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Independent Nonframeshift Deletions in the *MC1R* Gene Are Not Associated with Melanistic Coat Coloration in Three Mustelid Lineages

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Sequence variation within the 5′ flanking (about 240 bp) and exon regions (426 bp) of the melanocortin-1 receptor (*MC1R*) gene was examined to determine the potential role of this protein in the melanistic coat coloration of 17 mustelid species in four genera: *Gulo* (wolverines), *Martes* (martens), *Mustela* (weasels), and *Meles* (badgers). Members of the genera *Mustela* and *Meles*, together with *Martes flavigula* and *Martes pennanti*, were shown to have intact gene sequences. However, several ''in frame'' deletions of the *MC1R* gene region implicated in melanism of other species were detected within members of the genera *Martes* and *Gulo*. For instance, *Gulo gulo* possessed a 15 bp deletion in the second transmembrane domain coding region, while *Martes americana*, *Martes melampus*, *Martes zibellina*, and *Martes martes* shared a 45 bp deletion overlapping this area. In addition, *Martes foina* was found to possess a 10 bp insertion followed closely by a 28 bp deletion immediately downstream of the deletion found in other martens. Notably, none of these indels was associated with a melanistic phenotype. Phylogenetic analysis revealed that each of these nonrandomly distributed deletions arose independently during the evolution of this family. Specific indel-neighboring motifs appear to largely account for the biased and repeated occurrence of deletion events in the *Martes*/*Gulo* clade.

Mammals display conspicuous variation in pelage coloration. Not surprisingly, several genes have been linked to the differential expression of pigments within the melanocytes of a broad range of species (Eizirik et al. 2003; Rieder et al. 2001). One such locus, encoding the transmembrane melanocortin-1 receptor (*MC1R*), is thought to play a major role in red-yellow (phaeomelanin) and black-brown (eumelanin) melanization (MacDougall-Shackleton et al. 2003). Indeed, numerous amino acid substitutions within the coding region of this gene have been reported to alter the coat coloration of laboratory mice (Robbins et al. 1993), pocket mice (Nachman et al. 2003), horses (Johansson et al. 1994; Marklund et al. 1996; Rieder et al. 2001), cattle (Joerg et al. 1996; Klungland et al. 1995), foxes (Våge et al. 1997), cats (Eizirik et al. 2003), dogs (Everts et al. 2000; Newton et al. 2000; but see Kerns et al. 2003), and several species of birds (Mundy et al. 2004). These *MC1R* gene polymorphisms are classified as either loss-of-function or gain-of-function mutations, each resulting in red-yellow or black-brown coat colorations, respectively (Robbins et al. 1993). For example, point mutations in the second (Glu92Lys) and third (Cys125Arg) transmembrane domains are associated with melanism in mice (Robbins et al. 1993) and red foxes (Våge et al. 1997), respectively. Similarly Eizirik et al. (2003) suggested that 15 bp (codons 100–105) and 24 bp (codons 95–102) deletions within this region of the gene are responsible for intraspecific gain-of-function mutations in jaguars and jaguarundies, respectively. Notably, an eight amino acid deletion identical to that found in jaguarundies has also been linked to the melanic phenotype of golden-headed lion tamarins (Mundy and Kelly 2003).

Within the family Mustelidae, weasels, minks, and martens exhibit remarkable intra- and interspecific pelage color variations (Anderson 1970; Thomas 1897). For example, two distinct populations of Japanese marten (*Martes melampus*) are easily identified by coat coloration: a yellow phenotype more common to the Japanese islands and a dark brown phenotype particular to populations in the Tsushima and Shikoku Islands and the Kii peninsula (Hosoda and Oshima 1993). In addition to these color variants, most weasels undergo seasonal molts that involve dramatic alterations in

color, though this trait tends to be limited to northern populations within each species (Nowak 1999). Although numerous studies have been completed on the genetic systems that affect coat coloration [see Rees (2003) for a review], less attention has been focused on the evolution of melanistic pelage traits (Eizirik et al. 2003; Mundy and Kelly 2003). In addition, almost nothing is known regarding the genetic basis of seasonal changes in pelage coloration common to several north-temperate mammalian groups.

Here we examined sequence variations in a portion of the *MC1R* gene in 17 mustelid species as an initial step for such genetic investigation. Evolution of the *MC1R* gene within each of these lineages was inferred by mapping coat coloration and genetic traits onto a molecular phylogenetic tree constructed from mitochondrial (Hosoda et al. 2000) and nuclear (Sato et al. 2003) gene sequences.

