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Padrões de variabilidade do gene *ASIP* (*Agouti Signaling Protein*) em mamíferos

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ÍNDICE

1. RESUMO

O melanismo em mamíferos decorre principalmente da atividade de dois genes: *MC1R* (*Melanocortin-1 Receptor*), cujo produto induz a produção de eumelanina (preto-marrom); e *ASIP* (*Agouti Signaling Protein*), que codifica um peptídeo antagonista que promove a produção de feomelanina (pigmento claro). A combinação do efeito destes dois locos faz com que o pêlo cresça escuro com bandas subapicais amarelas. No presente estudo investigamos a diversidade nucleotídica e os padrões de variabilidade presentes no gene *ASIP*, principalmente nas regiões codificadoras dos éxons 2 e 3 e em regiões não codificadoras dos íntrons 2 e 3 em alguns mamíferos, com ênfase em felídeos (Mammalia, Carnivora, Felidae). Através do alinhamento entre as espécies analisadas nesse estudo foi possível construir três bases de dados que foram divididas em diferentes blocos conforme as regiões de alinhamento. A análise comparativa de seqüências permitiu a caracterização de diferentes blocos de seqüência conservada, assim como a identificação de uma inserção SINE presente apenas no gato doméstico, de uma região repetitiva hipervariável em todos os felídeos analisados, e também de variantes moleculares (SNPs) em *Felis catus* e *Leopardus geoffroyi*. Nenhum dos polimorfismos identificados nesta espécie estava localizado em regiões exônicas ou apresentou associação a fenótipos de coloração, indicando que as regiões analisadas não estão envolvidas na indução do melanismo nesta espécie.

Palavras-chave: melanismo, *ASIP*, eumelanina, feomelanina, antagonista, felídeos e mamíferos.

2. APRESENTAÇÃO DO TEMA

2.1. Bases genéticas da coloração do pêlo em mamíferos

A diversidade de cores exibidas por mamíferos, e suas bases genéticas, atraíram ao longo da história a atenção de muitos cientistas (Darwin 1883; Beddard 1895; Fisher 1930; Cott 1940). Variações fenotípicas naturais foram usadas para propor teorias clássicas sobre a adaptação de mamíferos, e sua relevância comportamental e ecológica tem sido explorada em vários aspectos (Cott 1940; Ortolani & Caro 1996). Por muito tempo, variantes de coloração naturais e induzidas foram utilizadas como marcadores genéticos em modelos animais na era pré-molecular, tendo importância crucial em esforços iniciais para o mapeamento de genes e a caracterização de processos genômicos (Silvers 1979). Por exemplo, variantes na cor do pêlo constituíram a primeira característica de mamíferos analisada com genética Mendeliana (Castle e Allen 1903).

O uso dessas características como marcadores levou ao acúmulo de uma grande quantidade de conhecimentos sobre a genética, bioquímica, fisiologia e biologia molecular dos processos envolvidos na determinação da cor dos pêlos em camundongos (*Mus musculus*) (Silvers 1979; Jackson 1994; Barsh 1996). Vários genes envolvidos na produção e distribuição de pigmentos em camundongos foram caracterizados em nível molecular (Jackson 1994; Barsh 1995, 1996). Demonstrou-se que eles participam de diversos processos celulares, fisiológicos e do desenvolvimento embriológico, e em muitos casos estão implicados em doenças como anemia, esterilidade e problemas neurológicos (Silvers 1979; Fleischman 1993; Jackson 1994). Homólogos humanos de muitos desses genes foram identificados e caracterizados, e em vários casos estão

associados com condições patológicas como hipopigmentação (Fleischman 1993) e albinismo oculocutâneo (Manga et al. 1997). Seu papel na variação da cor da pele e dos cabelos em humanos é ainda pouco conhecido, mas as evidências disponíveis indicam que interações complexas e formas diferentes de seleção envolvendo alguns desses mesmos genes podem ser responsáveis pela variação fenotípica observada em nossa espécie (Barsh 1996; Box et al. 1997; Rana et al. 1999; Harding et al. 2000; Rees 2000; Sturm et al. 2001). Homólogos para esses genes têm sido também identificados e caracterizados em outras espécies, e relacionados com a cor de pêlos e/ou fenótipos patológicos semelhantes aos observados em camundongos e humanos (p. ex. Klunglund et al. 1995; Joerg et al. 1996; Kijas et al. 1998, 2001; Metallinos et al. 1998; Marklund et al. 1999). Atualmente diversos estudos abordam as bases moleculares dos fenótipos ligados à coloração dos pêlos em múltiplas espécies, tentando investigar aspectos da sua história evolutiva e significância adaptativa. Para obter-se um conhecimento mais detalhado, estão sendo feitos estudos sobre a estrutura, função, regulação e integração desses genes envolvidos na pigmentação.

2.2. Genes envolvidos na regulação do melanismo

O melanismo é um fenômeno presente em muitas formas de vida e tem sido amplamente definido como uma situação em que ocorre uma acentuada produção de melanina escura, gerando uma coloração escurecida no tegumento do organismo (Majerus 1988). A pigmentação de mamíferos é regulada por mais de 120 genes, os quais codificam proteínas envolvidas na migração e diferenciação dos melanócitos (células especializadas localizadas na base da epiderme e nos folículos pilosos) bem como na regulação de diferentes pontos da rota melanogênica. A melanogênese folicular envolve seqüencialmente a atividade melanogênica dos melanócitos foliculares, e a

transferência de grânulos de melanina para dentro dos queratinócitos. Esta atividade é regulada por um conjunto de enzimas, proteínas estruturais e regulatórias, transportadoras e receptores com seus ligantes, ativando o desenvolvimento de estágios celulares e níveis foliculares do pêlo (fig. 1) (Prota, 1992).

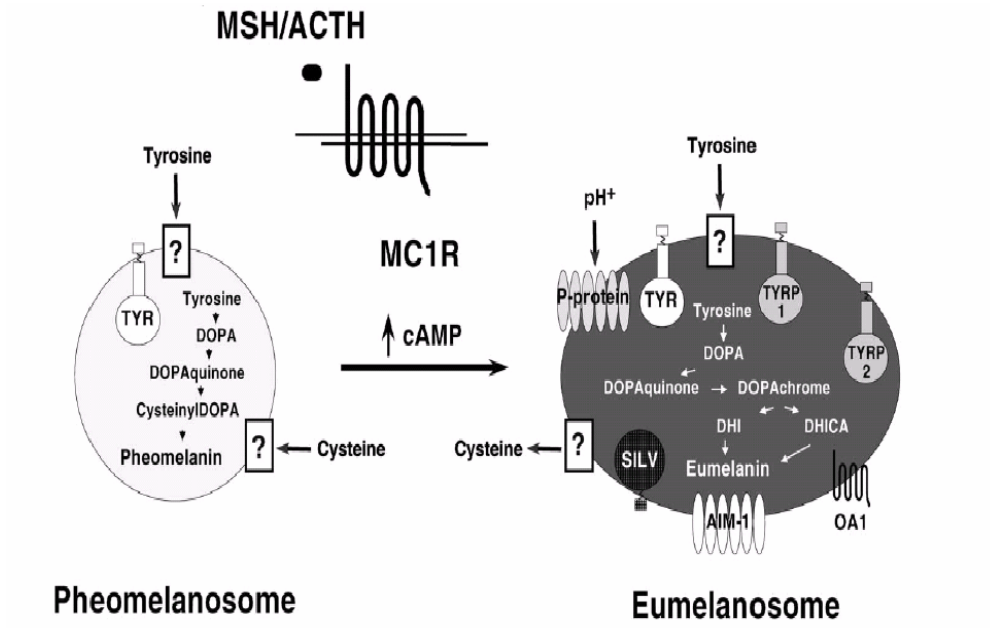


Fig. 1 – Rota melanogênica – Estrutura, identificação e funcionamento de organelas responsáveis pela produção de diferentes pigmentos no folículo piloso, (Sturm et al. 2001).

A principal enzima atuando neste processo é a tirosinase, que catalisa a hidrolização da tirosina para 2,3,4 dihidroxifenilalanina, e a posterior oxidação para dopaquinona. Os padrões de velocidade e especificidade das reações são reguladas pela tirosinase e duas proteínas relacionadas (*tyrosinase related proteins* [TRP] 1 e 2) (Korner e Pawelek, 1982; Hearing, 1999). A estrutura da proteína tirosinase é altamente conservada entre diferentes espécies e existe alta similaridade com as outras TRPs. Em camundongos, a TRP1 age na oxidação do 5,6 dihidroxindole ácido carboxílico (DHICA), e parece ser particularmente importante para a eumelanogênese (formação de pigmento escuro). A TRP2 pode agir como Dopacromo Tautomerase, catalizando a

transformação da dopacromo para DHICA, e também parece atuar de forma predominante na síntese do pigmento escuro. Assim sendo, o aumento na expressão e atividade destas enzimas do grupo TRP parece ser fundamental para a acentuação preferencial da rota eumelanogênica, podendo culminar na ocorrência do melanismo.

