PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL

FACULDADE DE BIOCIÊNCIAS

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

TAIZ LEONOR LOPES SIMÃO

AVALIAÇÃO DE GENES NUCLEARES COMO MARCADORES FILOGENÉTICOS EM DUAS LINHAGENS RECENTES DE CARNÍVOROS NEOTROPICAIS

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Dissertação apresentada ao Programa de Pós-Graduação em Biologia Celular e Molecular como requisito para a obtenção do grau de Mestre.

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RESUMO

A região Neotropical abriga aproximadamente 30% da diversidade de espécies das famílias Felidae (subordem Feliformia) e Canidae (subordem Caniformia) (Eisenberg & Redford 1999), as quais migraram para a América do Sul após a formação do istmo do Panamá, há cerca de 3 milhões de anos. Devido ao recente processo de especiação que caracteriza estes grupos, alguns aspectos de sua estrutura filogenética permanecem controversos, especialmente no que tange às relações evolutivas entre espécies pertencentes a duas linhagens, o gênero *Leopardus* (Feliformia, Felidae) e o gênero *Lycalopex* (Caniformia, Canidae). O objetivo do presente estudo é caracterizar de forma comparativa a história evolutiva dos gêneros *Leopardus* e *Lycalopex*, empregando seqüências de múltiplos segmentos nucleares e múltiplos indivíduos por espécie, avaliando a eficácia deste tipo abordagem para a resolução de processos recentes de diversificação através do programa **BEAST*. Para cada um dos genes analisados, observamos a ocorrência de variação interespecífica e intra-específica em ambas as linhagens. Discrepâncias genealógicas consideráveis foram constatadas entre os segmentos, evidenciando a complexidade da tarefa de reconstruir a filogenia destes grupos com marcadores nucleares. As genealogias estimadas demonstraram que em muitos casos as espécies não se apresentam monofiléticas, o que ocorre em paralelo com o compartilhamento de haplótipos entre espécies. Não obstante, para *Leopardus*, obtivemos uma *species tree* com alta resolução. Para *Lycalopex*, entretanto, a maior parte dos nós internos permaneceu com baixo suporte, indicando que um número maior de genes será provavelmente necessário para que se busque uma resolução consistente da filogenia deste grupo empregando estratégias multi-locus. De forma geral, nossos resultados demonstraram de forma empírica a ocorrência de discordância genealógica em ambas as linhagens, e ilustraram o potencial de análises multi-locus na resolução de filogenias que envolvam processos recentes de diversificação.

Palavras-chave: **BEAST*, *Species tree*, Análises Multilocus, Processo recente de diversificação, Gênero *Leopardus*, Gênero *Lycalopex*.

ABSTRACT

The Neotropical region holds approximately 30% of the current species diversity in the carnivoran families Felidae (subordem Feliformia) and Canidae (subordem Caniformia), which migrated to South America after the closure of the Panamanian Isthmus, *ca.* 3 million years ago. Due to the recent speciation process that characterizes each of these groups, some aspects of their phylogenetic structure remain controversial, especially those related to the evolutionary relationships among species belonging to two lineages, the genus *Leopardus* (Felidae) and the genus *Lycalopex* (Canidae). The objective of the present study was to perform a comparative characterization of the evolutionary history of these genera, using sequences of multiple independent nuclear gene loci and multiple individuals per species to investigate the occurrence of genealogical discordance, as well as to infer a 'species tree' for each lineage using the program **BEAST*. We observed both intra-specific and interspecific variation for all the surveyed segments. Genealogical discordance was identified among segments, highlighting the complexity of the task of reconstructing the phylogeny of such groups by employing nuclear markers. The estimated genealogies demonstrated that species were often not monophyletic, while there were several cases of inter-specific haplotype sharing. Nevertheless, the species tree reconstructed for *Leopardus* was highly resolved and supported, indicating that our data set contained sufficient genealogical information to retrieve this phylogeny. However, in the case of *Lycalopex*, most of the internal nodes received low support, indicating that a larger number of genes will likely be necessary to consistently resolve its phylogenetic structure using this type of approach. Overall, our results have empirically demonstrated the occurrence of genealogical discordance in both lineages, and illustrated the potential of multi-locus analyses to resolve phylogenies underlying recent diversification processes.

