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Who's behind that mask and cape? The Asian leopard cat's *Agouti* (*ASIP*) allele likely affects coat colour phenotype in the Bengal cat breed

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Summary

Coat colours and patterns are highly variable in cats and are determined mainly by several genes with Mendelian inheritance. A 2-bp deletion in *agouti signalling protein* (*ASIP*) is associated with melanism in domestic cats. Bengal cats are hybrids between domestic cats and Asian leopard cats (*Prionailurus bengalensis*), and the charcoal coat colouration/pattern in Bengals presents as a possible incomplete melanism. The complete coding region of *ASIP* was directly sequenced in Asian leopard, domestic and Bengal cats. Twenty-seven variants were identified between domestic and leopard cats, and were investigated in Bengals and Savannahs, a hybrid with servals (*Leptailurus serval*). The leopard cat *ASIP* haplotype was distinguished from domestic cat by four synonymous and four non-synonymous exonic SNPs, as well as 19 intronic variants, including a 42-bp deletion in intron 4. Fifty-six of 64 reported charcoal cats were compound heterozygotes at *ASIP*, with leopard cat *agouti* (A^{Pbe}) and domestic cat non-*agouti* (*a*) haplotypes. Twenty-four Bengals had an additional unique haplotype (*A2*) for exon 2 that was not identified in leopard cats, servals or jungle cats (*Felis chaus*). The compound heterozygote state suggests the leopard cat allele, in combination with the recessive *non-agouti* allele, influences Bengal markings, producing a darker, yet not completely melanistic coat. This is the first validation of a leopard cat allele segregating in the Bengal breed and likely affecting their overall pelage phenotype. Genetic testing services need to be aware of the possible segregation of wild felid alleles in all assays performed on hybrid cats.

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Keywords

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The Bengal cat breed consists of hybrid animals developed from crosses between the domestic cat (*Felis silvestris catus*) and the Asian leopard cat (*Prionailurus bengalensis*) (Johnson 1991). An unusual pelage type involving a darker face 'mask' and a dark dorsal stripe, commonly referred to as a 'cape', is unique to the breed. This 'charcoal' pattern does not produce a fully melanistic cat but bestows darker and more extended markings (Fig. 1), suggesting unusual interactions between melanism and patterning genes in the hybrid cats.

Coat colours and patterns are highly variable in cats but are determined mainly by a few genes with simple modes of inheritance (Lyons 2012). Two genes are associated with felid melanism: *melanocortin-1 receptor* [*MC1R; Extension (E,e)*] and *agouti signalling protein* [*ASIP; Agouti (A,a)*] (Eizirik *et al.* 2003; Schneider *et al.* 2012). In domestic cats, a 2-bpdeletion in exon 2 of *ASIP* causes the recessive melanistic *non-agouti* allele (*a*) (Eizirik *et al.* 2003). Given that the charcoal coat pattern inheritance in Bengals appears to affect eumelanin production, *ASIP* was investigated as a candidate gene for causing this unique Bengal phenotype.

Archival wild felid DNA, including 11 Asian leopard cats (*P.b. bengalensis* and *P.b. euptilura*) (Menotti-Raymond *et al.* 1999), four African servals (*Leptailurus serval*) and two jungle cats (*Felis chaus*) were used in the analysis. Six non-Bengal domestic shorthair or pedigree cats with different *agouti* genotypes were used as controls for the domestic cat sequence. One hundred and forty-eight Bengal cats were included, 64 were reported as charcoal, six had unknown phenotypes, two were solid black and the remaining 76 Bengal cats presented non-charcoal tabby patterns, mostly spotted, rosetted or marbled. Six Savannah cats were also examined. Details of all samples tested are shown in Table S1. A description of the cats' coat colour and pattern were verified with pictures when available. For examples see Fig. 1. The coding region of *ASIP* was amplified by PCR using primers designed with the NETPRIMER software (PREMIER Biosoft International) based on the genomic sequence of the domestic cat available at Ensembl (Accession number AY237394.1) (Table S2). Exons 2 and 3 were amplified in 20 μl containing 1.75 mM of $MgCl_2$, 0.2 mM of dNTPs, 1μM each of the forward and reverse primers and 0.75 U of Choice-Taq™ DNA Polymerase (Denville Scientific Inc.). A touchdown profile was used for thermal cycling with annealing temperature decreasing from 62–58 °C in nine cycles, followed by 40 cycles with annealing at 57 °C and a final extension at 72 °C. Exon 4 was amplified using one forward primer and two reverse primers. One reverse primer was designed to be specific to the leopard cat allele, as the domestic cat reverse primer used for PCR overlapped a variable region in the leopard cat sequence, resulting in preferential amplification of the domestic cat allele in hybrid cat samples. The final volume of 20 μl contained 2.3 μl of 10x PCRx Enhancer Solution (Invitrogen,), 2.0 mM of $MgCl_2$, 0.7 mM of dNTPs, 0.2 μM each of the forward and reverse primers and 1.3 U of Choice-Taq™ DNA Polymerase (Denville Scientific Inc.). PCR products were purified, directly sequenced and analysed as previously described (Gandolfi