Dos diversos *loci* envolvidos na pigmentação, dois deles atuam primariamente na regulação da troca entre eumelanina (escura) e feomelanina (clara), os quais eram originalmente conhecidos como *agouti* e *extension* (quando identificados por estudos clássicos em camundongos), e hoje são identificados como *ASIP* e *MC1R*, respectivamente. Ambos foram bem caracterizados em nível molecular em camundongos (Bultman et al. 1992; Mountjoy et al. 1992; Robbins et al. 1993; Perry et al. 1996). Nesta espécie, a sinalização pelo Hormônio Estimulador de Melanócitos- α (α-MSH) ou *ASIP* através do *MC1R* regula o tipo de melanina produzida, sendo que a interação do α-MSH com *MC1R* resulta em produção de eumelanina (fig. 2) (Voisey e Daal, 2002).

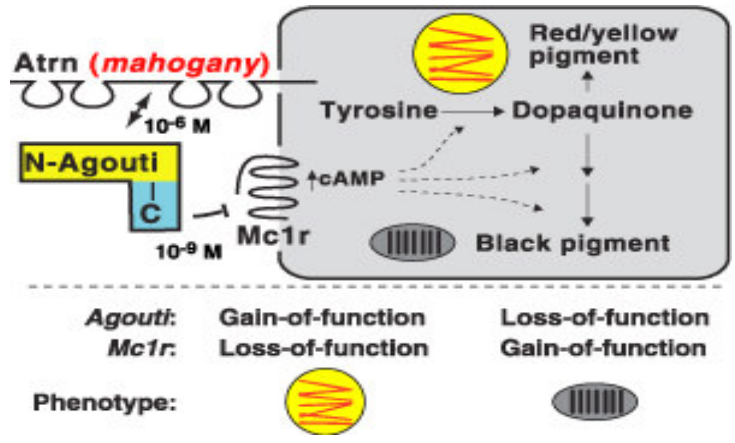


Fig.2 – Esquema demonstrando os dois genes principais – *ASIP* e *MC1R* envolvidos na rota melanogênica (Barsh SG. et al. 2002).

O gene *agouti* (*ASIP*) em camundongos está localizado no cromossomo 2, e é descrito como codificador de uma proteína que possui 131 aminoácidos consistindo de um peptídeo sinal, um domínio central básico e uma região C-terminal rica em cisteína. A troca entre eumelanogênese e a feomelanogênese envolve efeitos opostos de *ASIP* e α-MSH como ligantes para o receptor MC1R (Rouzaud e Hearing 2004). O gene *agouti* do camundongo é composto de três éxons codificadores (contendo 170pb, 65pb e 385pb, respectivamente), ocupando uma região genômica de aproximadamente 5Kb, ficando cerca de 3 a 4 éxons adicionais distantes da região 5' do sítio de iniciação (Siracusa1994). A região gênica total consiste de cerca de 110Kb (fig.3) (Bultman et al. 1994).

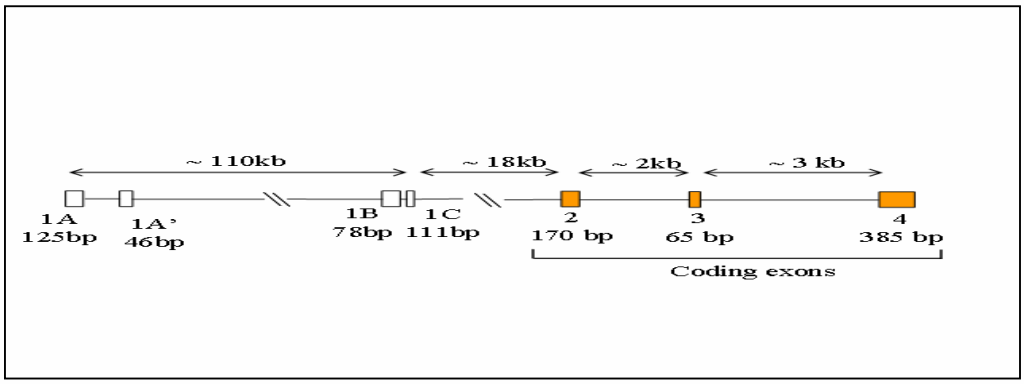


Fig. 3- Estrutura do gene *ASIP* em camundongos (modificado a partir de Bultman 1992, 1994).

Em outras espécies já estudadas, o gene *ASIP* varia quanto ao número de aminoácidos codificados, porém todos apresentam três éxons codificadores, em uma estrutura geral semelhante à descrita para o camundongo. No gato doméstico (*Felis catus*), este gene está localizado no cromossomo A3, e codifica uma proteína com 135 aminoácidos (Eizirik et al. 2003).

A proteína *ASIP* regula a pigmentação em folículos pilosos de camundongo produzindo um pêlo preto com uma banda subapical amarela que pode também ser

observado em muitos outros mamíferos incluindo a família de felídeos. Alelos recessivos na região codificadora do *agouti* ocasionam pêlo de cor escura e alelos dominantes, resultam em cor amarela e, no caso de expressão ectópica, fenótipos como obesidade, diabetes tipo II, aumento somático no crescimento e tumorigênese (Yen et al. 1994).

ASIP foi seqüenciado e estudado em algumas espécies de mamíferos e em vários casos a identificação de seqüências variantes foi associada a fenótipos envolvidos no melanismo ou outras características fisiológicas (p. ex. Joerg et al. 1996; Marklund et al. 1996; Vage et al. 1997; Everts et al. 2000; Newton et al. 2000). Variantes do gene *ASIP* foram implicadas em fenótipos melânicos em raposas vermelhas (Vage et al. 1997), ratos (Kuramoto bet al. 2001) e cavalos (Rieder et al. 2001), além das mutações originalmente descritas em camundongos. A abundância de exemplos de alterações nas mutações de cor de pêlos fazem destes genes bons candidatos para estudos das bases genéticas do melanismo em outras linhagens de mamíferos.

2.3. Variação genética da cor de pêlos na família Felidae

Entre os mamíferos, a família Felidae é um grupo de extrema importância para o estudo da cor de pêlos e sua história evolutiva. Variações marcantes entre espécies e polimorfismo intra-específico são observados em gatos domésticos e felinos selvagens, o que serviu de base para hipóteses de adaptação, biogeografia e associações ecológicas (Beddard 1895; Cott 1940; Weigel 1961; Kitchener 1991; Ortolani & Caro 1996). A ocorrência de pigmentação polimórfica é comum em gatos domésticos, incluindo variação na cor de fundo (do branco ao amarelo, cinza, vermelho ou marrom escuro) e também na presença, forma, coloração e distribuição de manchas (p. ex. pintas, listas, rosetas ou ocelos). Em várias espécies de felídeos a aparente segregação geográfica de

cores polimórficas foi utilizada para descrever diferentes "subespécies" ou populações locais historicamente distintas. Em vários casos é plausível supor que estas variações sejam produzidas por adaptação local a ambientes distintos, sendo assim importantes componentes na história de vida dessas espécies.

Das 37 espécies de felídeos, 12 comprovadamente apresentam melanismo como uma variante de coloração (Eizirik 2002). Até o momento o gene *ASIP* foi somente seqüenciado no gato doméstico (*Felis catus*) e o *MC1R* em *F. catus*, onça pintada (*Panthera onca*) e jaguarundi (*Puma yagouaroundi*), nos quais foram identificadas variantes moleculares associadas ao melanismo (Eizirik et al. 2003). Entre os animais observados, o que pode constatar-se é que ocorreu uma deleção de dois nucleotídios no gene *ASIP* em *Felis catus* pretos. No gene *MC1R*, duas deleções foram identificadas: uma deleção de 5 códons (sem alteração no quadro de leitura) em *Panthera onca* melânicas, as quais podem ser homozigotas ou heterozigotas (o que permitiu verificar que esse fenótipo apresenta herança dominante); e a segunda em *Puma yagouaroundi* de pelagem escura, onde notou-se a deleção de 8 códons (com padrão de herança semi-dominante).