Keywords: **BEAST*, Species tree, Multilocus analysis, Recent diversification process, *Leopardus*, *Lycalopex*

SUMÁRIO

CAPÍTULO 1

INTRODUÇÃO E OBJETIVOS

1.1 INTRODUÇÃO

A Ordem Carnivora é atualmente classificada em 16 famílias que agrupam 286 espécies, com ampla variação morfológica, ecológica, fisiológica e comportamental (Eizirik & Murphy 2009), distribuídas em praticamente todos os ambientes (Nowak 2005). Seus representantes são agrupados em duas subordens monofiléticas, Feliformia e Caniformia, com a divergência estimada em 53 milhões de anos (Ma) (Eizirik & Murphy 2009) (Figura 1).

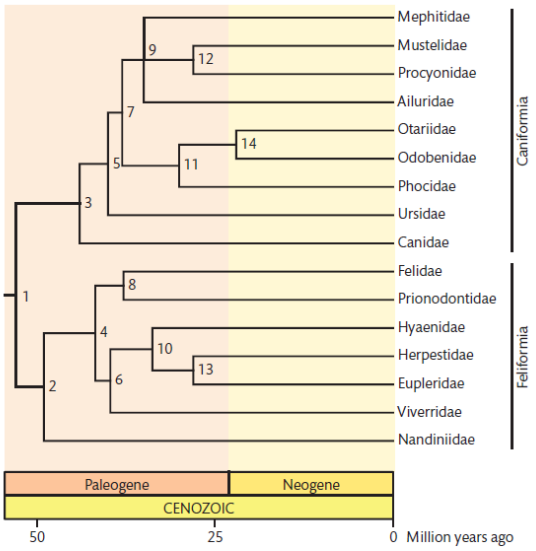

Figura 1: Relações filogenéticas da ordem Carnivora, apresentada com a estimativa do tempo de divergência entre as linhagens (fonte: Eizirik & Murphy 2009).

A região Neotropical abriga aproximadamente 30% da diversidade de espécies das famílias Felidae (subordem Feliformia) e Canidae (subordem Caniformia) (Eisenberg & Redford 1999), as quais migraram para a América do Sul após a formação do istmo do Panamá, há cerca de 3 Ma (Johnson et al. 2006; Eizirik [no prelo]). Evidências moleculares indicam que ambas sofreram processos de radiação adaptativa endêmica, os quais podem ter sido

facilitados ou promovidos por características geológicas e ecológicas desta região. Por exemplo, pode-se mencionar a hipótese de que flutuações climáticas ocorridas durante o Pleistoceno promoveram modificações na cobertura vegetal, conduzindo ao isolamento de populações em áreas de refúgio e, resultando em altas taxas de especiação na fauna tropical (Haffer 1969). Devido ao recente processo de especiação que caracteriza estes grupos, alguns aspectos de sua estrutura filogenética permanecem controversos, especialmente no que tange às relações evolutivas entre espécies pertencentes a duas linhagens, o gênero *Leopardus* (Feliformia, Felidae) (Figura 2) e o gênero *Lycalopex* (Caniformia, Canidae) (Figura 3).

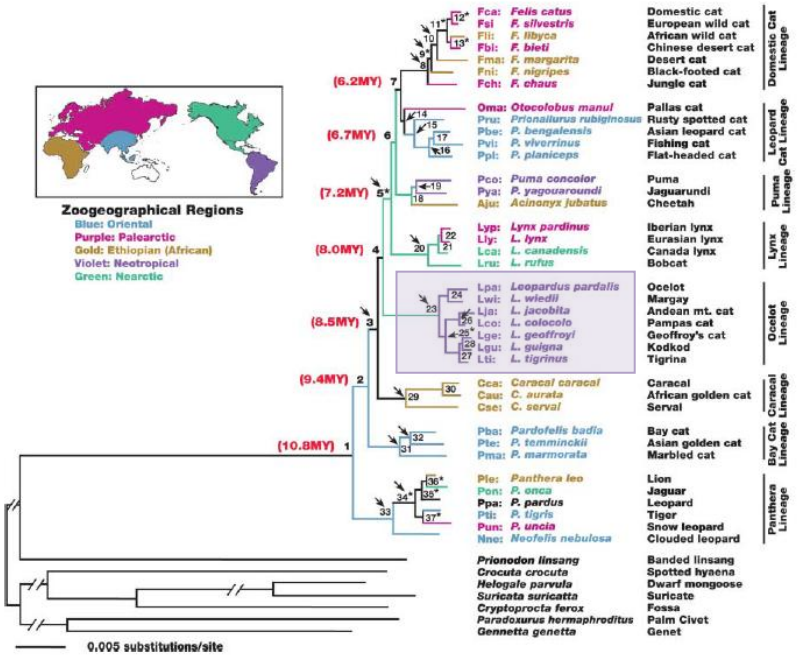

Figura 2: Relações filogenéticas da Família Felidae. O nó 23 (salientado por um retângulo roxo) representa as espécies pertencentes ao gênero *Leopardus*. (fonte: Johnson et al. 2006). As cores indicam a distribuição geográfica de cada uma das linhagens, conforme a legenda exibida na porção superior.

