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Molecular Genetics and Evolution of Melanism in the Cat Family

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Summary

Melanistic coat coloration occurs as a common polymorphism in 11 of 37 felid species and reaches high population frequency in some cases but never achieves complete fixation [1–3]. To investigate the genetic basis, adaptive significance, and evolutionary history of melanistic variants in the Felidae, we mapped, cloned, and sequenced the cat homologs of two putative candidate genes for melanism (*ASIP* [*agouti*] and *MC1R*) and identified three independent deletions associated with dark coloration in three different felid species. Association and transmission analyses revealed that a 2 bp deletion in the *ASIP* gene specifies black coloration in domestic cats, and two different "in-frame" deletions in the *MC1R* gene are implicated in melanism in jaguars and jaguarundis. Melanistic individuals from five other felid species did not carry any of these mutations, implying that there are at least four independent genetic origins for melanism in the cat family. The inferred multiple origins and independent historical elevation in population frequency of felid melanistic mutations suggest the occurrence of adaptive evolution of this visible phenotype in a group of related free-ranging species.

Results and Discussion

The cat family (Felidae) exhibits a wide diversity of coat colors and patterns, including melanism in at least 11 species and different color "phases" in several others. For example, in the small Neotropical jaguarundi (*Herpailurus yaguarondi*), coloration varies from dark brown/gray (the most common form, widely regarded as the wild-type) to light reddish [1, 2].

Molecular genetic studies in mice have identified several genes involved in pigmentation phenotypes [4, 5], including loci involved in melanism, such as *agouti/ASIP* (*Agouti Signaling Protein*) [6, 7] and *extension/MC1R* (*Melanocortin-1 receptor*) [8, 9]. Recessive variants of *agouti* cause melanism, which is also induced by dominant mutations in *MC1R* [4–9]. Melanism in the domestic cat (*Felis catus*) is inherited as a recessive trait, suggesting *agouti/ASIP* as a candidate gene [1, 2], whereas a dominant inheritance pattern has been reported for melanism in the jaguar (*Panthera onca*) [3], suggesting involvement of *extension/MC1R*. To date, little is known about the molecular or adaptive basis of coat color variation in free-ranging mammals, and so far no study has addressed this issue in multiple polymorphic species from the same family.

We first mapped, cloned, and sequenced the domestic cat homologs of *ASIP* and *MC1R* ([10]; Eizirik et al., unpublished data). The domestic cat *ASIP* gene maps to chromosome A3, and *MC1R* maps to chromosome E2; in both cases, the location corresponds to the homologous genomic position of their human counterparts [10]. The feline *ASIP* gene consists of three coding exons, as in other mammals, but comprises 405 bp (135 codons) as opposed to 393–396 in other species [6, 11–13], due to a three-residue insertion after codon 84 (see the Supplementary Material [available with this article online and at http://lgd.nci.nih.gov as a link to this paper] for the full sequence of both genes). The cat *MC1R* gene consists of an intron-less 951 bp (317 codons) open reading frame, similar in structure to other mammalian homologs [8, 9, 11, 12, 14–17].

Three novel microsatellite markers linked to *ASIP* were isolated from a domestic cat BAC clone containing this gene and were used to perform linkage analyses in a pedigree of 89 domestic cats that segregated for melanism. LOD scores obtained from these loci indicated the existence of highly significant linkage with no recombination between these markers and melanism (maximum LOD scores were at 0.0 cM: 16.39, 11.35, and 11.03 for FCA708, FCA718, and FCA719, respectively). Sequence characterization of the *ASIP* gene in multiple domestic cats revealed that black individuals were homozygous for an allele (named *ASIP*-Δ2) in which a 2 bp deletion at nucleotide positions 123–124 induces a frame shift in the inferred protein, predicting a complete loss of the C-terminal active domain (Figure 1A).