Materials and Methods

Amplification and Sequencing of DNA

Seventeen mustelid species (36 individuals) were examined in this study (Table 1). We designed primers for the 5′ upstream and exon regions of the *MC1R* gene using dog (Everts et al. 2000) and fox (Våge et al. 1997) sequences. Base pair numbers in primer names refer to nucleotide positions of the genomic canine *MC1R* sequence in GenBank (AF064455; Everts et al. 2000). In the 5′ flanking region, primers used for the first round of amplification were 5′MC1R-70 (5′-AAACGTACGTCTAACCTGAGCAA-3′) and 3′MC1R-469 (5′-GCTCACCAGCCCCAGGCTGAGGAA-3′). The second primer pair, for polymerase chain reaction (PCR), was 5′MC1R-70 and 3′MC1R-339 (5′-GTTGGGAATGGACACCTCCAGGCA-3′). For exon amplification, primers used in the first PCR were 5′MC1R-302 (5′-GATGAGCTGAGCGGGACGCCTG-3′) and 3′MC1R-772 (5′-GGTATCGCAGCGCGTAGAAGATG-3′). The primers for the second PCR were 5′MC1R-322 (5′-CTGCGAGTGAGGACCCCTTTCTG-3′) and 3′MC1R-772. Following an initial denaturation cycle (94°C for 3 min), cycling conditions were 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C (30 cycles), followed by a final 5 min extension period at 72°C. Products of the second PCR were sequenced directly according to the manufacturer's instructions with a Big Dye Terminator Cycle Sequencing Kit on an ABI 3100 Genetic Analyzer. Sequences obtained in this study were deposited in international DNA databases (Table 1).

Data Analyses

Maximum parsimony (MP; Swofford and Olsen 1990) phylogenetic trees were constructed using PAUP* version 4.0b10 (Swofford 2001) from a concatenated dataset incorporating published sequences of the mitochondrial cytochrome *b* (cyt *b*; 1140 bp) and nuclear interphotoreceptor retinoid binding protein (IRBP; 1185 bp) genes of the 17 mustelid species (Hosoda et al. 2000; Sato et al. 2003, 2004). The Eurasian badger (*Meles meles*) was employed as an outgroup on the basis of previous phylogenetic hypotheses (Sato et al. 2003, 2004) and the analysis was conducted using 100 heuristic tree-bisection reconnection searches in which the input order of taxa was randomized and based on the following character weighting: equally weighted nucleotide substitutions (IRBP), transversions only at the third codon positions, and all nucleotide substitutions at the first and second codon positions (cyt *b*). Bootstrap proportions (BS; Felsenstein 1985) were obtained by generating 1000 heuristic replicates with PAUP, each consisting of 100 heuristic tree-bisection reconnection searches in which the input order of taxa was randomized. Finally, synonymous and nonsynonymous nucleotide changes along the *MC1R* exon were mapped onto the tree with the aid of MacClade version 4 (Maddison and Maddison 2000). Although the cyt *b*/IRBP tree supported a monophyletic relationship between *Mustela altaica* and *Mustela nivalis* with moderate bootstrap values (75%), this relationship was not parsimonious after accounting for the *MC1R* gene sequences of each species. Consequently the topology was modified slightly (see dashed lines of Figure 1) to minimize the number of substitution events required.

The number of synonymous substitutions per synonymous sites (dS) and nonsynonymous substitutions per nonsynonymous sites (dN) among coding region sequences were computed with MEGA2 (Kumar et al. 2001) using a modified Nei-Gojobori method (Nei and Gojobori 1986; Zhang et al. 1998) with the Jukes and Cantor (1969) model. The 10 bp insertion in the *Martes foina MC1R* sequence was excluded from this analysis.

Results and Discussion

Nucleotide sequences of the upstream flanking (238–241 bp) and the 5′ exon region (382–427 bp, 127–142 codons) of the *MC1R* gene were determined from 17 mustelid species (Table 1). Unfortunately we were unable to obtain sequences upstream of the *MC1R* gene for the yellow-throated marten (*Martes flavigula*). Among the mustelids examined, intraspecific variations were found in some species, but not in others. For instance, gene sequences for the stone marten (*M. foina*) from Primorye, Russia ($n = 2$) and south China ($n = 1$) differed at three nucleotide positions: 29, 73, and 229 (site numbers counted from A of the initiation codon, ATG, in the coding region). The *MC1R* sequences of three sables (*Martes zibellina*) from Hokkaido possessed three variable sites, one in the upstream region (site –180) and two in the coding region (sites 104 and 203), while the coding sequences of the two *M. flavigula* specimens exhibited one base substitution (site 363). In contrast, the five individual pine martens (*Martes martes*) possessed matching sequences. Similarly the 11 Japanese martens showed no variation along the region of the *MC1R* gene examined, despite exhibiting seasonal differences in coat color (yellow, $n = 6$; dark brown, $n = 5$).