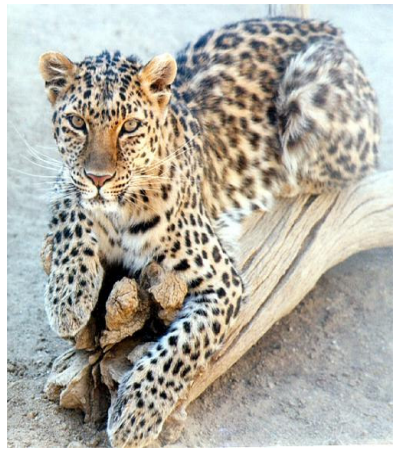
Estudos semelhantes ainda não foram realizados para outras espécies de felídeos; para algumas delas, inclusive em casos de ocorrência de melanismo, pode-se excluir a ocorrência das mutações descritas acima (Eizirik et al. 2003); entretanto a caracterização completa destes genes candidatos não foi realizada até o momento. Cinco espécies de felídeos foram o alvo principal deste estudo: o gato-doméstico, o gato-do-mato-pequeno (*Leopardus tigrinus*), o gato-do-mato-grande (*L. geoffroyi*), o gato-palheiro (*L. colocolo*) e o leopardo (*Panthera pardus*). Fenótipos melânicos são observados em todas estas espécies, e a ocorrência desta variação não pode ser atribuída às mutações já descritas, exceto no caso do gato doméstico (Eizirik et al. 2003). Assim

sendo, é relevante investigar a variabilidade de seqüência nos genes candidatos para este fenótipo, a fim de buscar possíveis variantes associadas ao mesmo.

Leopardus geoffroyi
(www.kedi.ws)

Leopardus tigrinus
(procosara.org/images/leopardus_tigrinus)

Leopardus colocolo
(www.kedi.ws)



Panthera pardus

Felis catus

(http://home.vicnet.net.au/~neils/africa/leopard.htm) (www.sierrabaza.org)

3. OBJETIVOS

O presente estudo teve como finalidade principal a caracterização do gene *ASIP* em alguns mamíferos com ênfase em espécies de felídeos, buscando investigar a diversidade nucleotídica presente neste *locus*, seus padrões de variabilidade, e testar sua associação com fenótipos de coloração polimórficos observados nestes organismos.

3.1 OBJETIVOS ESPECÍFICOS

3.1.1. A partir de amostras de DNA genômico, amplificar por PCR e caracterizar em nível de seqüência os exons 2 e 3, juntamente a segmentos intrônicos associados, do gene *ASIP* em um ou mais indivíduos de *F. catus*, *P. pardus, L. tigrinus*, *L. geoffroyi* e *L. colocolo.*

3.1.2. Caracterizar a diversidade genética e os padrões de variabilidade presentes neste *locus* em algumas espécies de mamíferos, através de comparações evolutivas em diferentes níveis de profundidade filogenética, utilizando as seqüências de felídeos geradas nesse estudo em combinação com outras já disponíveis.

3.1.3. Comparar seqüências deste gene entre indivíduos melânicos e não-melânicos de *L. geoffroyi* e *L. colocolo*, buscando testar a ocorrência de polimorfismos associados ao melanismo.

4. ARTIGO

Genetics and Molecular Biology

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PATTERNS OF NUCLEOTIDE VARIABILITY IN THE *AGOUTI SIGNALING PROTEIN* (*ASIP*) GENE IN MAMMALS

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Abstract

The regulation of coat color in mammals is controlled mainly by the action of two genes, *MC1R (Melanocortin-1 receptor)*, responsible for inducing the production of dark pigment (eumelanin), and *ASIP (Agouti Signaling Protein)*, which codes for an antagonistic peptide whose action promotes the formation of light pigment (feomelanin). The understanding of the structural characteristics of these loci and of the evolutionary processes influencing their patterns of conservation is still in its infancy, with few species analyzed so far, and no broad comparison available. In this paper we analyzed different regions of the *ASIP* gene in several mammalian species, with emphasis on the family Felidae (Mammalia, Carnivora). Our goals were to characterize patterns of variability and conservation in the surveyed regions of *ASIP*, and also to investigate the possibility of molecular variants at this locus being associated with melanistic phenotypes in the Geoffroy's cat (*Leopardus geoffroyi*). Complete sequences of exons 2 and 3, intron 2, and part of intron 3, were obtained for a total of five different felid species, in some cases represented by multiple individuals each. These sequences were compared to each other and to two canid species (Carnivora, Canidae) and one representative each of the mammalian orders Cetartiodactyla and Perissodactyla. Our results allow the identification and characterization of several conserved sequence blocks in intronic regions, which present similar or higher levels of sequence conservation when compared to coding regions. The boundaries of these conserved blocks were characterized through comparisons at increasing levels of evolutionary depth, allowing the identification of core areas maintained over *ca.* 90 million years of

evolution, and possibly involved in regulatory activities. No exonic polymorphism was observed in *L. geoffroyi*, including melanistic individuals, indicating that exons 2 and 3 are not involved in this phenotype in this species.

Introduction

Melanism is a phenomenon present in many life forms and has been broadly defined as a situation where an overabundance of skin pigmentation occurs, generating a darkened external coloration (Majerus 1998). The pigmentation of mammals is the result of the action of more than 100 genes that influence the production and distribution of melanins in the skin, fur and eyes (Girardot et al. 2006). Biosynthesis of these pigments is a process predominantly controlled by the action of three enzymes of the tyrosinase family: TYR, TYRP-1 and TYRP-2. These enzymes are directly involved in the synthesis of dark pigment (eumelanin, with black or brown coloration), although the former (TYR) also plays a role in the genesis of feomelanin (light pigment: yellow or reddish). Both types of pigment are complex polymers that are produced and accumulated in specialized organelles present in melanocytes, known as melanosomes. Melanin-containing melanosomes are transferred from melanocytes to skin keratinocytes or to the growing hair shaft. The overall coat color depends mainly on the relative amount and distribution of eumelanin and pheomelanin in hair shafts throughout the body.

The synthesis of eumelanin in melanocytes is induced by the α-Melanocyte Stimulating Hormone (α-MSH), which is an agonistic ligand of the Melanocortin-1 Receptor (MC1R), a G-protein coupled receptor present on the melanocyte surface. Conversely, the synthesis of feomelanin in the melanocyte is induced by the interaction between MC1R and the paracrine antagonist peptide “agouti” (also known as ASIP

[Agouti Signaling Protein]), secreted by dermal papilla cells (Voisey et al. 2002). This system was first described in the mouse, in which the agouti peptide was found to consist of 131 amino acids, and to act as an MC1R antagonist, blocking its activation by α-MSH (Kwon et al. 1994). It is now well established that the switch between eumelanogenesis and feomelanogenesis involves opposite effects of *ASIP* and α-MSH as ligands of the same receptor (Rouzaud and Hearing 2004). A typical "agouti" hair in the mouse is black with a subapical yellowish band (the agouti band), which results from a pulse of expression of the *agouti* gene during hair growth.

Many other mammals present similar patterns of hair pigmentation, with one or more "agouti" bands placed on an overall darker hair shaft, suggesting that this pigmentation mechanism has been conserved throughout the evolution of this group. Interestingly, mutant alleles of the mouse *agouti* gene cause darker coat color (melanism) exhibiting recessive inheritance, while dominant mutant alleles result in yellow color and in some cases also pleiotropic effects such as obesity, type II diabetes, increased body size and tumorigenesis (Yen et al. 1994). It is now well understood that recessive melanism in these cases is due to loss of function of the agouti peptide, leading to constant eumelanogenesis. Likewise, the yellow coloration of dominant agouti mutants is due to gain of function leading to constant blocking of eumelanogenic signaling. Pleiotropic effects of dominant yellow mouse mutants have been found to be caused by ectopic expression of *agouti*, leading to interaction with other melanocortin receptors and thus interfering on other signaling pathways.

The mouse *agouti* gene (whose homologue is called *ASIP* in humans and most other species) is located on chromosome 2 and is composed of three coding exons (containing 170 bp, 65 bp and 385 bp, respectively) which span a genomic region of approximately 5 Kb. In addition, there are up to four non-coding exons, located more

than 100 kb upstream from the coding region, which are involved in the expression of alternative transcripts (Siracusa 1994). The total genomic region consists of *ca.* 110 kb (Bultman et al. 1994). In other species studied to date, the *ASIP* gene varies in the exact number of codons, but is always composed of three coding exons, with a general structure similar to that described originally for the mouse. In the domestic cat *(Felis catus)*, this gene is located on chromosome A3, and codes for a protein containing 135 amino acids (Eizirik et al. 2003).

