C..P..LPFT.AP---.R..Q.E.....	
C_familiaris	.VW.GPQRLL..L.G.SP...FE.A.---...R.E...N.....V....	
E_caballus	..L.GPQRLL..L..LP...Y.G.TT---...E.P.E.....	
H_sapiens	.AV.GSQRLL..L.....I.Q.G.A.---...AR.E...S.....A	
C_intermedius	..M.EPQRLL.PF...R.GVP.E.---...H.....	
	61	120
T_bottae_cons	LVISIAKNRNLHSPMYFICCLALSDDLVSIVLETTVILVLEAGV	LATRVTVVQWLD
S_scrofa	...AA.....V...V.....N...A.L.L...A.AQAA...Q..	
B_taurus	...AA.....V.....N...A.MPL.....QAA...Q..	
C_familiaris	...AA.....G...V.....TN...A.M.LV...A.AQAA...Q..	
E_caballus	...TA.....V.....M.N...MAIL.L.....QAS.L.Q..	
H_sapiens	...AT.....C.....G.N...A...L...A.VA.AA.L.Q..	
C_intermediusQ.....L.....A.....Q..	
	121	180
T_bottae_cons	EVIDVLICGSMVSSLGFLGAIADVDRYISIFYALRYHSIVTLPR	RWAMVAI WVTSILSST
S_scrofa	N.M.....C.....V.....GR.IA...AG.V...	
B_taurus	N.....C.....V.....WRIIA...A...T.L	
C_familiaris	DI.....C.....L.....WR.IS...A.V...	
E_caballus	NI.....C...S.....MM...VWR.I...V.V...	
H_sapiens	N...IT.S.L...C.....R.VA...A.VVF..	
C_intermedius	N.....C.....I...A...S...	
	181	240
T_bottae_cons	LFVAYYNHTAVLLCLVTFPLAMLALMAVLYVHMLSRARQHAQV	IAQLHKRQHPVHQGRFL
S_scrofa	..I...H.....G..S..V.....A..C..GRH..R...T..TR..CG.	
B_taurus	..IT...KVI...GL.I.....A..C...RG..R.Q...R.I...G.	
C_familiaris	..I.....S..V..V.....A.....RG..R.R...S...G.	
E_caballus	..I.....V..V.....A..C...RG..R.....I...G.	
H_sapiens	..I...D.V.....V...V.....A..C...G..R...R...G.	
C_intermediusT...V.....A..H...A.....L.....	
	241	300
T_bottae_cons	KGAATLITLLGIFFLCWGPFLLHLTLTVLCPKHPTCS	CFKLNLFALALIIFN
S_scrofaV.L..A.....S.V...Q...G.V...V...V.C.....	
B_taurusV.....S.I...Q...G.I...F.....C.A.....	
C_familiarisS.M...Q...I.G.V.Q.F...T...C...I..F.	
E_caballusV.....S.LI...Q...G.V...FK...T...LCSA.....	
H_sapiens	..V.....I...E...G.I...F.....C.A.I...	
C_intermediusY..FI.....G.....	
	301	320
T_bottae_cons	YAFRSEELRMTLKEVLLCSW	
S_scrofaQ...K..Q...Q...	
B_taurusQ...K..Q...Q...	
C_familiarisQ...K..Q..V...	
E_caballusQ...K..Q.....	
H_sapiens	...H.Q...R.....T...	
C_intermediusQ.....	

(position 208 in the *T. bottae* sequence corresponds to position 218 in the consensus sequence).

Funding

This work was funded by National Science Foundation (DEB 9981810) and National Institutes of Health (R01 GM074245-01 A1) grants to MWN.

Acknowledgments

We especially thank J.L. Patton for suggestions. We thank the Museum of Vertebrate Zoology, University of California, Berkeley, and the Mammal Collection at the University of Arizona, Tucson for providing the tissue samples and skin samples used in this study.

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Received July 6, 2006

Accepted May 31, 2007

Corresponding Editor: Stephen J. O'Brien