PCR primers flanking the *ASIP*-Δ2 deletion were designed and used to screen a collection of 83 unrelated domestic cats, the 89-member domestic cat pedigree, and 56 wild felid individuals from 20 species. Family transmission analysis in the domestic cat pedigree demonstrated perfect cosegregation between the *ASIP*-Δ2 allele and black coloration, and this cosegregation conforms to the recessive mode of inheritance [1, 2] (see the Supplementary Material). In addition, 57 unrelated black domestic cats collected throughout the world were homozygous for the *ASIP*-Δ2 allele, whereas 26 non-black individuals carried at least one wild-type allele (Figure 2), demonstrating perfect association between coloration phenotype and molecular genotype ($p < 0.001$ in a Fisher's exact test using a recessive mode of inheritance). The survey of 20 other cat species (including melanistic individuals for 7 of them) representing all major lineages in the Felidae revealed that the *ASIP*-Δ2 allele was unique to domestic cats and absent among

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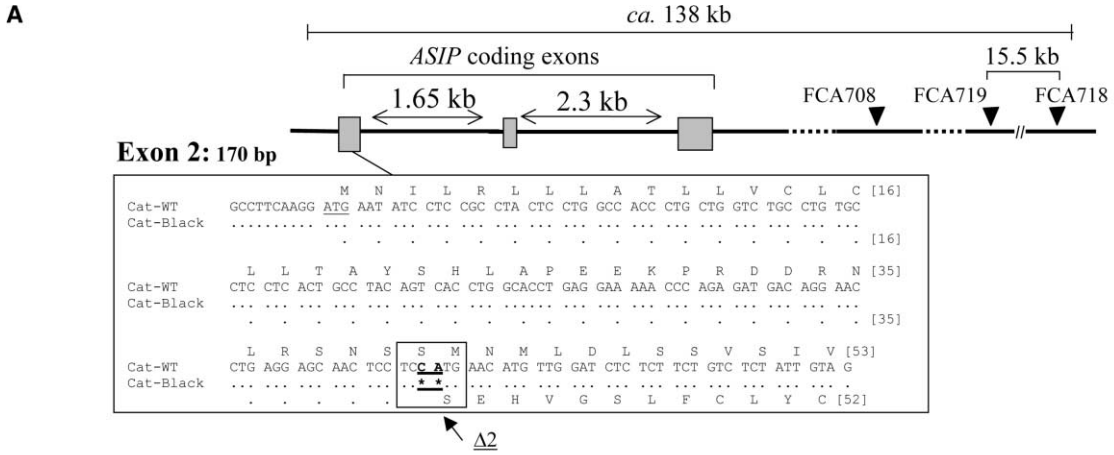


B *MC1R* 274(92) 324(108)

	V	L	E	T	A	V	M	L	L	L	E	A	G	A	L	A	G
D.cat	GTG	CTG	GAG	ACG	GCC	GTC	ATG	CTG	CTG	CTG	GAG	GCA	GGC	GCC	CTG	GCC	GGC
Jaguar-WT	...	...	...	...	...	...	...	...	...	...	...	..G	...	A..	...	...	...
Jaguar-Mel	...	...	...	...	...	...	...	...	...	---	---	---	---	---	**AC**.	...	...
Jaguarundi-Dark	...	...	...	---	---	---	---	---	---	---	---	...	...	A..	...	...	...
Jaguarundi-Red	...	...	...	...	...	...	...	...	...	...	...	...	...	A..	...	...	...
Dog	...	...	...	...	...	...	...	...	...	G..	...	...	...	...	T..	..T	.CG
Pig	...	...	...	...	...	..G	C..	...	...	...	...	..G	...	...	...	...	.C.
Cattle	...	...	...	...	..A	...	...	...	...	...	...	..C	..T	.T.	...	...	AC.
Horse	...	...	...	.T.	..A	A..	T..	...	...	...	...	..C	..A	.T.	...	...	AC.
Human	...	...	...	...	...	...	..C	..C	...	...	...	..C	..T	..A	...	.TG	.C.