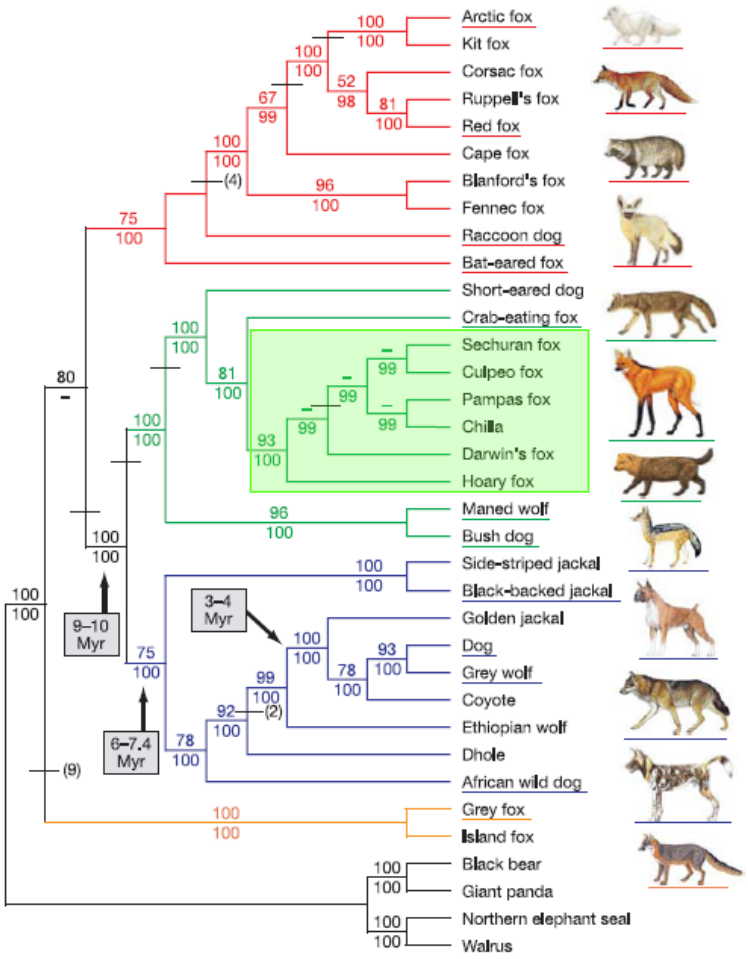

Figura 3: Relações filogenéticas entre espécies da família Canidae. O clado em verde representa as espécies neotropicais incluindo o gênero *Lycalopex* (salientado por um retângulo verde). Os nomes populares indicados se referem às seguintes espécies: Sechuran fox = *Lycalopex sechurae*; Culpeo fox = *L. culpaeus*; Pampas fox = *L. gymnocercus*; Chilla = *L. griseus*; Darwin's fox = *L. fulvipes*; Hoary fox = *L. vetulus* (fonte: Lindblad-Toh et al. 2005).

O gênero *Leopardus* inclui sete das dez espécies neotropicais da família Felidae. Seus representantes sofreram diversificação nos últimos 2.9 Ma e distribuem-se predominantemente na América de Sul. Enquanto algumas espécies apresentam distribuição restrita (por exemplo, *L. guigna* e *L. jacobita*) outras são observadas por áreas extensas e algumas vezes em simpatria (por exemplo, *L. pardalis* é simpátrico com *L. wiedii* ao longo de quase toda a sua

distribuição). As relações filogenéticas entre várias destas espécies já é bem caracterizada com base em marcadores moleculares (Eizirik et al. 1998; Johnson et al. 2006; Trigo et al. 2008), permanecendo incerta principalmente a posição de *L. jacobita*. Outra característica interessante deste grupo é a ocorrência documentada de hibridação entre *L. tigrinus* e duas espécies distintas, *L. geoffroyi e L. colocolo* (Johnson et al. 1999; Trigo et al. 2008), o que provavelmente reflete sua história recente de especiação, e oportuniza investigações interessantes no contexto de análises filogenéticas, genômicas e ecológicas.