The coding regions of *ASIP* and *MC1R* have been characterized in some species of mammals (and many other vertebrates, in the case of the *MC1R*), leading to the identification of molecular variants in one or both genes that were associated to melanistic or other coat color phenotypes (*e.g.* Joerg et al. 1996; Marklund et al. 1996; Väge et al. 1997; Everts et al. 2000; Newton et al. 2000). Variants of *MC1R* were associated to melanism in the cow (Kungland et al. 1995), red foxes (Väge et al. 1997), pigs (Kijas et al. 1998), sheep (Väge et al. 1999) and also in chickens (Takeuchi et al. 1996) and bananaquit birds (Theron et al. 2001). Fewer such studies have addressed *ASIP*, as this gene seems to be more difficult to characterize, due to its containing three coding exons instead of *MC1R*'s single contiguous Open Reading Frame, and exhibiting several isoforms composed of different combinations of non-coding exons. In addition to the pioneering mouse studies (Bultman et al. 1992) that characterized the *ASIP* coding region as well as various mutants involving genomic rearrangements and non-coding elements, only a few species have so far been sequenced for this locus. These include descriptions of the *ASIP* coding region in the red fox (Väge et. 1997), rat (Kuramoto et al. 2001), horse (Rieder et al. 2001) and cow (Girardot et al. 2005).

Among the mammals, the carnivoran family Felidae is an interesting system in which to study the evolution of coat color variation. The occurrence of polymorphic

pigmentation is common in domestic cats, including variation in the background color (from white to yellow, gray, red, brown or black) and also in the appearance, shape, coloration and distribution of markings (*e.g.* spots, stripes, and rosettes). Several other felids display polymorphic coloration, in some cases showing geographic discontinuities or gradients that have been used to describe different "subspecies" or historically distinct local populations (*e.g.* Pocock 1940; Garcia-Perea 1994). In some cases it is reasonable to assume that these variations are produced by local adaptation to distinct environments, thus being important components of these species' life history.

Of the 37 currently recognized felid species, 12 have been confirmed to exhibit melanism as a coat color variant (Eizirik 2002). In an effort to understand the evolution of this trait in the Felidae, the candidate genes *ASIP* and *MC1R* genes were mapped, cloned and sequenced in the domestic cat (*Felis catus*), providing tools for the direct investigation of these loci in other felids (Eizirik et al. 2003). That study identified three distinct mutants associated with melanism in different cat species. Black domestic cats are homozygous for a deletion of two nucleotides in exon 2 of *ASIP*, leading to a frameshift that likely abolishes the function of the coded protein, in agreement with a recessive inheritance of melanism in this species. Two different *MC1R* mutants were observed: melanistic jaguars (*Panthera onca*) carry a mutant allele bearing a five-codon deletion, while dark jaguarundis (*Puma yagouaroundi*) carry another allele lacking eight full codons (Eizirik et al. 2003). In the two latter cases the inheritance of melanism is dominant and semi-dominant, respectively, in agreement with a gain-of-function change in *MC1R*. The molecular basis of melanistic phenotypes remains to be uncovered the nine other felid species in which this trait has been observed. An initial step in this investigation is the characterization of the candidate genes *ASIP* and *MC1R* in multiple

species of the family, as well as related groups, so as to define regions of evolutionary conservation that may be particularly involved in gene function.

In this study we sequenced exonic and intronic regions of the *ASIP* gene in four felid species (*Leopardus geoffroyi, L. tigrinus, L. colocolo* and *Panthera pardus*) which had not been previously characterized for this locus. We also sequenced additional domestic cat individuals for the region under study, which were compared to our original *Felis catus ASIP* sequence (Eizirik et al. 2003), and used to evaluate intra-specific patterns of diversity in this genomic segment. In addition, melanistic and non-melanistic individuals of *L. geoffroyi* were compared for two exonic regions, in order to investigate the presence of molecular variants potentially associated to this phenotype in this species. Finally, we compared the felid sequences generated here to available data from other placental mammals belonging to the supra-ordinal clade Ferungulata (which contains the orders Carnivora, Pholidota, Cetartiodactyla and Perissodactyla - Springer et al. 2007). These comparisons, which span different levels of evolutionary depth, allowed a characterization of the patterns of sequence variability in this segment in mammals, contributing to the identification of conserved non-coding blocks potentially involved in regulatory activities.

Materials and methods

Genomic DNA from one to 13 individuals each of *Leopardus geoffroyi, L. colocolo, L. tigrinus, Felis catus* and *Panthera pardus* (Table 1) were amplified by the Polymerase Chain Reaction (PCR) using previously designed *ASIP* primers (Eizirik 2002). In most cases, external primers agoEx2-F2 and agoEx3-R1 (Figure 1, Table 2) were used for amplification of a single contiguous segment *ca.* 2 kb long. To investigate exonic variability in the *L. geoffroyi ASIP* gene, multiple individuals of this species

were analyzed for exon 2 and exon 3 alone (Table 1), using a separate PCR reaction for each fragment (employing primers agoEx2-F2 and agoEx2-R1 for Exon 2, and agoEx3-F1 and agoEx3-R1 for exon 3 – see Figure 1). PCR reactions were performed in a total volume of 20 μL, containing 1μL of empirically diluted DNA, 2 μL of 10X buffer II; 2 mM $MgCl_2$, 0.2 mM dNTP; 1 U AmpliTaq DNA polymerase (Invitrogen) and 0.2 μM of each primer. The PCR profile consisted of 3' initial denaturing at 94ºC, followed by 40 cycles of 94ºC for 45'', 55ºC for 1', 72ºC for 2', and a final extension of 72ºC for 10'. PCR fragments were verified through electrophoresis in a 1% agarose gel stained with ethidium bromide, and purified using either an enzymatic clean-up protocol (Shrimp alkaline phosphatase and Exonuclease I [Amersham]) or Polyethyleneglycol (PEG) 8000. Purified fragments were sequenced using either ABI Big Dye chemistry or the DYEnamic ET Terminator Sequencing Kit (GE/Amersham Biosciences), and read on an ABI 3700 or a MEGABACE 1000 (GE/Amersham) automated sequencer, respectively. Fragments were sequenced in both directions using the PCR primers, as well as multiple internal primers (see Table 2) in the case of long amplicons (flanked by agoEx2-F2 and agoEx3-R1). Sequence electropherograms were checked and edited visually using SEQUENCHER (Gene Codes) or MEGA 3.1 (Kumar et al. 2004), leading to the construction of a final contig for each individual composed of two to 23 separate reads. All sequences generated here were deposited in GenBank (accession numbers XXXX-XXX).

Orthologous *ASIP* sequences from additional species were obtained from GenBank using the BLAST algorithm (Altschul et al. 1990). These included sequences of two canids (*Canis familiaris* [NC_006606] and *Vulpes vulpes* [VVU250364]), and one representative each of the mammalian orders Cetartiodactyla (*Bos taurus* [X99691]) and Perissodactyla (*Equus caballus* [AF288358]). Sequences were aligned using the

ClustalW algorithm implemented in MEGA 3.1, and final alignments were produced via direct checking and editing by hand. Three different data sets were analyzed, whose variation in evolutionary depth led to differences in alignment reliability: (A) felids only; (B) Carnivora only (*i.e.* felids + canids); and (C) Ferungulata (Carnivora + Cetartiodactyla + Perissodactyla). For each of the data sets, sequence blocks that could be reliably aligned (*i.e.* for which homologous nucleotide positions could be recognized) across the included taxa were identified using MEGA, which was also utilized for subsequent analyses of these segments. Analyses included phylogenetic reconstruction of evolutionary relationships using different data partitions, as well as estimations of nucleotide variability for each block at each level of evolutionary depth.

Results

Two coding regions of the *ASIP* gene (exon 2 [160 bp] and exon 3 [62bp]) and two non-coding segments of the same locus (intron 2 [1360bp] and part of intron 3 [154bp]) were characterized in five felid species, and compared to available sequences of other mammals belonging to the Grandorder Ferungulata. The alignment of these sequences varied between 2,082 bp and 2,192 bp, depending on the included species.

The comparative analysis of the sequences was carried out using three distinct data sets (A, B and C), which differed in terms of phylogenetic depth. Data set A included only felid sequences, spanning the base of the extant diversity in this family (*ca.* 11 million years) taking into consideration the comparison between *P. pardus* and the remaining species (Johnson et al. 2006). In database B, two canids were included (*Canis familiaris* and *Vulpes vulpes*), allowing the assessment of nucleotide conservation throughout the evolution of the two main branches (Feliformia and Caniformia) of the order Carnivora, spanning a divergence of *ca.* 55 million years

(Springer et al. 2003). The third database (C) also included sequences of representatives of two other mammalian orders related to the carnivores: a horse, representing the Perissodactyla, and a cow, representing the Cetartiodactyla. The inclusion of these species allowed a comparative analysis spanning *ca.* 90 million years of evolutionary depth (Springer et al. 2003).