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et al. 2013). Sequences for the different wild felid alleles were submitted to GenBank (Accession numbers: KJ395758—KJ395774).

ASIP coding exons 2–4 were sequenced in the domestic, Bengal and leopard cats. Eight exonic variants distinguished Asian leopard cats from the domestic cat (Table S1). The amino acid translation and alignment is depicted in Fig. S1. Four SNPs, one in exon 3 and three in exon 4, were synonymous. Two SNPs in exon 2 (c.41G>C, p.Cys14Ser and c.142T>C, p.Ser48Pro) and two in exon 4 (c.251A>G, p.Gln84Arg and c.302A>G, p.Asp101Gly) were non-synonymous (Table 1). No leopard cat had the 2-bp deletion *non-agouti* mutation in exon 2. Leopard cats also showed 19 fixed intronic variants, including two single base deletions, one single base insertion, 15 SNPs and a 42-bp deletion in intron 4 (Table S1). All leopard cats tested were homozygous for these variants as well as the eight exonic SNPs, thus presenting a leopard cat-specific haplotype. An intron 2 SNP, c.160+86T>C, and an intron 3 SNP, c.222+85C>T, differentiated the two subspecies *P.b. bengalensis* and *P.b. euptilura* (Table S1). A synonymous SNP in exon 4, c.375C>T, was identified in Bengal cats ($n = 12$) only.

Fifty-six of 64 charcoal Bengals were heterozygous at *ASIP*, with one domestic cat *non-agouti* haplotype (*a*) and one leopard cat haplotype (A^{Pbe}). Eight Bengals, submitted as charcoal in coloration, had different haplotype combinations. Three cats were homozygous for the leopard cat haplotype and one was heterozygous with a domestic cat *agouti* haplotype (*A*), thus explaining their unique colouration, though not specifically conforming to the charcoal pelage phenotype. The coat colour and pattern of the four remaining discordant cats could not be verified through visual assessment. These cats were heterozygous for the domestic cat *agouti* haplotypes (*A, a*). Eight of 78 Bengal cats submitted as non-charcoal were homozygous or heterozygous for the leopard cat *ASIP* haplotype (A^{Pbe}) and the wild-type domestic haplotype (*A*).

Genotyping of Bengals by pyrosequencing for the *agouti* mutations, with primers given in Table S3, identified a previously unknown variant (c.127A>G). Sequencing of exon 2 in the variant Bengal cat identified non-synonymous SNPs c.41G>C, c.110-111GG>AA and c.127A>G, resulting in amino acid changes p.Cys14Ser, p.Arg37Lys and p.Asn43Asp respectively and a non-synonymous SNP at c.156T>A. These variants produced a unique haplotype for *ASIP* exon 2 (termed A2). Twenty-four non-charcoal Bengals and one Savannah carried the A2 with either the domestic cat wildtype *agouti* (*A*) or *non-agouti* (*a*) haplotypes. Because other hybrid cat breeds, such as Savannah and Chaussie, have been occasionally introgressed into the Bengal breed, exon 2 was sequenced in five servals and two jungle cats, the two wild felids used to produce these cat breeds respectively (Morris 1999). The A2 haplotype was not detected in the wild felids, therefore its origin is unknown.