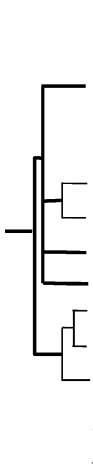
Figure 1. Nucleotide Variation in the *ASIP* and *MC1R* Genes Associated with Melanism in the Felidae

(A) Nucleotide sequence of domestic cat *ASIP* exon 2, shown for a wild-type (Cat-WT) and a black individual (Cat-Black) homozygous for the *ASIP*-Δ2 allele; amino acid sequences are given above (Cat-WT) and below (Cat-Black) the third position of each codon. Dots indicate identity to the top sequence. The genomic structure of *ASIP* and the location of the STR loci (arrowheads) used for the linkage analysis are indicated above the sequence; dashed lines indicate uncertain positions of different shotgun sequence contigs ([10]; Eizirik et al., unpublished data). The initiation codon is underlined, and the two (bold underlined) nucleotides deleted in the *ASIP*-Δ2 allele (indicated by asterisks) are boxed. A premature stop codon after residue 99 in *ASIP*-Δ2 removes the terminal portion of the peptide (see the Supplementary Material for more details).

(B) Partial nucleotide sequence (positions 274–324; codon positions in parentheses; see the Supplementary Material for full sequence) of the feline *MC1R* gene aligned with other mammalian homologs (GenBank accession numbers: dog, AF064455; pig, AF326520; cattle, U39469; horse, AF288357; human, AF326275). Sequences are shown from a domestic cat (D.cat), wild-type jaguar allele (Jaguar-WT), melanistic jaguar allele *MC1R*-Δ15 (Jaguar-Mel), dark-brown jaguarundi allele *MC1R*-Δ24 (Jaguarundi-Dark), and a reddish jaguarundi (Jaguarundi-Red). Dots indicate identity to the top sequence; the domestic cat amino acid sequence is given above the nucleotide alignment. The jaguar and jaguarundi melanistic deletions are shaded (dashes indicate deleted sites). Codons repeated at both ends of each deletion (possibly involved in the origin of these variants through replication slippage) are underlined. The conserved leucine codon 3′ of the jaguar deletion is boxed, and the two nonsynonymous changes in the *MC1R*-Δ15 allele are marked with a double underline.

all other sampled felids regardless of their coloration (Figure 2).

We confirmed the dominant mode of inheritance of melanism in jaguars [3] by performing phenotype transmission analysis in a 116-individual captive pedigree (see the Supplementary Material). Given the dominant inheritance and the absence of the *ASIP*-Δ2 mutation in melanistic jaguars (Figure 2), we tested whether the *MC1R* gene is implicated in jaguar melanism by obtaining its full coding sequence in several wild-type (yellow with dark rosettes) and black individuals. Melanistic animals were found to carry at least one copy of a mutant *MC1R* sequence allele bearing a 15 bp (five-codon) in-frame deletion at positions 301–315 (Figure 1B). This allele (designated *MC1R*-Δ15) displayed two nonsynonymous nucleotide substitutions immediately adjacent to the deletion (changing a CTG codon to ACG), and the substitutions result in a Leu/Thr replacement relative to the wild-type jaguar sequence at a codon that is otherwise conserved across mammals (Figure 1B). An additional variable site was identified at position 825, where a synonymous T/C polymorphism was observed. The nondeleted alleles contained either C or T at this position, whereas the surveyed *MC1R*-Δ15 alleles all had a T at that site. A total of 46 jaguars differing in coloration phenotype were screened for their *MC1R* genotypes by using specific PCR primers designed to detect the deletion allele. Ten unrelated melanistic jaguars were either homozygous or heterozygous for the *MC1R*-Δ15 allele, whereas all 36 wild-type coloration jaguars (sampled from Mexico to southern Brazil [18]) were homozygous for the wild-type allele ($p < 0.001$ for color-



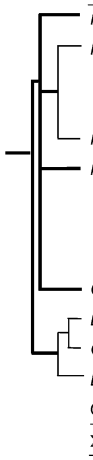
Felidae Species	n (mel)*	*ASIP* Genotypes			
		Δ2/Δ2	Δ2/+	+/+	f [Δ2]
Felis catus					**0.41**
Melanistic**	57 (57)	57	-	-	
Non-melanistic	26 (0)	-	15	11	
Panthera onca	10 (4)	-	-	10	0.00
Panthera pardus	4 (4)	-	-	4	0.00
Herpailurus yaguarondi	15 (8)	-	-	15	0.00
Catopuma temmincki	2 (1)	-	-	2	0.00
Leopardus tigrinus	3 (1)	-	-	3	0.00
Oncifelis geoffroyi	4 (2)	-	-	4	0.00
Lynchailurus colocolo	3 (1)	-	-	3	0.00
Other Felidae***	15 (0)	-	-	15	0.00
Σ	139 (78)	57	15	67	