De maneira similar à família Felidae, também são descritas dez espécies de canídeos na região neotropical, as quais apresentam ampla variação ecológica. Alguns autores sugerem que a migração para a América do Sul tenha ocorrido em múltiplos episódios (p.ex. Wang et al. 2004), enquanto outros defendem que a radiação neotropical desta família foi predominantemente endêmica, sendo derivada de apenas dois eventos de fundação (p.ex. Perini et al. 2010). Tendo em vista os resultados mais recentes de datação molecular, permanece possível que toda a radiação de canídeos neotropicais seja endêmica da região, derivando de apenas um episódio de colonização (Eizirik [no prelo]). Dentro desta linhagem, um caso extremo de radiação endêmica é aquele do gênero *Lycalopex,* composto por seis espécies cuja diversificação ocorreu há aproximadamente um milhão de anos (Wang et al. 2004; Perini et al. 2010), e cujas relações filogenéticas ainda não foram bem resolvidas (p.ex. Zrzavy & Ricankova 2004; Lindblad-Toh et al. 2005; Perini et al. 2010).

A análise de seqüências do DNA mitocondrial (mtDNA) tem sido uma abordagem muito importante em estudos filogenéticos cujo objetivo é determinar as relações evolutivas entre linhagens. O mtDNA apresenta características como a herança matrilinear, recombinação baixa ou ausente e taxas de mutação geralmente mais altas do que aquelas observadas no DNA nuclear (nDNA) (p.ex. Brown et al. 1979; Ladoukakis & Zouros 2001). A comparação entre seqüências do nDNA desenvolveu-se ao longo da década de 90, em conjunto com as análises *multilocus*, onde regiões diversas do genoma são amostradas. Isto possibilitou a resolução de diferentes níveis taxonômicos, pois, o genoma é heterogêneo e os processos de substituição, tal como, as taxas de mutação, variam entre os genes (Cummings et al. 1995; Brito & Edwards 2009).

Atualmente, diversos autores apontam os marcadores nucleares como uma ferramenta inovadora que expressa de forma mais precisa a diversidade genética, o tamanho populacional, a divergência entre espécies e a datação destes eventos (Bazin et al. 2006; Degnan & Rosenberg 2009; Yang & Rannala 2010). Frequentemente a análise individual de segmentos nucleares produz genealogias conflitantes, sendo insuficiente para determinar o padrão de coalescência ancestral em cada um dos nós de uma filogenia (Figura 4) (Edwards et al. 2007; Brito & Edwards 2009). Neste contexto, torna-se relevante a amostragem de múltiplos locos e múltiplos indivíduos por espécie, pois, em virtude da variação intra-específica e/ou manutenção do haplótipo ancestral, inferências a partir de um único loco e/ou um único indivíduo podem

não expressar de forma confiável a relação entre as linhagens (Degnan & Rosenberg 2009; Yang & Rannala 2010).

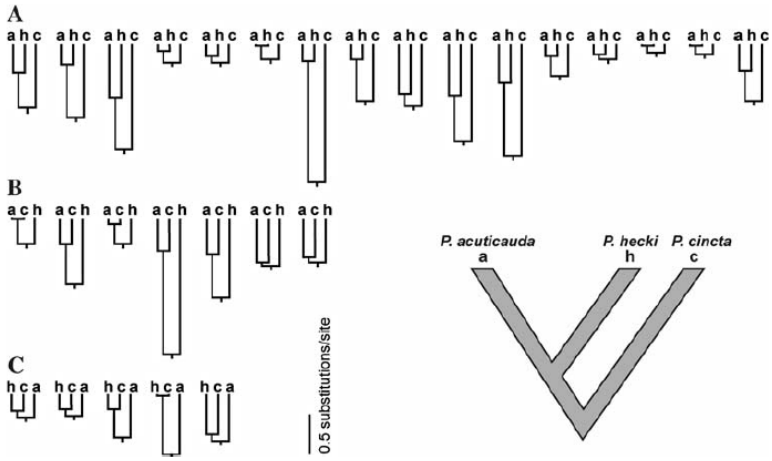

Figura 4: Exemplo da heterogeneidade na topologia e tempos de coalescência entre diferentes regiões genômicas em uma análise filogeográfica multilocus (cada árvore indica a genealogia estimada por um segmento distinto). As letras *a*, *h* e *c* representam as espécies *Poephila acuticauda*, *P. hecki* e *P. cincta*, respectivamente. A árvore filogenética no canto inferior direito representa as relações filogenéticas entre as espécies inferidas pelo método de 'species tree' multilocus de Liu & Pearl (2007). As genealogias indicadas na porção 'A' da figura estão de acordo com a filogenia das espécies, enquanto 'B' e 'C' indicam topologias alternativas (fonte: Brito & Edwards 2009).