Comparative analyses among sequences of the 17 mustelid species revealed remarkable divergence in both the upstream and coding regions of the *MC1R* gene with respect to

Table 1. List of species used in this study

Species (common name)	Sample number	Source	Coat color in winter	5′ flanking		Coding	
				Accession no.	length (bp)	Accession no.	length (bp)
Gulo gulo (wolverine)	TH150HS1603	Sakhalin, Russia	blackish brown[a]	AB189797	241	AB189828	412
Martes americana (American pine marten)	HS990	USA	chestnut brown[a]	AB189787	241	AB189818	382
Martes flavigula	HS844	China	dark brown[a]	–	–	AB189814	427
(yellow-throated marten)	HS1224	Russia	brownish yellow	–	–	AB189815	427
Martes foina	HS1396	Kunming, China	pale gray[a]	AB189793	241	AB189824	409
(stone marten)	HS1751	Turingia, Germany	pale gray[a]	AB189794	241	AB189825	409
	HS1752	Turingia, Germany	pale gray[a]	AB189795	241	AB189826	409
Martes martes	AK702HS1356	Moscow, Russia	chestnut brown[a]	AB189790	241	AB189821	382
(European pine marten)	AK718HS1393	Tver′ region, Russia	chestnut brown[a]	AB189791	241	AB189822	382
	TH220HS1754	Germany	chestnut brown[a]	AB212935	241	AB212938	382
	TH221HS1755	Germany	chestnut brown[a]	AB212936	241	AB212939	382
	TH222HS1756	Germany	chestnut brown[a]	AB212937	241	AB212940	382
Martes melampus	TH048HS646	Hokkaido, Japan	yellow	AB214368	241	AB214370	382
(Japanese marten)[b]	TH017HS515	Tochigi, Japan	yellow	AB189800	241	AB189831	382
	TH018HS516	Shimane, Japan	yellow	AB189801	241	AB189832	382
	TH020HS517	Wakayama, Japan	brown	AB189802	241	AB189833	382
	TH006HS518	Niigata, Japan	yellow	AB189803	241	AB189834	382
	TH010HS519	Tokushima, Japan	brown	AB189804	241	AB189835	382
	TH007HS520	Tsushima, Japan	brown	AB189805	241	AB189836	382
	TH004HS521	Tsushima, Japan	brown	AB189806	241	AB189837	382
	TH005HS522	Tsushima, Japan	brown	AB189807	241	AB189838	382
	TH012HS523	Kumamoto, Japan	yellow	AB189808	241	AB189839	382
	HS1041	Miyazaki, Japan	yellow	AB189809	241	AB189840	382
Martes pennanti (fisher)	HS2588	Canada	dark brown[a]	AB189799	241	AB189830	382
Martes zibellina (sable)	TH043HS641	Hokkaido, Japan	gray brown	AB214369	241	AB214371	382
	TH047HS645	Hokkaido, Japan	light yellow	AB189783	241	AB189812	382
	TH053HS806	Hokkaido, Japan	gray brown	AB189784	241	AB189813	382
Mustela eversmanii (steppe polecat)	HS826	Novosibirsk, Russia	dark brown[a]	AB189786	240	AB189817	427
Mustela putorius furo (ferret)	TH027HS449	Experimental animal	dark brown[a]	AB189781	240	AB189810	427
Mustela sibirica (Siberian weasel)	HS1121	Primorye, Russia	straw yellow[a]	AB189788	241	AB189819	427
Mustela lutreola (European mink)	HS1225	Novosibirsk, Russia	dark brown[a]	AB189789	240	AB189820	427
Mustela altaica (mountain weasel)	AK803HS1516	Cherga, Russia	straw yellow[a]	AB189796	240	AB189827	427
Mustela nivalis (least weasel)[b]	AK719HS1394	Rostov region, Russia	white[a]	AB189792	240	AB189823	427
Mustela erminea (stoat)[b]	HEG305HS1759	Iwate, Japan	white	AB189798	239	AB189829	427
Mustela vison (American mink)	HS647	Hokkaido, Japan	chocolate brown	AB189785	238	AB189816	427
Meles meles (badger)	TH002HS525	Miyazaki, Japan	gray brown	AB189782	240	AB189811	427

[a] Pelage of specimen not examined. Coat colors during the winter were obtained from the literature (Partridge, 1995; Heptner et al. 2002).