Figure 2. Genotyping Results for the *ASIP*-Δ2 Deletion Allele Identified in Domestic Cats

The cladogram indicates evolutionary relationships of species with melanistic forms included in this study; thick branches on the tree indicate major lineages in the Felidae [19]. Only unrelated individuals from each species were included. The number of melanistic individuals assayed is given in parentheses (see asterisk). Melanistic individuals include black domestic cats from Botswana (n = 1), Brazil (n = 10), Israel (n = 5), Mongolia (n = 4), and USA (n = 37) (see double asterisk); USA black cats include random-bred individuals as well as representatives of the following breeds: Bombay, Maine Coon, Norwegian Forest Cat, Cornish Rex, Turkish Van, and Sphynx. The other Felidae include one or two individuals from each of the following cat species: black-footed cat (*Felis nigripes*), sand cat (*Felis margarita*), lion (*Panthera leo*), tiger (*Panthera tigris*), snow leopard (*Panthera uncia*), clouded leopard (*Neofelis nebulosa*), ocelot (*Leopardus pardalis*), puma (*Puma concolor*), cheetah (*Acinonyx jubatus*), leopard cat (*Prionailurus bengalensis*), caracal (*Caracal caracal*), African golden cat (*Profelis aurata*), and bobcat (*Lynx rufus*) (see triple asterisk). f [Δ2] represents the frequency of the *ASIP*-Δ2 allele in each species; the domestic cat frequency was calculated exclusively from the nonmelanistic genotype frequencies, assuming Hardy-Weinberg equilibrium.

genotype association using a dominant model, Figure 3). Transmission analysis in an eight-individual captive jaguar pedigree demonstrated exact cosegregation between the deletion genotype and the melanistic phenotype (see the Supplementary Material), supporting a dominant mode of inheritance mediated by the *MC1R*-Δ15 allele.

Neither *ASIP*-Δ2 nor *MC1R*-Δ15 were seen in a screen of 29 jaguarundis that varied in coat color from very dark brown/gray to red (Figures 2 and 3). However, a second in-frame deletion in *MC1R* (designated *MC1R*-Δ24), which removed 24 bp (8 codons) at a position adjacent to, but distinct from, the deletion seen in the jaguar *MC1R*-Δ15 allele, was discovered in jaguarundis (Figure 1B). The jaguar *MC1R*-Δ15 and the jaguarundi *MC1R*-Δ24 deletions likely derive from independent mutational events, given the sequence homology of adjacent nucleotides, the fact that each species belongs to a separate lineage in the Felidae [19], and the persistence of nondeleted (ancestral) alleles in both of them (Figures 1B and 3). In addition to the presence of the deletion, the jaguarundi *MC1R*-Δ24 allele also differed



Felidae Species	n (mel)*	*MC1R* Genotypes						
		Δ15/Δ15	Δ15/+	Δ24/Δ24	Δ24/+	+/+	f [Δ15]	f [Δ24]
Felis catus	43 (28)	-	-	-	-	43	0.00	0.00
Panthera onca							**0.12**	0.00
Non-melanistic	36 (0)	-	-	-	-	36		
Melanistic	10 (10)	1	9	-	-	-		
Panthera pardus	8 (4)	-	-	-	-	8		
Herpailurus yaguarondi							0.00	**0.50**
Very dark	7 (7)	-	-	6	1	-		
Dark brown / gray	12 (12)	-	-	3	9	-		
Red / reddish	10 (0)	-	-	-	4	6		
Catopuma temmincki	2 (1)	-	-	-	-	2	0.00	0.00
Leopardus tigrinus	3 (1)	-	-	-	-	3	0.00	0.00
Oncifelis geoffroyi	4 (2)	-	-	-	-	4	0.00	0.00
Lynchailurus colocolo	4 (1)	-	-	-	-	4	0.00	0.00
Other Felidae**	22 (0)	-	-	-	-	22	0.00	
Σ	172 (66)	1	9	12	16	134		

