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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£be) 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.

Keywords charcoal, domestic cat, felid, Felis silvestris catus, hybrid, jungle cat, pelage, serval

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-bp deletion 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. 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 MgCl₂, 0.2 mM of dNTPs, 1 µM 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 to 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 10× PCRx Enhancer Solution (Invitrogen), 2.0 mM of MgCl₂, 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 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-syn-
ononymous (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 exon 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 colouration, 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, although 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>A and c.127A>G, resulting in amino acid changes p.Cys14Ser, p.Arg37Lys and p.Asp101Gly.

### Table 1 Non-synonymous ASIP variants observed in domestic cats, wild felids and hybrid cats.

| Felid    | Phenotype | No. | Genotype^2 | Exon 2                                      | Exon 4                                      |
|----------|-----------|-----|------------|---------------------------------------------|---------------------------------------------|
|          |           |     | c.41G=C    | c.110-111                                    | c.129-134                                   |
|          |           |     | p.Cys14Ser | GG→AA                                       | 2-bp del^1                                  |
|          |           |     |            | AA c.123-124                                  | 2-p.Ter48Pro                                 |
| Domestics| Wild type | 2   | A/a        | G/G                                          | A/A                                         |
|          |           |     |            | GG/GG                                        | T/T                                         |
|          | Solid     | 1   | a/a        | G/G                                          | A/A                                         |
|          |            |     |            | GG/GG                                        | T/T                                         |
| British SH| Wild type | 1   | a/a        | G/G                                          | A/A                                         |
|          |            |     |            | GG/GG                                        | T/T                                         |
| Siamese  | Solid     | 1   | a/a        | G/G                                          | A/A                                         |
|          |            |     |            | GG/GG                                        | T/T                                         |
| Australian Mist| Leopard cat | Wild type | 11 | A^Pbe/A^Pbe | C/C                                       | G/G                                         |
| Serval   | Wild type | 5   | A^Lew/Lew | C/C                                          | A/C                                         |
| Jungle   | Wild type | 2   | A^Ch/A^Ch | C/C                                          | T/T                                         |
| Cat      | Bengal    | Solid | 2   | a/a        | G/G                                          | A/A                                         |
|          | Bengal    | Charcoal | 56  | A^Pbe/A^Pbe | C/C                                       | A/A                                         |
|          | Bengal    | Non-charcoal | 5 (3) | A^Pbe/A^Pbe | C/C                                       | A/A                                         |
|          | Bengal    | Non-charcoal | 7 (1) | A^Pbe/A^Pbe | C/C                                       | A/A                                         |
|          | Bengal    | Non-charcoal | 24 (4) | A/a       | G/G                                          | A/A                                         |
|          | Bengal    | Unknown | 1   | A/a        | G/G                                          | A/A                                         |
|          | Bengal    | Non-charcoal | 26  | A/A        | G/G                                          | A/g                                         |
|          | Bengal    | Unknown | 3   | A/A        | G/G                                          | A/A                                         |
|          | Bengal    | Unknown | 2   | A/A2       | G/C                                          | A/A                                         |
|          | Bengal    | Non-charcoal | 17^4 | A/A2      | G/C                                          | A/A                                         |
|          | Bengal    | Non-charcoal | 5  | a/A2       | G/C                                          | A/A                                         |
| Savannah | Wild type | 1   | A/A2       | G/G                                          | A/A                                         |
| Savannah | Wild type | 2   | A/A        | G/G                                          | A/A                                         |
| Savannah | Wild type | 2   | a/A^Lew    | G/G                                          | A/A                                         |
| Savannah | Wild type | 1   | A^Lew/Lew  | C/C                                          | A/C                                         |
| TOTAL    |           |     |            |                                              |                                              |

^1Wild type implies a tabby pattern that is most common to the given felid species. Dominestics could be mackerel, blotched or spotted. Leopard cats and servals are spotted; jungle cats have no pattern.

^2The 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 colours and patterns, which may alter tones in colouration and extent of tabby markings, especially genes such as MC1R.

^3These SNPs form a consistent haplotype and are considered the wild-type (wt) agouti alleles for leopard cat (A^Pbe), serval (A^Lew) and jungle cat (A^Ch). 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.

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

^5Cats 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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alleles, such as agouti (A) or non-agouti (a) haplotypes. Because other hybrid cat breeds, such as Savannah and Chausie, have been occasionally intergressed into the Bengal breed, epoxy 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 recognise Benga, and some registries recognise the Savannah and Chausie hybrid breeds. For competition in cat shows, hybrid cats cannot have a parent from a non-domestic species within four generations (TICA 2013), suggesting ≤6.125% genome contribution from any wild felid species. However, because early generation male 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 colouration 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^t) (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 support the charcoal presentation as a compound heterozygote (A^Phe/a) of the leopard cat agouti allele (A^Phe) and the domestic cat non-agouti melanism allele (a). The allelic relationships of ASIP-A^Phe 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^Phe 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 Benga, 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 (Kuelin & 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.

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**Supporting information**

Additional supporting information may be found in the online version of this article.  
Table S1. ASIP genotypes of individual cats.  
Table S2. Primers for analysis of ASIP in hybrid cats and wild felids.  
Table S3. Pyrosequencing primers for ASIP analysis.  
Figure S1. Agouti-signaling protein (ASIP) alignment for domestic and wild felids.