Análises sofisticadas desenvolvidas recentemente (p.ex. Liu & Pearl 2007 e Heled & Drummond 2010) viabilizam o uso combinado de múltiplos locos para estimar a filogenia das espécies, o seu tempo de divergência e/ou o tamanho populacional de ancestrais representados em nós da árvore de relacionamentos. Este tipo de análise pode considerar características como recombinação, sendo mais eficiente do que o método de concatenação, permitindo a delimitação de espécies derivadas de eventos recentes de diversificação (Edwards et al. 2007).

1.2 OJBETIVOS

1.2.1 Objetivo geral:

- Caracterizar de forma comparativa a história evolutiva dos gêneros *Leopardus* e *Lycalopex*, empregando seqüências de múltiplos segmentos nucleares e múltiplos indivíduos por espécie, avaliando a eficácia deste tipo abordagem para a resolução de processos recentes de diversificação.

1.2.2 Objetivos específicos:

- Identificar marcadores moleculares nucleares que apresentem variabilidade em grupos de espécies de carnívoros recentemente divergidas.
- Caracterizar a diversidade genética destes segmentos em múltiplos indivíduos das espécies selecionadas para este estudo.
- Estimar a genealogia das espécies e indivíduos amostrados para cada segmento, comparando os padrões observados entre cada um dos marcadores
- Estimar genealogias de espécies empregando diferentes métodos analíticos, comparando seus resultados e contribuindo para a resolução da filogenia interna de cada um dos gêneros investigados.

CAPÍTULO 2

ARTIGO CIENTÍFICO

An empirical test of nuclear genealogical discordance in two parallel radiations of Neotropical carnivores

(Artigo submetido ao periódico *Biology Letters*)

An empirical test of nuclear genealogical discordance in two parallel radiations of Neotropical carnivores

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Genealogical discordance describes cases in which different genomic regions produce discrepant phylogenies of the same group of organisms. Such occurrences may result from several processes, including incomplete lineage sorting affecting multiple successive rounds of speciation. Although such processes may be prevalent in recently diversified lineages and seriously hamper accurate phylogenetic reconstruction, there are still few empirical studies documenting and analyzing this problem. Moreover, many recent phylogenetic studies continue to use a single individual to represent each species, and to concatenate multiple nuclear segments, without an assessment of possible biases resulting from this approach in the case of recent radiations.

Here we investigate this issue in two recently diversified lineages of Neotropical carnivores: the genera *Leopardus* (Felidae) and *Lycalopex* (Canidae). We sequenced multiple nuclear segments in multiple individuals of these species and analyzed their genealogical patterns. We observed cases of intra-specific variation coupled with lack of species monophyly in all genes for both genera, indicating the pervasive occurrence of incomplete lineage sorting in these recent radiations. We estimated a species tree for each lineage using the program **BEAST*, and obtained good resolution for genus *Leopardus*, but not for the more recently diversified *Lycalopex*. Overall, our results demonstrate the potential problems derived from concatenating nuclear segments and employing a single individual per species when attempting to reconstruct the phylogenetic history of recent radiations.

Running Head: Genealogical discordance in carnivores

Keywords: *BEAST, Species tree, multi-locus analysis, gene genealogies, recent diversification, *Leopardus, Lycalopex*.

1. INTRODUCTION

The Neotropical region holds approximately 30% of the species diversity of the carnivoran families Felidae (subordem Feliformia) and Canidae (subordem Caniformia) [1], which migrated to South America after the closure of the Panama Isthmus, *ca.* 3 million years ago [2,3]. Molecular evidence indicates that both went through processes of endemic adaptive radiation, which may have been facilitated or promoted by geological and environmental features of this region. Due to the recent speciation processes that occurred in these groups, some aspects of their phylogenetic structure remain controversial, especially those related to evolutionary relationships among species belonging to two lineages, the genera *Leopardus* (Felidae) and *Lycalopex* (Canidae).