Figure 3. Genotyping Results for the Deletions Identified in the *MC1R* Gene of Jaguars and Jaguarundis

Only unrelated animals from each species were included. The tree on the left indicates phylogenetic relationships of species with melanistic forms included in this study; thick branches on the tree indicate major lineages in the Felidae [19]. Alleles are coded as follows: Δ15 is the jaguar deletion allele *MC1R*-Δ15, Δ24 is the jaguarundi deletion allele *MC1R*-Δ24, + indicates an ancestral-type (nondeleted) allele. The number of melanistic individuals assayed is given in parentheses (see the asterisk). The other Felidae include one or two individuals from each of the following cat species: *Felis silvestris*, *Felis nigripes*, *Felis margarita*, *Panthera leo*, *Panthera tigris*, *Panthera uncia*, *Neofelis nebulosa*, *Leopardus pardalis*, *Puma concolor*, *Acinonyx jubatus*, *Prionailurus bengalensis*, *Prionailurus planiceps*, *Caracal caracal*, *Profelis aurata*, *Lynx rufus*, *Catopuma badia*, and *Leptailurus serval* (see double asterisk). f [Δ15] and f [Δ24] are the frequencies of the *MC1R*-Δ15 and *MC1R*-Δ24 alleles, respectively, calculated from the available sample for each species.