The analysis carried out with these three databases allowed the identification and characterization of different sequence blocks, with distinct properties (Fig. 2). In database A we observed the existence of six conserved blocks (Table 3), consisting of the two exons along with four well-aligned and fairly long intronic regions. In addition to these conserved segments, two distinct regions could be identified within intron 2. One of them was a SINE element insertion (spanning 240 bp), present only *Felis catus*, and the other one was a complex repetitive region (spanning *ca.* 83 bp) observed in all felids, and consisting of a series of short tandem repeats dominated by the motifs (TG), (TTTG), (TTTTG) and (TTTTTG). Another observation was the occurrence of a well-defined 14-bp deletion (positions 285-298) in intron 2 (Sequence block 2 - see Fig. 2) of all *Felis catus* individuals, but not in any of the other species. Within the domestic cat SINE element insertion, all *F. catus* individuals were identical except for one Single Nucleotide Polymorphism (SNP) at position 724 (counting from the start of the initiation codon in exon 2 – see Fig. 2 and Table 3), in which A/A, T/T and A/T genotypes were observed. Three other SNPs were identified in intron 2, at positions 281 (A/G), 833 (T/C) and 1599 (T/C). Homozygotes for both alleles were observed in each of these SNPs, and heterozygotes were recorded for site 833 only. Among the sampled *L. geoffroyi* individuals, five SNPs were identified, but none was located in the surveyed exons, and none exhibited evidence of allelic association with melanistic

phenotypes. The two *L. colocolo* individuals analyzed here (one of which was melanistic) did not bear any nucleotide difference from each other.

The analyses performed with data set B allowed the identification of seven conserved blocks, due to the division of block 3 into two sub-blocks (Fig. 2, Table 4). The available *Vulpes vulpes* sequence was shorter than the overall data set, so that the first 134 bp (most of exon 2) and the final 216 bp (intron 3 and part of exon 3) could not be compared to the other species. Database C consisted of a longer alignment (2,192 bp) relative to A and B, due to several indels inferred to have occurred when more distantly related mammals were included in the analysis. The available *Equus caballus* sequence was also shorter than those of most other species, precluding comparisons involving the initial 32 bp (beginning of exon 2) and the final 210 bp (intron 3 and part of exon 3). This alignment allowed the identification of 11 well-defined (*i.e.* conserved) sequence blocks, including the two exons (Table 5). The increased number of blocks resulted from the subdivision of block 3 into 4 sub-blocks (3.1.1, 3.1.2, 3.2.1 and 3.2.2 – See Table 5 and Fig. 2) and of block 4 into 3 sub-blocks (4.1, 4.2 and 4.3). Other general observations gleaned from this data set included the presence in *Bos taurus* of an extra codon (AAA, positions 1987-1989) in exon 3, and an 11-bp insertion (positions 2052 - 2062) in intron 3, relative to the remaining species.

In order to characterize the evolutionary conservation across this genomic segment, we analyzed the level of nucleotide variability in each defined block for each of the data sets. In data set A, the least variable segment was the sampled portion of intron 3 (block 6), with 3.2% variable sites, while the most variable was exon 2 (5.6% variable sites). Overall, intronic blocks were found to exhibit similar or lower variability than the examined exons (Table 3). The inclusion of canid sequences (Data Set B) allowed a more refined analysis of these blocks (Table 4). At this increased level

of evolutionary depth, intronic block 3.1 and exon 3 (block 5) were found to be the most conserved segments (6.9% and 7.0% variable sites, respectively). Exon 2 remained quite variable at this level (13.7% variable sites), but was surpassed in diversity by intronic blocks 2 and 3.2. At the same time, the available portion of intron 3 (block 6) remained considerably conserved (8% variable sites) (Table 4).

The same approach was then extended to Data Set C, the deepest phylogenetic level contained in our alignment, so as to identify core areas that represent well-defined blocks of conserved sequence (Table 5). In this case, the most conserved of all segments was block 4.3 (5.5% variable sites), a 36 bp-long region of intron 2 immediately adjacent to exon 3. At this level, both of the analyzed exons were found to be quite variable (23.7% and 15.6% variable sites for exons 2 and 3, respectively). Variability in alignable areas of introns was even greater (e.g. 50% in block 3.1.1), not to mention intervening intronic segments in which similarity among taxa could no longer recognized (i.e. they were not part of the identified blocks). As expected, the defined blocks became shorter as we analyzed deeper phylogenetic divergences (e.g. compare block 2 in Tables 3, 4 and 5), even though in some cases the length was affected in the opposite direction by the larger number of indels in this data set.

On the basis of these initial observations, we performed a more comprehensive comparative analysis of sequence conservation patterns using the refined blocks delimited with Data Set C (Table 6). Here the conservation of block 4.3 became really apparent, with no variable sites among the sampled carnivores, and a single difference in each of the comparisons between Carnivora and the other two orders. A trend of increased variability correlating with evolutionary depth could be clearly seen for all surveyed segments. This standardized comparison showed that exon 2 is consistently more variable than exon 3, at all phylogenetic levels, in many cases exhibiting a similar

amount of variation as the intronic blocks (Table 6). This is especially true when the more shallow comparisons are considered (Data sets A and B), but intronic segments become consistently less conserved than exons when inter-ordinal pairs are contrasted.

Interestingly, we observed a trend in which, when separate comparisons were performed between Carnivora and each of the other two orders, the cow was almost always more different from the former than the horse. This pattern is reflected in a phylogeny built with our data set, showing the cow to be relatively accelerated relative to the horse (Fig. 3), or else to be more distantly related from carnivores than the perissodactyl included here. When transitions (Ts) and transversions (Tv) were analyzed separately (Table 6), again we observed that pairwise comparisons involving Carnivora *vs.* Perissodactyla exhibited a smaller amount of transversions than equivalent comparisons involving Cetartiodactyla. Within the Felidae, *F. catus* tended to exhibit more mutations in the exonic segments than the other species (Fig. 3), although this pattern was not apparent in the surveyed intronic regions.

Given the observations of varying levels of variability among intronic segments (e.g. extreme conservation of block 4.3), we performed another set of analyses dividing introns into two classes based on location: marginal regions were defined as the 30 bp immediately adjacent to an exon, hypothesized to contain regulatory motifs important for appropriate splicing. The remaining intronic segments (considering only defined blocks) were categorized as internal regions. We surveyed variation in these two intronic classes at two levels (Data Sets A and B), since the intron 3 segment was not sampled in the horse sequence. We observed that, within felids, the exons are indeed quite variable, exhibiting less conservation than even internal regions of the introns (Table 7). This pattern is reversed when the two major carnivoran branches are compared by adding canids to the assessment; at that level exons became the most

conserved class, followed by marginal intronic regions and leaving internal segments as the most variable.

Discussion

To investigate the patterns of nucleotide conservation in the mammalian gene *ASIP*, a sequential phylogenetic approach was used in this study. The initial divergence among the extant lineages of placental mammals occurred approximately 106 million years ago (MYA) (Springer et al. 2003). Within this group, the mammalian orders analyzed here (Carnivora, Cetartiodactyla and Perissodactyla) belong in the group Ferungulata of the Magnorder Laurasiatheria, and diverged from each other in a rapid sequence which is still not fully resolved, *ca.* 90 MYA (Springer et al. 2007). Within the order Carnivora, representatives of its two suborders, Feliformia and Caniformia (whose divergence occurred *ca.* 55 MYA), were included in this study. Moving further into the carnivoran tree, we focused on the family Felidae, in which we sampled species belonging to three different genera: *Panthera*, *Leopardus* and *Felis*. We have recently achieved a good understanding of the branching sequence among these genera (Johnson et al. 2006), with *Panthera* being the first to diverge (*ca.* 11 MYA) from the remaining lineages, and the separation between *Leopardus* and *Felis* occurring *ca.* 5 – 6 MYA.

Assessments of patterns of genetic variability may benefit from evolutionary comparisons at varying phylogenetic depths. The initial divergence between sister-taxa leads to the accumulation of sporadic differences across the genome, which gradually increases as evolutionary time elapses. Therefore, many regions that appear conserved when closely related taxa are compared may eventually become very variable or even

unalignable at increased phylogenetic depth, revealing that the broad conservation at shallow levels was more likely due to evolutionary inertia (lack of sufficient time to accumulate mutations). Inertia thus obscures patterns of sequence conservation that are caused by functional constraints, so that only a subset of segments found to be conserved among taxa are actually subjective to negative selection due to functional relevance.