[b] Species that undergo seasonal changes in coat coloration.

both nucleotide substitutions and insertion/deletion (indel) events (Table 1 and Figure 1). The sequence alignment revealed that, excluding indels, 18.6% of sites (45 of 242 bp) were variable in the 5′ flanking region. Unexpectedly a high percentage of variable sites (68 of 427 bp, 15.9%) were observed in the exon sequence, even in regions encoding functional domains. Excluding indels, 29 nucleotide substitutions were identified among the eight species of the *Martes/Gulo* clade (Figure 1). Notably substitutions at the first (8 sites) and second (15 sites) codon positions occurred more frequently than the third position (6 sites), leading to amino acid substitutions at 23 residues. Remarkably all but one intraspecific nucleotide variation identified within this clade was found to be nonsynonymous (white bars of Figure 1). Among the eight species of the *Mustela* clade, 31 nucleotide substitutions were detected, with 12, 8, and 11 substitutions at the first, second, and third codon positions, respectively, leading to amino acid substitutions at 21 residues (Figure 1). Considering the large proportion (74% to 79%) of nucleotide substitutions leading to amino acid changes, nonsynonymous

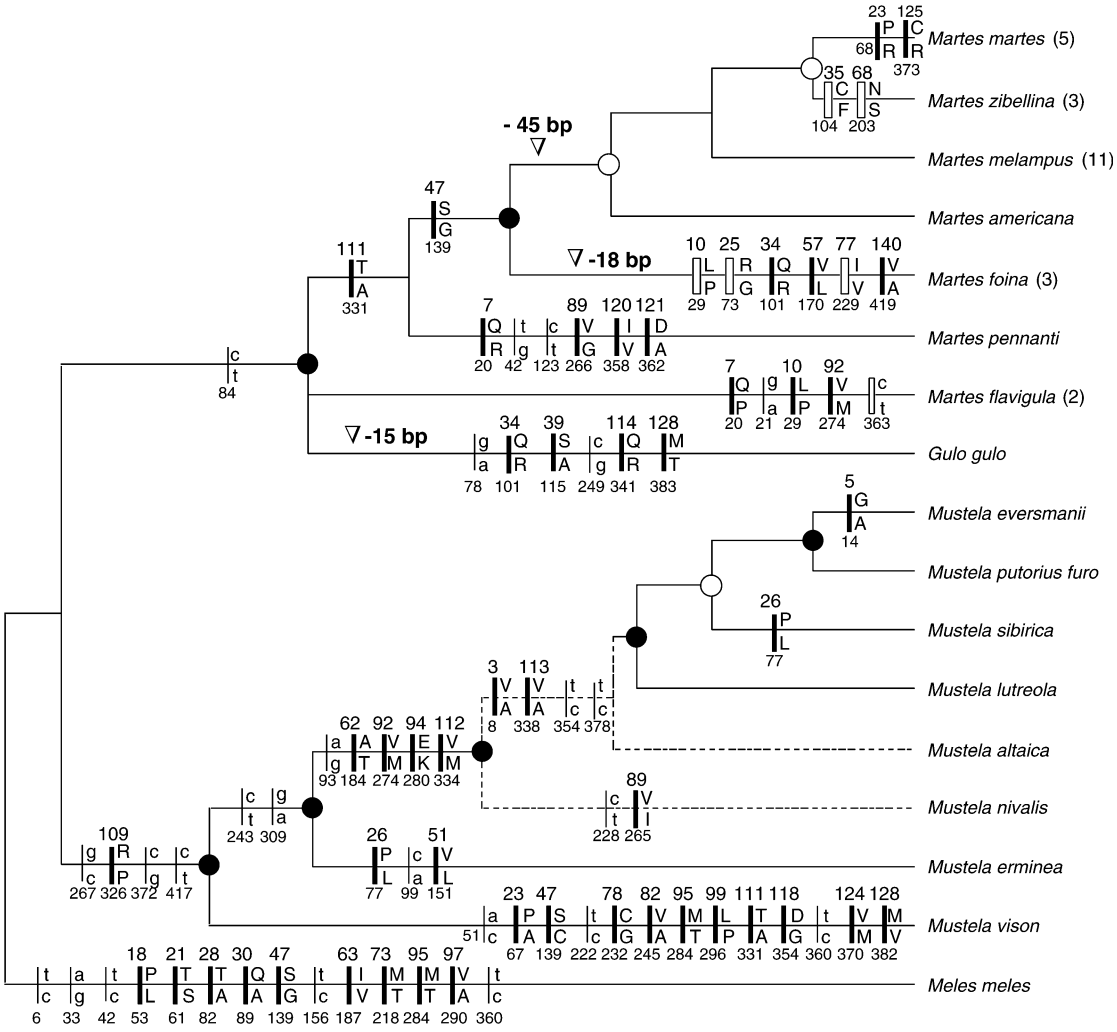

Figure 1. Evolution of the *MC1R* gene in 17 species of the family Mustelidae (numbers of specimens examined for each species are indicated in parentheses). Nucleotide substitutions and indels of the *MC1R* exon were mapped on a phylogenetic tree constructed with the maximum parsimony method from a concatenated sequence of the cytochrome *b* (1140 bp) and interphotoreceptor retinoid binding protein (1185 bp) genes. Although the tree suggested the monophyly of *M. altaica* and *M. nivalis* (Sato et al. 2003), it was altered (denoted by dashed lines) to account for the *MC1R* gene sequences of each species (see text for details). Inverted triangles indicate