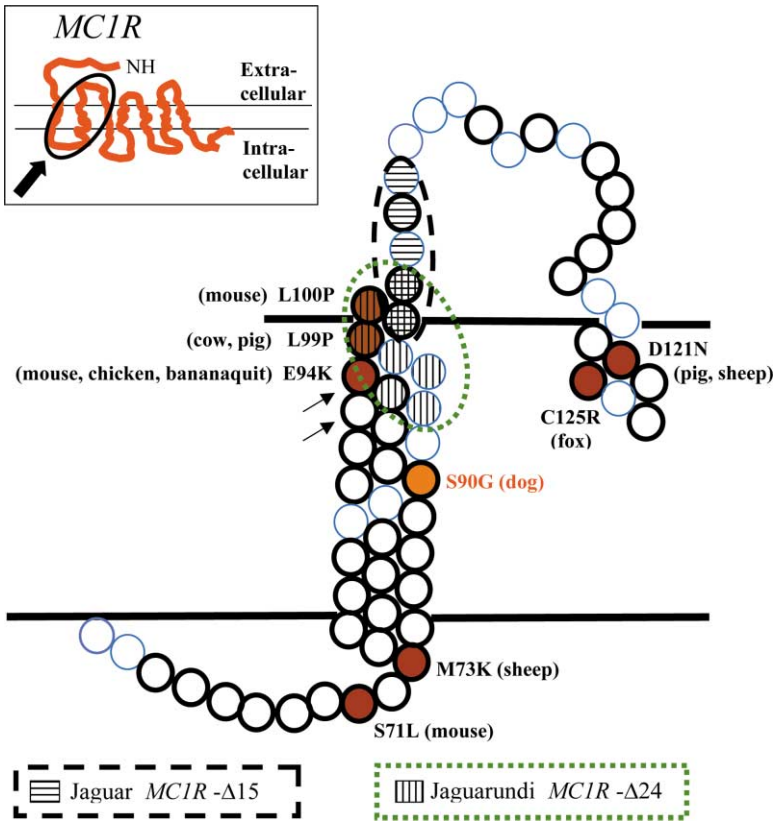


Figure 4. Spatial Distribution of Melanistic Mutations in *MC1R*

Partial diagram of the MC1R protein in the jaguar (*Panthera onca*) *MC1R*-Δ15 allele and the jaguarundi (*Herpailurus yaguarondi*) *MC1R*-Δ24 allele, focusing on the region in which deletions were identified in these variants. The inset in the top left corner shows a schematic view of the whole protein in the melanocyte membrane, and the enlarged segment is defined by an ellipse. The detailed view starts in the first intracellular loop and ends at the beginning of the third transmembrane domain (residues 63–125 in the cat; spatial arrangement adapted from [9], with site numbers based on the cat sequence). Circles bordered by a thick black line represent amino acid residues conserved between the mouse [9] and the deletion alleles shown here; those with a thin blue border are different in at least one of them (see the Supplementary Material for full alignment). Amino acids shaded in brown represent all of those at which substitutions have been previously reported to cause dominant melanistic phenotypes in other species: S71L (E^{tob}), E94K ($E^{so\text{-}3J}$), and L100P (E^{so}) in the mouse [9] (E94K has also been found in melanistic chickens and bananaquits [22, 23]); L99P in cattle [17]; C125R in the red fox [11]; L99P and D121N in the pig [14]; and M73K and D121N in sheep [15]. The replacement S90G (in orange) is potentially associated with melanism in the domestic dog [16]. The hatched residues are those that are deleted in melanistic jaguars (horizontal pattern, deletion encompassed by dashed black line) and jaguarundis (vertical pattern, deletion encompassed by green dotted line). Two residues (grid pattern) are included in both deletions under the alignment scheme shown in Figure 1B. Arrows indicate two residues that are included in the jaguarundi *MC1R*-Δ24 deletion under an alternative alignment option (see Figure 1B and text).

from the ancestral-type sequence at three amino acid positions (P22L, I63V, and Q310R; see the Supplementary Material). These substitutions may influence MC1R activity in the jaguarundi, but their conservative nature (P22L, I63V) and/or the occurrence of identical or similar residues at homologous positions in other mammals (22L in cattle [17]; 310K in humans and mice [8, 9]) suggest that their impact on the protein structure and function is not as significant as that of the deletion itself.

Genotyping of the *MC1R* gene among 29 unrelated jaguarundis sampled across the geographic range of the species (Mexico to Argentina) revealed widespread occurrence of the *MC1R*-Δ24 variant and a dramatic semidominant pattern of association with coat color (Figure 3). Individuals bearing the *MC1R*-Δ24 allele, and particularly those homozygous for it, were consistently darker than the ancestral-type homozygotes, which were exclusively red/reddish in coloration. Using a simplified dominant model (i.e., a 2×2 contingency table) to allow statistical testing with the small available sample size, this association was found to be highly significant ($p < 0.001$, Fisher's exact test). This significance of the association would be even higher under a semidominant model. These results implicate *MC1R*-Δ24 as a derived melanistic variant responsible for jaguarundi coat color polymorphism. Interestingly, the recessive reddish color, which heretofore was considered as mutant among jaguarundis due to its lower incidence, is actually the ancestral-type presentation, based on its *MC1R* genotype.

The results from this study strongly suggest that the *ASIP* and *MC1R* deletions identified here have causative effects on the occurrence of melanism in three different Felidae species, although functional assays will be required to directly establish the biological effects of these variants. The *ASIP*-Δ2 deletion identified in the domestic cat likely leads to complete loss of function, as the protein sequence is totally modified after that position (Figure 1A and the Supplementary Material) and an early stop codon after residue 99 removes most of the biologically critical [20] C-terminal domain. Complete loss of function at *ASIP* has also been associated with recessively inherited extreme melanism in the mouse, rat, and horse [7, 12, 13].

Distinct *MC1R* deletions identified in melanistic jaguars and jaguarundis are associated with a dominant or semidominant effect, respectively (Figures 1 and 3), similar to dominant "gain-of-function" melanistic *MC1R* mutations reported for other mammals [9, 15]. Previously described operative mutations in nonfelid species have been missense substitutions located in the same region of the gene (Figure 4), and they were shown or inferred to cause constitutive activation or increased basal signaling for eumelanin, likely due to conformational changes [9, 11, 14, 17]. In-frame deletions in this region of *MC1R* have not been reported for any species,