In this study, we aimed to characterize the patterns of nucleotide conservation across intronic and exonic segments of a gene implicated in melanistic phenotypes in several mammalian groups. Previous observations revealed that this gene is quite variable, and that its coding regions seem to be subjected to relaxed functional constraints (Eizirik 2002). In contrast, some intronic blocks appeared quite conserved when comparisons among felids were performed (Eizirik 2002), prompting the question of whether they might be involved in regulatory activities. To evaluate the extent of intronic conservation across a broader taxonomic comparison, and to attempt to define more precisely intronic blocks that appear to be constrained, we used three levels of phylogenetic depth, as well as separate comparisons between Carnivora and two of the mammalian orders most closely related to it. The results indicate that much of the intronic conservation observed among felids is due to evolutionary inertia, but some of the conserved blocks are indeed remarkably similar even among mammalian orders that diverged *ca.* 90 MYA. This is especially the case of block 4.3, whose conservation surpasses that of exons in all comparisons performed here (Table 6). The conservation of this block likely contributes to the pattern observed in Table 7, where marginal intronic regions appear as substantially conserved, likely due to functional relevance in splicing regulation. No such extreme conservation was observed in the other two marginal intronic segments (blocks 2 and 6); however, the levels of variation in these

area were also rather low compared to the rest of the surveyed introns (see Table 6). It is also noteworthy that block 2 was very large, appearing as the longest segment remaining definable in Data Set C (Table 6 and Fig. 2). The size of the segment leads to a lower estimate of conservation if it is actually a mosaic of sub-segments varying in functional constraint. On the other hand, the fact that it remains as a large identifiable block across three mammalian orders in itself suggests that it contains functionally relevant motifs. Although alternative isoforms of *ASIP* are known (e.g. Bultman et al. 1994), so far none has been found to involve different combinations of the three coding exons (2, 3 and 4), so that splicing in this area of the gene seems to be conserved among placental mammals. The conserved intronic segments observed here are likely involved in this regulation, but may also influence other aspects of *ASIP* expression. Since the transcriptional regulation of *ASIP* appears to be complex (pulse-like coupled with hair growth in the mouse dorsal area – Bultman et al. 1994) and has not been fully characterized in any species, it is relevant to identify conserved regions among taxa so as to target these potentially regulatory segments for further scrutiny. In particular, variations in the deployment of lighter coat areas are rampant among mammals, as well as cases of multiple agouti bands per hair shaft (implying multiple pulses of *ASIP* expression). It is thus clear that additional species must be characterized in terms of *ASIP* structure and function to complement the data derived from the mouse, and to identify patterns that are common across mammals or lineage-specific. The results provided here point to some intronic areas that deserve further investigation using a broader taxonomic sample and more sophisticated analytical methods.

Exonic regions of *ASIP* are remarkably variable (see Tables 3-6), corroborating the view that functional constraints are relaxed in this locus (Eizirik 2002). This may be associated to the relatively common involvement of this gene in mammalian coat color

variants, as pleiotropic effects of loss-of-function seem to be absent or limited. Nevertheless, when deeper comparisons are performed, it can be observed that exons do present higher conservation than most intronic segments, as would be expected if they were indeed subjected to some level of functional constraint.

Intra-specific polymorphisms in *ASIP* are also interesting to investigate, as they provide information for population-based analyses and may be associated with coloration variants (e.g. Rieder et al. 2001; Eizirik et al. 2003). So far few studies have addressed this topic, but in every case where intra-specific variation was surveyed (all restricted to laboratory, domestic or farmed animals) mutants could be identified in this gene. This is the case of horses, where four mutantions were identified in this region, including an 11-bp deletion in exon 2 that was associated with black color (Rieder et al. 2001). An indel variant associated with dark coat color was also detected in farmed red foxes, where all of exon 2 is lost within a 1.2 kb deletion in this genomic region (Vage et al. 1997). Broader surveys of segregating variants (e.g. SNPs) in this genomic segments are lacking, and could provide interesting tools for evolutionary studies of this locus. The SNPs identified here in *F. catus* and *L. geoffroyi*, as well as the microsatellite-like hypervariable segment observed in all felids, may be used in further studies on the intra-specific patterns of variability in this locus, as well as association studies with coat color variants.

As for melanism in *L. geoffroyi*, nothing is currently known regarding the molecular basis of this phenotype in this species. Given the common involvement of *ASIP* in melanism in other mammals, this locus should be considered a plausible candidate. The results obtained here indicate that *ASIP* exons 2 and 3 are not implicated in this characteristic, as no variable sites were identified differentiating melanistic *vs.* non-melanistic animals. Intronic SNPs were also not obviously associated with

melanism, although increased sample sizes might detect association due to linkage disequilibrium if this locus is indeed implicated. Further studies aiming to identify candidate mutations involved in melanism in *L. geoffroyi* should concentrate their attention on *ASIP* exon 4, which was not covered here, as well as interrogate other genes involved in the regulation of melanogenesis, such as *MC1R*. An enhanced understanding of the molecular changes underlying melanistic phenotypes in multiple felid species should shed light onto the evolutionary processes influencing the recurrent origin and maintenance of this characteristic.

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Table 1. Biological samples utilized in this study.

Individual ID	Color	Geographic Origin/Breed
Geoffroy's cat		
(*L. geoffroyi*)		
*bLge08**	non-melanistic	Cachoeira do Sul, RS, Brazil
*bLge10**	non-melanistic	Cachoeira do Sul, RS, Brazil
*bLge11**	non-melanistic	Cachoeira do Sul, RS, Brazil
*bLge13**	non-melanistic	Eldorado do Sul, RS, Brazil
*bLge71**	melanistic	Pelotas, RS, Brazil
*bLge74**	melanistic	Pinheiro Machado, RS, Brazil
*bLge76**	non-melanistic	Arroio Grande, RS, Brazil
*bLge77**	melanistic	Dom Pedrito, RS, Brazil
*bLge78**	non-melanistic	Rio Grande, RS, Brazil
Oge26	non-melanistic	Córdoba, Argentina
bOge4	melanistic	Cachoeira do Sul, RS, Brasil
bOge5	melanistic	Cachoeira do Sul, RS, Brasil
bOge6	melanistic	Cachoeira do Sul, RS, Brasil
Domestic cat		
(*Felis catus*)		
Fca273	blotched tabby	USA
Fca802	blotched tabby	USA
Fca934	blotched tabby	USA
Fca938	mackerel tabby	USA
Fca1902	abyssinian	Abyssinian
Fca2096	black	Frederick, MD, USA
Fca2348	silver mackerel tabby	Scottish Fold
Fca2641	black	Israel
Fca2685	black	Porto Alegre, RS, Brazil
Fca2976	abyssinian	Domestic shorthair
Fca3004	silver spotted	Egyptian Mau
Pampas cat		
(*L. colocolo*)		
Lco13	non-melanistic	Brazil
Lco30	melanistic	USA
Oncilla		
(*L. tigrinus*)		
Lti43	non-melanistic	Brazil
Leopard		
(*Panthera pardus*)		
Ppa281	non-melanistic	Thailand

* Sequenced only for exons 2 and 3.

Table 2. PCR primers utilized in this study.

Primer	Sequence (5'-3')
agoF1	GTCATCCGCCTACTCCTGGC
agoR1	TCCGCTTCTTTTCTGCTGATC
agoR4	CATGCTGACAGTACTGCTTG
agoR7	TTCCCTGCTCCTTCCCTGAT
agoRH-F1	GGGCCTGACATTGAACATCT
agoISB-F1	CCGAGAGACCCTGAAGTCAA
agoISB-R1	CTCACTTCCCAGTGCCTAGC
agoEx2-F2	TTCTCTCTTCCACTCAGGCC
agoEx2-R1	ACTCCGGTCCATGAGAAGG
agoEx3-F1	TCCACTCCTCCACTTTACTG
agoEx3-R1	CCCTTAGCTCTCTGGGCTTC

Table 3. Sequence blocks identified with Data Set A (Felidae only).

Sequence Block	Size (bp)	Genomic Region	No. of Variable Sites
1	160 bp (1-160)	Exon2	9 (5.6%)
2	340 bp (161-500)	Intron2	19 (5.5%)
3	567 bp (741-1307)	Intron2	26 (4.5%)
4	453 bp (1391-1843)	Intron2	21 (4.6%)
5	62 bp (1844-1905)	Exon3	3 (4.8%)
6	177 bp (1906-2082)	Intron3	5 (3.2%)

Table 4. Sequence blocks identified with Data Set B (Felidae + Canidae).

Sequence Block	Size (positions)	Genomic Region	No. of Variable Sites
1	160 bp (1-160)	Exon2	21 (13.7%)
2	288 bp (161-449)	Intron2	58 (20.1%)
3.1	389 bp (743-1132)	Intron2	70 (6.9%)
3.2	140 bp (1171-1310)	Intron2	21 (15%)
4	303 bp (1575-1877)	Intron2	38 (12.8%)
5	62 bp (1878-1939)	Exon3	4 (7%)
6	177 bp (1940-2116)	Intron3	14 (8%)

Table 5. Sequence blocks identified with Data Set C (carnivores + cow + horse).