The genus *Leopardus* contains seven species that diverged from a common ancestor within the last 2.9 million years. The phylogenetic relationships among these species are well characterized on the basis of molecular sequence data [2,4,5], except for the placement of *L. jacobita*, which remains poorly supported. The canid genus *Lycalopex* is a more extreme case of endemic radiation, as it is composed of six extant species that seem to have undergone a very rapid and recent diversification process, likely within the last 1 Ma [6,7]. As a consequence, their phylogenetic relationships remain largely unresolved [7,8,9].

Many authors have recently pointed out that nuclear gene loci may be used as innovative molecular markers that allow more precise estimates of

phylogenetic relationships and divergence times among species, as well as genetic diversity and demographic history [10,11,12]. Still, analyses employing single gene sequences often lead to discordant genealogies, thus appearing to be insufficient to consistently determine the pattern of the ancestral coalescence [13,14]. In this context, it becomes relevant to analyze multiple gene loci and multiple individuals per species when assessing evolutionary parameters, as this allows better estimates that take into account intraspecific variation and/or incomplete lineage sorting. Inferences from a single locus and/or a single individual may be unable to reliably express the relationship between lineages [11,12]. Novel analytical methods that reconstruct species trees from gene genealogies have been recently developed, enabling the analysis of data sets containing multiple loci and multiple individuals [15,16]. These methods promise to enable much better estimation accuracy for species tree topology than concatenation, especially in closely related species that have gone through a rapid radiation, because they accommodate the discrepancy between species trees and gene trees.

The objective of the present study was to comparatively characterize the evolutionary history of genera *Leopardus* and *Lycalopex*, using sequences of multiple independent nuclear gene loci and multiple individuals per species to test the occurrence and prevalence of genealogical discordance in these recently diversified lineages. In addition, we assessed the performance of a species tree approach for phylogenetic reconstruction in these lineages, and observed contrasting achievements that are possibly related to the age and speed of these recent evolutionary radiations.

2. Material and Methods

We generated sequences for six species of the genus Lycalopex (L. culpaeus, L. fulvipes, L. griseus, L. gymnocercus, L. vetulus and L. sechurae) and five species of the genus Leopardus (L. colocolo, L. geoffroyi, L. pardalis, L. tigrinus and L. wiedii). In most cases, multiple individuals per species were sequenced, so as to test species-level monophyly as well as to improve the inter-species resolution (See Table S1 [Supplementary Material] for the number of sequences generated per species for each gene segment). For the species Leopardus jacobita and Leopardus guigna, we used sequences deposited in GenBank. As outgroups we utilized Cerdocyon thous for the genus Lycalopex and Felis catus and Puma concolor for the genus Leopardus (see Tables S2 and S3 [Supplementary Material] for Genbank accession numbers).

Genomic DNA was extracted from tissue and blood samples following a standard phenol/chloroform protocol [17]. We analyzed eight gene segments (ATP7AE4, BTK, CHRNA1, CYP1A1, GHR, PLP1I2, SILV, TCP1) for genus Leopardus, and seven segments (ATP7AE3, CHRNA1, CYP1A1, FES, GHR, TCP1, VTN) for genus Lycalopex. Segments were amplified by the Polymerase Chain Reaction (PCR) using primer sets designed here or in previous phylogenetic studies (Table S4, Supplementary Material). PCR products were examined on a 1% agarose gel stained with GelRed (Biotium, Hayward, CA), purified with shrimp alkaline phosphatase and exonuclease I, sequenced with the DYEnamic ET Dye Terminator

Sequencing Kit (GE Healthcare), and analyzed in a MegaBACE 1000 automated sequencer (GE Healthcare). Sequences were deposited in GenBank under accession numbers xxx – xxx.

Sequences were visually checked and edited by hand using FinchTV (Geospiza, Seattle, WA) and final contigs (integrating two or more reads) were constructed using the software package containing PHRED, PHRAP, and CONSED (http://www.phrap.org/phredphrapconsed.html). We aligned final contigs with the CLUSTALW algorithm implemented in MEGA 4.1 [18]. The program Phase [19] was used to identify the gametic phase of segments bearing complex patterns of heterozygosity. Haplotype networks depicting the evolutionary relationships among sequences were built using the median-joining approach [20] implemented in Network 4.2.0 (www.fluxus-engineering.com). To determine the appropriate model of nucleotide sequence evolution, we used the Akaike information criterion as implemented in MrModeltest 2.3 [21]. Species trees were inferred using the program *BEAST [16] (see Table S5 [Supplementary Material] for parameters of the *BEAST run).