deletion events. Nonsynonymous substitutions are indicated by thick bars, while synonymous substitutions are indicated by thin bars. The numbers above the thick bars indicate the amino acid position, with letters below each line denoting the amino acid substitution from the ancestral state (above line). The numbers below the thin bars indicate the nucleotide position, with letters below each line representing the nucleotide substitution from the ancestral state (above line). Substitutions in polymorphic state within a species are indicated by white boxes. Circles associated with each node represent the strength of support for the clade as evaluated by bootstrap values (black, greater than 75% support; white, 50%–74% support). The duplicated region in the *M. foina* sequence (see Figure 2) was excluded from the analysis.

substitutions appear to dominate the history of the *MC1R* gene in mustelids. However, while the mean dN/dS ratios were 1.47 and 0.97 in the *Martes/Gulo* and *Mustela* lineages, respectively, we could not detect a clear trend for positive selection ($dS < dN$) at the $P < 0.05$ level. These results suggest that the *MC1R* coding region of mustelids has been subjected to either diversifying selection, supporting the previous notion based on human *MC1R* gene sequences (Rana et al. 1999), or relaxation of functional constraints in the *MC1R* gene.

Numerous amino acid substitutions have been associated with changes in *MC1R* function in a broad range of mammal species. For instance, Glu92Lys is thought to induce black coat coloration in mice (Robbins et al. 1993). We found an identical substitution at the corresponding site (codon 94) in the six derived species of *Mustela* (Figure 1). However, none of the six individuals examined showed signs of melanism (Table 1), suggesting the substitution Glu94Lys in mustelids is unlikely to lead to the same functional change as that of the murine rodent. Similarly *M. martes* possessed