Sequence Block	Size (position)	Genomic Region	No. of Variable Sites
1	160 bp (1 - 160)	Exon2	38 (23.7%)
2	270 bp (161 - 430)	Intron2	102 (37.7%)
3.1.1	38 bp (719 - 756)	Intron2	19 (50%)
3.1.2	228 bp (757 - 984)	Intron2	101 (41.2%)
3.2.1	78 bp (1082 -1159)	Intron2	22 (28.2%)
3.2.2	131 bp (1226-1356)	Intron2	38 (29%)
4.1	193 bp (1592-1784)	Intron2	60 (31%)
4.2	48 bp (1785-1832)	Intron2	15 (31.2%)
4.3	36 bp (1901-1936)	Intron2	2 (5.5%)
5	65 bp (1937-2001)	Exon3	10 (15.6%)
6	191 bp (2002-2192)	Intron3	48 (24.8%)

Table 6 – Number of variable sites (V) and mean pairwise distance (p-distance, expressed as percentage) of ASIP nucleotide sequences compared at different levels of evolutionary depth. Block boundaries were defined based on the analysis of Data Set C.

	Bloco de seqüência										
	1	2	3.1.1	3.1.2	3.2.1	3.2.2	4.1	4.2	4.3	5	6
Felidae											
V [a]	9 (5.6%)	12 (4.4%)	2 (5.2%)	13 (5.7%)	2 (2.5%)	4 (3%)	9 (4.6%)	1 (2.1%)	0%	3 (4.6%)	5 (2.6 %)
P-Dist-Ts [b]	2%	2%	0%	2%	0%	1%	1%	1%	0%	2%	1%
P-Dist-Tv [c]	1%	0%	3%	1%	1%	1%	1%	0%	0%	0%	0%
Felidae x Canidae											
V [a]	21 (13.7%)	51 (18.8%)	6 (15.7%)	45 (19.7%)	7(8.9%)	19 (14.5%)	27 (13.9%)	6 (12.5%)	0%	4 (6.1%)	15 (7.8%)
P-Dist-Ts [b]	8%	15%	9%	9%	4%	9%	9%	10%	0%	3%	4%
P-Dist-Tv [c]	2%	3%	4%	5%	4%	5%	4%	5%	0%	0%	3%
Carnivora x Cetartiodactyla											
V [a]	33(20.6%)	88 (32.6%)	17(44.7%)	83 (36.4%)	19(24.3%)	34 (25.6%)	53 (27.4%)	12 (25%)	1 (2.8%)	7 (10.7%)	46 (23.4%)
P-Dist-Ts [b]	9%	22%	29%	19%	17%	18%	12%	16%	3%	7%	14%
P-Dist-Tv [c]	6%	7%	21%	16%	4%	4%	12%	8%	0%	1%	14%
Carnivora x Perissodactyla											
V [a]	27(16.8%)	79 (29.2%)	9(23.6%)	69(30.2%)	16(20.5%)	29 (22.1%)	44 (22.8%)	11 (23%)	1 (2.8%)	8 (12.3%)	n/c [d]
P-Dist-Ts [b]	13%	15%	4%	15%	15%	14%	11%	14%	3%	9%	n/c [d]
P-Dist-Tv [c]	1%	8%	18%	8%	2%	4%	5%	7%	0%	2%	n/c [d]
Carnivora, Cetartiodactyla, Perissodactyla											
V [a]	38 (23.7%)	102 (37.7%)	19(50%)	101(42.4%)	22(28.2%)	38 (29%)	60 (31.1%)	15 (31.2%)	2 (5.6%)	10 (15.6%)	48 (24.8%)

[a] V = Number of variable sites (% given in parentheses relative to the size of each block).
[b] P-Dist-Ts = Mean pairwise p-distance (transitions only)
[c] P-Dist- Tv = Mean pairwise p-distance (transversions only)
[d] n/c= not calculated due to the lack of this segment in the available *Equus caballus* sequence.

Table 7 - Percentage of variable sites in exonic, marginal intronic (30 bp adjacent to an exon) and internal intronic regions of the *ASIP* gene in felids and canids. Only core conserved blocks identified in Data Set C were are included in the analysis, with further subdivision of marginal blocks when required to fit the criterion of 30-bp long boundary areas.

	Exon	**Intron (Marginal Region)**	**Intron (Internal Region)**
Felidae	5.3%	3.6%	4.3%
Felidae + Canidae	10.6%	13.2%	15.3%

Figure Legends

Fig. 1. Location of the PCR primers used in this study, relative to exons and introns of the *ASIP* gene.

Fig. 2. Sequence blocks identified in the surveyed region of the *ASIP* gene, based on comparisons among Felids (Data Set A, top line), felids and canids (Data Set B, middle line), and ferungulates (felids, canids, cow and horse) (Data Set C, bottom line – see text for details). Numbers followed by asterisks indicate the size of each block in base pairs (bp); numbers at the bottom of each line identify the sequence blocks, while numbers on top of line C indicate the starting position of each block (see Tables 3-5 for precise location). Dotted lines connect equivalent points in the genomic segment analyzed here, whose position changed among data sets due to alignment adjustments.

Fig. 3. Phylogenetic relationships among *ASIP* sequences investigated in this study.

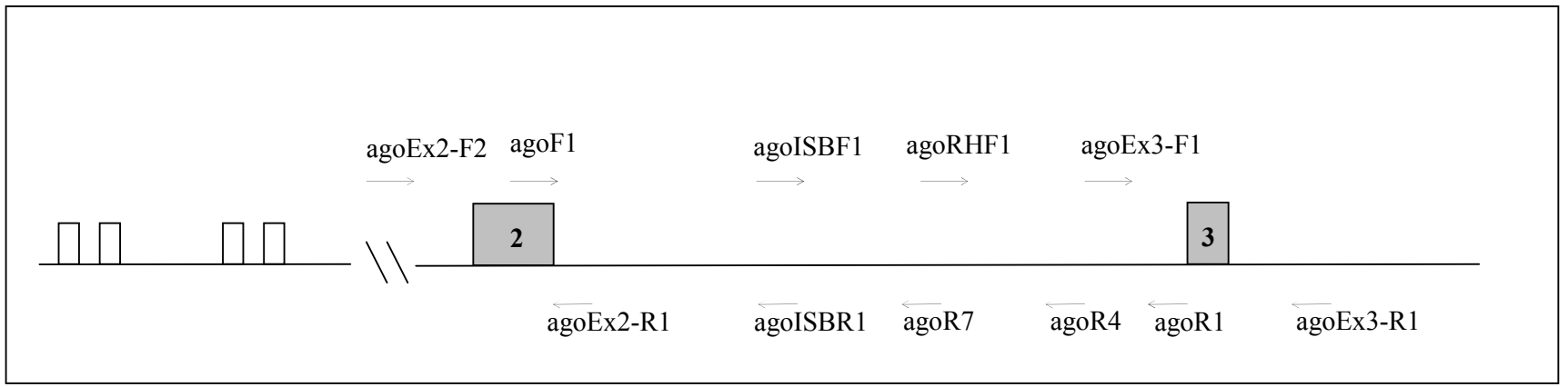
agoEx2-F2
agoF1
agoISBF1
agoRHF1
agoEx3-F1
2
3
agoEx2-R1
agoISBR1
agoR7
agoR4
agoR1
agoEx3-R1

Fig.1.

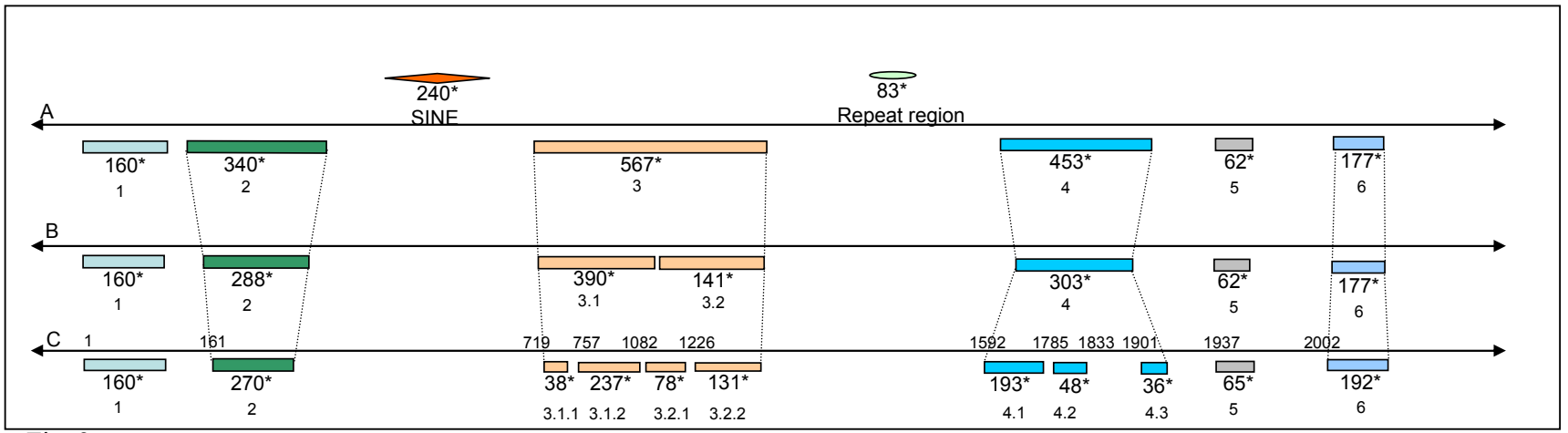
240*
SINE
83*
Repeat region
A
160*
1
340*
2
567*
3
453*
4
62*
5
177*
6
B
160*
1
288*
2
390*
3.1
141*
3.2
303*
4
62*
5
177*
6
C
1
161
719
757
1082
1226
1592
1785
1833
1901
1937
2002
160*
1
270*
2
38*
3.1.1
237*
3.1.2
78*
3.2.1
131*
3.2.2
193*
4.1
48*
4.2
36*
4.3
65*
5
192*
6