3. RESULTS

The monophyletic condition of the *Leopardus* and *Lycalopex* genera received significant statistical support, with 1.0 and 0.97 Bayesian posterior probabilities (BPP), respectively (Figure 1). All genes showed interspecific and intraspecific variation, which could be clearly observed in haplotype networks (Figures S1 and S2, Supplementary Material). We also observed considerable sharing of haplotypes between two or more species in all genes, and several cases in which species were not monophyletic.

For genus *Leopardus*, we estimated the species trees for two data sets. In the first one, only the species represented by multiple individuals (*L. colocolo, L. geoffroyi, L. pardalis, L. tigrinus* and *L. wiedii*) were analyzed. With this data set, we obtained a high resolution for the species tree, and all nodes bore more than 0.96 BPP (Figure S3). In the second analysis, all the species contained in the genus were included (Figure 1A). The species *L. jacobita* grouped with *L. colocolo* (with strong support) and *L. guigna* was placed as a sister-group to the (*L. geoffroyi* + *L. tigrinus*) group (albeit with lower support). For genus *Lycalopex*, we did not obtain strong support for most internal nodes (Figure 1B). In this group, only the clade that unites the species *L. gymnocercus* + *L. griseus* received high support (0.91 BPP).

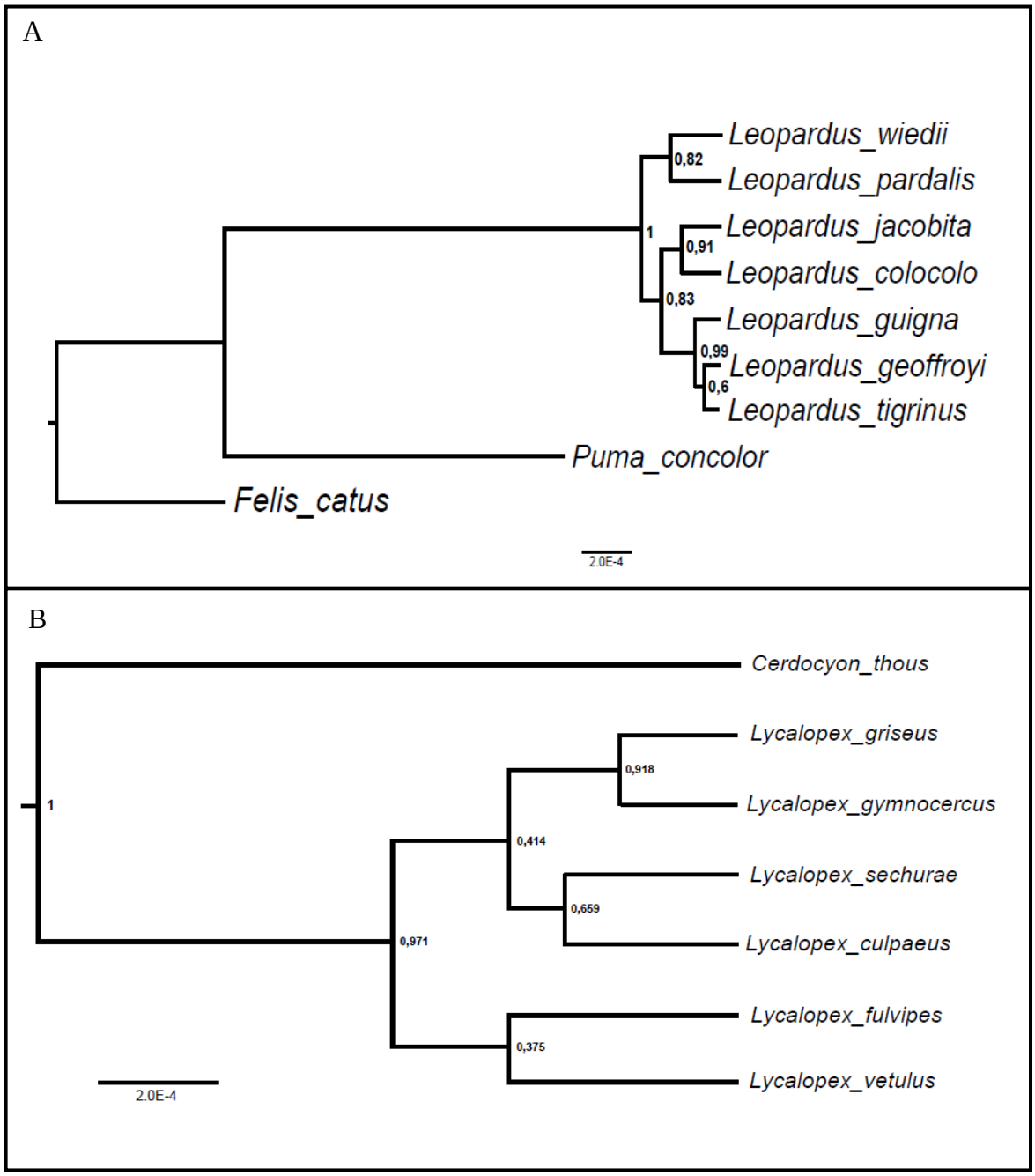

Figure 1: Species trees inferred with *BEAST for genera *Leopardus* (A) and *Lycalopex* (B). The Bayesian posterior probability of each clade is indicated next to the defining node.

4. Discussion

Most phylogenetic methods estimate gene trees and assume a complete correspondence between the topology of these gene trees, which would reflect the underlying species tree. This assumption is often correct, especially in deep coalescences, where events of interespecific gene flow or incomplete lineage sorting are rare [22]. On the other hand, recent and rapid radiations may lead to

departures from this pattern. Our results from the genera *Leopardus* and *Lycalopex* show rampant discordance among independent gene loci, along with lack of species monophyly and inter-specific haplotype sharing. The occurrence of intraspecific variation and sharing of haplotypes between species gene can severely mislead phylogenetic reconstruction, depending on the individuals included in the analysis. The differential sharing of haplotypes observed among species and the low support observed for some internal nodes in the multi-locus analyses are probably a result of variable sorting patterns of ancestral polymorphisms, which is