The Bengal cat breed was developed in the early 1970s (Johnson 1991; Morris 1999) and has become one of the most popular breeds worldwide. Most, but not all, worldwide cat associations recognize Bengals, and some registries recognize the Savannah and Chaussie hybrid breeds. For competition in cat shows, hybrid cats cannot have a parent from a non-domestic species within four generations (TICA 2013), suggesting less than 6.125% average genomic contribution from any wild felid species. However, because early generation male

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hybrids are infertile, females are backcrossed to either domestic cats or, more commonly, male hybrids of later generations. The hybrid to hybrid crosses, combined with no mandatory parentage verification in cat breeding, makes the prediction of wild felid 'blood' in these hybrids difficult. Although selection within the breed for colourations similar to the wild felids is strong, many domestic alleles, such as *Inhibitor* (*I*) (Turner & Robinson 1980), Siamese (c^s) and Burmese points (c^b) (Lyons *et al.* 2005b), brown variants (*b*, b^l) (Lyons *et al.* 2005a) and dilution (*d*) (Ishida *et al.* 2006), have entered the Bengal breed, thereby producing unique colourations not characteristic of wild felids. These colouration genes often confound proper phenotyping. The novel combination of alleles under artificial selection by domestic breeders with those from other felid species offer the possibility to detect interesting and unique colourations in hybrid cats not seen in other domesticated animals.

The data presented strongly supports the charcoal presentation as a compound heterozygote (A^{Pbe}/a) of the leopard cat *agouti* allele (A^{Pbe}) and the domestic cat *non-agouti* melanism allele (*a*). The allelic relationships of *ASIP-*A^{Pbe} with *ASIP-A* and *ASIP-a* are not fully understood, and more systematic studies are needed to determine the mode of inheritance for charcoal. Based on the cats used in this study, *ASIP-*A^{Pbe} is not fully dominant over *ASIP-a*, as both alleles appear to contribute to the charcoal phenotype. An additional unique colouration is also possible, given that 24 Bengals and one Savannah submitted for colour testing had *ASIP* exon 2 mutations not found in domestic or wild felids examined. Therefore, other interesting phenotypes may occur when the hybrid cats have *agouti* haplotypes from different species. The unique exon 2 haplotype (*ASIP-A2*) may have originated from another species or subspecies, or be a highly divergent domestic cat haplotype. This report presents the first validation of a leopard cat allele segregating in the Bengal cat breed that is likely to affect the overall phenotype of the pelage. Other wild felid specific variants will likely affect tabby patterning (Kaelin & Barsh 2010) as well as other aspects of colouration and morphological variation, each potentially affecting the accuracy of genetic tests. If used as a genetic test, Bengal breeders can more efficiently select for the desired charcoal colouration. All genetic testing in hybrid cats should be performed with caution, as the wild felid alleles could be present and disrupt the accuracy of test assays.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Charcoal colouration pattern in the Bengal cat breed. Charcoal markings (A and C) in a silver (Inhibitor; I-) and (B) a brown bengal cat (ii). Note the dark face mask with white or nearly white lines around the eyes and very dark dorsal cape. (Pictures courtesy of Terra Sinclair, Pocket Bengals)

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Table 1

Non-synonymous *ASIP* variants observed in domestic cats, wild felids and hybrid cats