Fig. 2

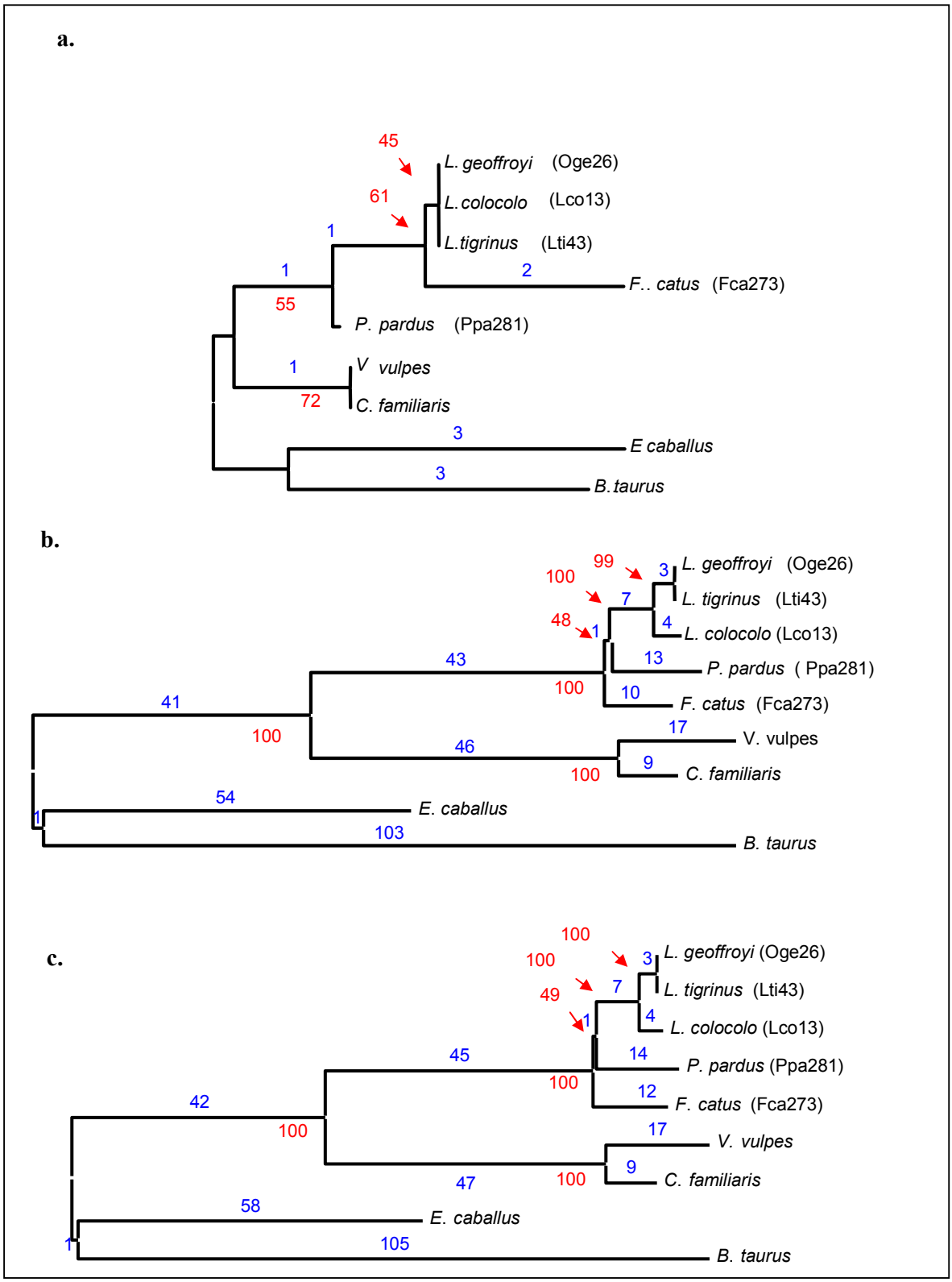
a.
45
L. geoffroyi (Oge26)
61
L. colocolo (Lco13)
1
L. tigrinus (Lti43)
1
2
F.. catus (Fca273)
55
P. pardus (Ppa281)
1
V vulpes
72
C. familiaris
3
E caballus
3
B. taurus
b.
99
100
3
L. geoffroyi (Oge26)
7
L. tigrinus (Lti43)
48
1
4
L. colocolo (Lco13)
43
13
P. pardus (Ppa281)
100
10
F. catus (Fca273)
41
17
V. vulpes
100
46
9
C. familiaris
100
54
E. caballus
1
103
B. taurus
c.
100
100
3
L. geoffroyi (Oge26)
49
7
L. tigrinus (Lti43)
1
4
L. colocolo (Lco13)
45
14
P. pardus (Ppa281)
100
12
F. catus (Fca273)
42
17
V. vulpes
100
9
C. familiaris
47
100
58
E. caballus
1
105
B. taurus

Fig.3

5. CONSIDERAÇÕES FINAIS

O peptídeo "agouti" produzido pelo gene *ASIP* (*Agouti Signaling Protein*) regula a pigmentação no folículo piloso dos mamíferos produzindo um pêlo preto com uma ou mais bandas subapicais amarelas. Esse efeito na pigmentação é obtido pela antagonização da ligação do hormônio estimulador do melanócito-α (α-MSH), que leva à inibição do MC1R (Receptor da melanocortina 1), mudando a síntese da melanina de eumelanina (preto/marrom) para feomelanina (vermelho/amarelo) (Voisey et al. 2002; Rouzaud e Hearing 2005; Yen et al. 1994). Em relação ao gene *MC1R*, poucos estudos até o momento abordaram a variabilidade genética do gene *ASIP* em mamíferos, possivelmente por tratar-se de um gene mais complexo, consistindo de 3 exons codificadores, apresentando *splicing* alternativo e exibindo pouca conservação entre grupos, o que dificulta o desenho de ensaios moleculares para a sua investigação. Entre as espécies estudadas estão as raposas vermelhas (Vage et al. 1997), ratos (Kuramoto et al. 2001), cavalos (Rieder et al. 2001), camundongos (Miltenberger et al. 2002; Bultman et al. 1992; Duhl 1994), bovinos (Klungland et al. 1995), cachorro (Berryere et al. 2004) e zebrafish (Geldmacher-Voss et al. 2003). Entre os felídeos, alvo principal deste estudo, até o momento o gene *ASIP* havia sido seqüenciado somente em *Felis catus*, o qual possui uma deleção de dois nucleotídeos no éxon 2 associada ao melanismo (Eizirik et al. 2003).

No presente estudo, foi possível caracterizar os níveis e padrões espaciais de variabilidade presentes na porção 5' da região codificadora do gene *ASIP* de várias espécies de mamíferos. Observou-se um relaxamento da seleção natural em regiões exônicas, levando a uma alta variabilidade, e uma conservação considerável em alguns blocos intrônicos, possivelmente envolvidos em atividades regulatórias. Não foram encontrados polimorfismos nas regiões codificadoras analisadas (éxons 2 e 3) em *L.*

geoffroyi ou *L. colocolo*, incluindo indivíduos melânicos e não-melânicos, indicando que estes segmentos não estão envolvidos na geração deste fenótipo de pigmentação. Os resultados deste estudo podem servir como base para futuras investigações sobre a variabilidade do gene *ASIP* em mamíferos, tanto no que tange ao estudo do melanismo em felídeos (focando esforços no exon 4 no caso de *L. geofforyi* e *L. colocolo*), como na caracterização da relevância funcional dos segmentos conservados aqui identificados.

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