and our results indicate that they can have a similar effect on MC1R function. Under the alignment scheme presented in Figure 1B, the observation that the jaguar *MC1R*-Δ15 and the jaguarundi *MC1R*-Δ24 deletions overlap by two amino acids (101L, 102E) suggests a critical role for these residues in mediating MC1R inactivation. Additionally, the adjacent two amino acids (99L, 100L) deleted in the jaguarundi *MC1R* have been previously implicated in melanistic phenotypes in mice, cattle, and pigs (Figure 4), suggesting critical roles for these residues as well. Under an alternative alignment scheme, conserved residues 93L and 94E would have been deleted in this jaguarundi allele (instead of 101L, 102E; see Figures 1B and 4). In this case, the deletion would include three residues in which melanism-implicated substitutions have been previously identified in other species (Figure 4). Given the functional studies performed in other species [9, 15], we infer that these deleted residues are important to maintain an inactive conformation of the MC1R protein, and/or they are critical for binding of the antagonist peptide agouti (ASIP).

The three distinctive melanistic deletions identified here appear to be species specific, as no other surveyed felid was found to carry any of them (Figures 2 and 3). The absence of these variants in melanistic individuals from five other felid species (leopard [*Panthera pardus*], Geoffroy's cat [*Oncifelis geoffroyi*], oncilla [*Leopardus tigrinus*], pampas cat [*Lynchailurus colocolo*], and Asian golden cat [*Catopuma temmincki*]) would suggest that melanism arose independently at least four times in the family Felidae (the three deletions identified here and at least one additional origin for the remaining species) and rose to high population-level frequencies in many cases [1–3]. The elevation of independent gene variants in parallel Felidae lineages raises the possibility of an adaptive advantage of melanistic mutants under certain ecological circumstances. An interesting example is the jaguarundi, whose "wild-type" dark coloration is here shown to be a derived condition, having replaced the ancestral reddish form throughout its continental range. The prospect of directly inspecting gene variants that specify phenotypic variation potentially subject to natural selection will allow the direct study of such traits in free-ranging populations. These and other applications of such integrated genetic approaches will hopefully enhance our understanding of species survival, diversification, and adaptive evolution over space and time.

Experimental Procedures

Conserved PCR primers for the *ASIP* and *MC1R* genes were designed on the basis of available mammalian sequences (see the Supplementary Material [available with this article online and at http://lgd.nci.nih.gov as a link to this paper] for a detailed description of all methods and primary data, including primer sequences, PCR conditions, a full list of samples used in this study, and deletion genotypes for all animals). A domestic cat BAC library [21] was screened to identify clones containing *ASIP* and *MC1R*: BAC clone RPCI86-188e3 was used to characterize the *ASIP* coding region and to identify closely linked STR loci; *MC1R* was characterized from an 8.7 kb subfragment of clone RPCI86-248I10. New primer sets were designed to span each *ASIP* exon and the whole *MC1R* coding region of felids.

Linkage analysis of melanism in domestic cats was performed by using three STRs tightly linked to *ASIP* and an 89-individual pedigree comprising 109 melanism meioses. This pedigree is a subset of the Purina Pedigree, a 259-individual kindred of domestic cats that we have characterized and expanded for use in the construction of reference linkage maps and in the study of coat color genes. Sequencing of the *ASIP* coding region revealed a 2 bp deletion in black domestic cats, and a new primer set (containing a fluorescent label) was designed around its location to allow large-scale genotyping of its presence.

The *MC1R* coding region was sequenced in four melanistic and four nonmelanistic jaguars, as well as two jaguarundis of different colorations. Cloning of the PCR products from melanistic jaguars revealed that they carried a 15 bp deletion allele. Direct sequencing of the jaguarundi *MC1R* revealed that the dark individual was homozygous for a 24 bp deletion adjacent to that found in melanistic jaguars. A single fluorescent genotyping assay for both *MC1R* deletions was developed and applied to a broad sample of each species and other felids.

Supplementary Material

Supplementary Material including a detailed description of the Experimental Procedures (including all PCR primers and conditions), tables with all samples and genotypes included in this study, and figures with ASIP and MC1R alignments is available at http://images.cellpress.com/supmat/supmatin.htm.

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Accession Numbers

The DNA sequences reported here have been deposited in GenBank (accession numbers AY237394–AY237399).