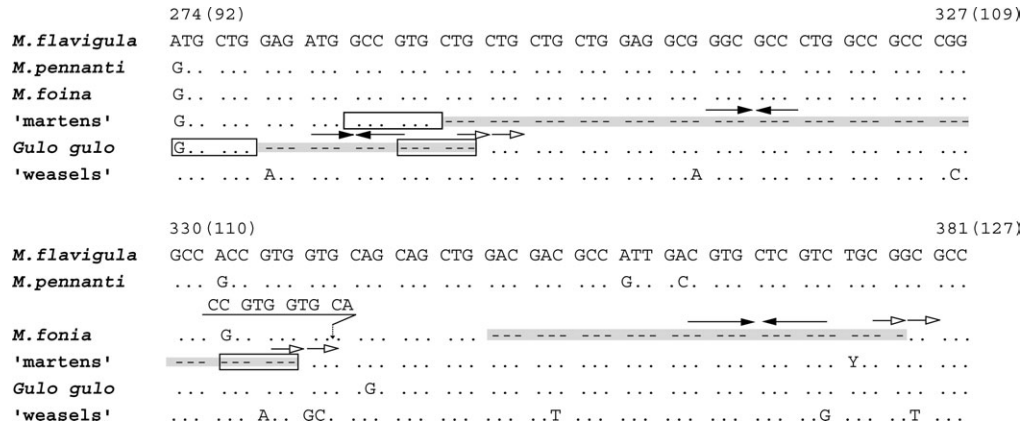

Figure 2. Partial nucleotide sequence (positions 274–381; codon positions in parentheses) of the mustelid *MC1R* exon. Dots indicate identity to the top sequence (*M. flavigula*). Sequences from *M. americana*, *M. martes*, *M. melampus*, and *M. zibellina* were identical except for position 373 (*M. martes* possessed a C while the other three species possessed a T) and given the term "martens." Similarly sequences from *M. altaica*, *Mustela eversmanii*, *Mustela lutreola*, *Mustela putorius*, and *Mustela sibirica* were grouped together and termed "weasels." Shaded regions indicate deleted regions (dashed lines denote deleted nucleotides), while underlined sequences represent nucleotide insertions. Hexanucleotide repeats at both ends of each deletion are boxed. Open arrows indicate repeated triplets at the 3' side of each deletion, while closed arrows indicate a short palindrome near the middle of each deleted segment.

a single substitution at codon 125 (Cys125Arg) that is known to cause a functional change in red fox coloration (Våge et al. 1997). However, there seems to be no substantial similarity in coat colors of *M. martes* with the variant color (silver) of *Vulpes vulpes* (Våge et al. 1997). Two additional substitutions implicated in the dominant melanistic phenotypes of cattle and pigs (Leu99Pro) (Kijas et al. 1998; Klungland et al. 1995) and sheep (Met73Thr) (Våge et al. 1999) were detected in our American mink (*Mustela vison*) and badger (*M. meles*) sequences, respectively. Again, neither of these mustelids showed signs of melanism (Table 1). In contrast, amino acid changes that may be associated with a loss-of-function mutation in the *MC1R* gene were observed in one *M. zibellina* specimen. This homozygous individual (TH047HS645), with a rare light yellow coat color, was found to have two substitutions (Cys35Phe and Asn68Ser) compared to one of the wild-type individuals (TH043HS641), while the second, gray brown sable (TH053HS805), was heterozygous at site 68 (photographs comparing the rare type with the common dark brown phenotype are available upon request).

In addition to the high rate of nonsynonymous substitutions within the *Martes/Gulo* clade, our *MC1R* sequence alignment revealed that at least four independent indel events occurred in the coding region of this gene during the evolution of this group (Figure 1). The first, a 15 bp deletion found in the second transmembrane domain (codons 94–98) of the *Gulo gulo MC1R*, presumably followed the divergence of wolverines from the *Martes* lineage (Figure 2). A second 45 bp in-frame deletion overlapping this region (codons 98–112) occurred before the radiation of *Martes americana*, *M. martes*, *M. melampus*, and *M. zibellina* (Figure 2). Finally, two indels were detected in the stone marten *MC1R* gene sequence: a 10 bp duplication immediately downstream of that found in the four other marten species, followed closely by a 28 bp deletion (Figure 2). Interestingly, these indels all occur in a gene region implicated to be involved in melanism of jaguarundies and golden-headed lion tamarins (codons 95–102) (Eizirik et al. 2003; Mundy and Kelly 2003) and jaguars (codons 101–105) (Eizirik et al. 2003). However, none of the mustelid *MC1R* indels appear to represent melanistic gain-of function mutations. Consequently our data suggest that deletions near the end of the second transmembrane domain are not always associated with phenotypic changes in coat coloration.

Our multiple alignment (Figure 2) of the *MC1R* exon further suggests that certain nucleotide motifs are associated with the relatively high incidence of indels that we detected. Thus it is reasonable to predict that the independent deletion events observed in the three mustelid lineages are related to specific nucleotide arrangements within the *MC1R* gene sequences. We observed three notable features associated with the observed deletion events. First, two of the three deletion events were associated with hexanucleotide direct repeats at both ends of each deletion (Figure 2). A similar pattern was found in the *MC1R* gene of jaguarundies (Eizirik et al. 2003), supporting the hypothesis of polymerase slippage due to slipped-strand mispairing (Nishizawa and Nishizawa 2002; Taylor et al. 2004). The second feature relates to the deletion of a repeated triplet at the 3′ end of each deletion event, while the third is the presence of short palindromic regions near the middle of each deleted segment. Notably one or two of these possible structural prerequisites favoring a deletion event are not found in the corresponding sequence region of the weasel lineages (Figure 2). Thus the consistent patterns around the deleted regions may provide useful insight into the molecular basis of deletion events that have occurred during the evolution of mammalian genomes.

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