Felid	Phenotype[1]	No.[2]	Genotype[3]	Exon 2					Exon 4	
				c.41G>C	c.110-111 GG>AA	c.123-124 2-bp del[4]	C.127A>G	c.142T>C	c.251A>G	C.302 A>G
				p.Cys14Ser	p.Arg37Lys		p.Asn43Asp	p.Ser48Pro	p.Gln84Arg	p.Asp101Gly
Domestic	Wildtype	2	*A/a*	G/G	GG/GG	CA/–	A/A	T/T	A/A	A/A
Domestic	Solid	1	*a/a*	G/G	GG/GG	–/–	A/A	T/T	A/A	A/A
British SH	Wildtype	1	*A/a*	G/G	GG/GG	CA/–	A/A	T/T		
Siamese	Solid	1	*a/a*	G/G	GG/GG	–/–	A/A	T/T		
Australian Mist	Wildtype	1	*A/A*	G/G	GG/GG	CA/CA	A/A	T/T		
Leopard cat	Wildtype	11	A^{Pbe}/A^{Pbe}	C/C	GG/GG	CA/CA	A/A	C/C	G/G	G/G
Serval	Wildtype	5	A^{Lse}/A^{Lse}	C/C	GG/GG	CA/CA	A/A	C/C		
Jungle Cat	Wildtype	2	A^{Fch}/A^{Fch}	C/C	AG/AG	CA/CA	A/A	T/T		
Bengal	Solid	2	*a/a*	G/G	GG/GG	–/–	A/A	T/T		
Bengal	Charcoal	56	a/A^{Pbe}	G/C	GG/GG	CA/-	A/A	T/C	A/G	A/G
Bengal	Non-charcoal	5 (3)	A^{Pbe}/A^{Pbe}	C/C	GG/GG	CA/CA	A/A	C/C	G/G	G/G
Bengal	Non-charcoal	7 (1)	A/A^{Pbe}	G/C	GG/GG	CA/CA	A/A	T/C	A/G	A/G
Bengal	Non-charcoal	24 (4)	*A/a*	G/G	GG/GG	CA/–	A/A	T/T	A/A	A/A
Bengal	Unknown	1	*A/a*	G/G	GG/GG	CA/–	A/A	T/T	A/A	A/A
Bengal	Non-charcoal	26	*A/A*	G/G	GG/GG	CA/CA	A/A	T/T	A/A	A/A
Bengal	Unknown	3	*A/A*	G/G	GG/GG	CA/CA	A/A	T/T	A/A	A/A
Bengal	Unknown	2	*A/A2*	G/C	GG/AA	CA/CA	A/G	T/T		
Bengal	Non-charcoal	17[5]	*A/A2*	G/C	GG/AA	CA/CA	A/G	T/T		
Bengal	Non-charcoal	5	*a/A2*	G/C	GG/AA	CA/–	A/G	T/T		
Savannah	Wildtype	1	*A/A2*	G/C	GG/AA	CA/CA	A/G	T/T		
Savannah	Wildtype	2	*A/A*	G/G	GG/GG	CA/CA	A/A	T/T		
Savannah	Wildtype	2	a/A^{Lse}	G/C	GG/GG	CA/–	A/A	T/C		
Savannah	Wildtype	1	A^{Lse}/A^{Lse}	C/C	GG/GG	CA/CA	A/A	C/C		
TOTAL		178								

[1] Wildtype implies a tabby pattern that is most common to the given felid species. Domestics could be *mackerel, blotched* or *spotted*. Leopard cats and servals are spotted, jungle cats have no pattern.

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[2]The number of cats reported as charcoal but having different genotypes is presented in parentheses; eight cats were discordant, including three as A^{Pbe}/A^{Pbe}, one as A/A^{Pbe}, and four as *A/a*. Other Leopard cat alleles could be segregating in these cats and further modifying their coat colors and patterns, which may alter tones in coloration and extent of *Tabby* markings, especially genes such as *MC1R*.

[3]These SNPs form a consistent haplotype and are considered the wildtype (wt^+) *agouti* alleles for leopard cat (A^{Pbe}), serval (A^{Lse}), and jungle cat (A^{Fch}). The '*A2*' allele represents the unique haplotype identified in Bengals and Savannahs that does not appear to be domestic or from the three wild felids examined. The two adenine mutations, c.110 and c.111, cause an amino acid change from arginine to lysine in the *A2* allele.

[4]Deletion causes frameshift and downstream stop codon in domestic cats causing the common non-*agouti* allele (*a*) in domestic cats (Eizirik *et al.* 2003).

[5]Cats were genotyped by pyrosequencing in exon 2 for c.41G>C, the indel, c.127A>G and c.142T>C only (Table S1).

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