consistent with expectations for a recent diversification process. In the case of *L. tigrinus,* who shared haplotypes with *L. geoffroyi* and *L. colocolo,* we can also raise the alternative hypothesis of hybridization among these species [5]. Distinguishing between these two causes will remain a challenge for multi-locus analyses in this lineage, and will likely require an expanded genomic sample and better understanding of haplotype structure in target regions.

The species tree inferred for *Leopardus* was strongly supported and has a concordant topology relative to a previous study [2]. We corroborate with high support the placement of *L. jacobita* as a sister-group of *L. colocolo*. We concluded that the selected markers, along with the species tree method employed here, are effective for robust phylogenetic resolution in this group. In contrast, for the more recent *Lycalopex* radiation, we did not obtain a well-resolved species tree. The reconstructed consensus topology was very similar to the maximum parsimony tree of Lindblad-Toh *et al.* (2005). However, in our analysis, *L. vetulus* grouped with *L. fulvipes* (weakly supported), instead of being placed at a basal position in the genus, as was the case in that study. Fully resolving the relationships within *Lycalopex* will likely demand the addition of more nuclear loci, which is not surprising given the very recent and rapid radiation of this genus [3,7]. Overall, our results illustrate the potential and challenges for multi-locus phylogenetic approaches targeting recent radiations, and highlight the importance of using multiple individuals per species to better assess the resolution and robustness of such phylogenies.

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SUPPLEMENTARY MATERIAL

Table S1: Number of sequences generated here per species for each gene segment.

Species \ Gene	*ATP7AE4*	*BTK*	*CHRNA1*	*CYP1A1*	*GHR*	*PLP1I2*	*SILV*	*TCP1*
Leopradus colocolo	7	15	10	9	3	10	9	3
Leopardus geoffroyi	7	18	10	10	2	9	11	6
Leopardus pardalis	7	15	13	10	2	8	11	2
Leopardus tigrinus	6	13	10	10	3	11	10	5
Leopardus wiedii	6	15	12	9	4	10	10	5
Leopardus guigna	1	0	1	0	1	1	1	1
Leopardus jacobita	1	0	1	0	1	1	1	1
Puma concolor	3	1	2	1	1	3	3	1
Felis catus	3	2	2	2	2	2	2	1
Total	**41**	**79**	**61**	**51**	**19**	**55**	**58**	**25**

Species \ Gene	*ATP7AE3*	*CHRNA1*	*CYP1A1*	*FES*	*GHR*	*TCP1*	*VTN*
Lycalopex culpaeus	3	8	6	2	1	6	4
Lycalopex fulvipes	5	7	6	3	2	8	4
Lycalopex griseus	4	9	9	4	2	9	5
Lycalopex gymnocercus	3	7	7	2	4	6	6
Lycalopex sechurae	2	4	5	1	2	3	4
Lycalopex vetulus	5	4	5	3	4	4	8
Cerdocyon thous	0	4	2	4	2	3	2
Total	**22**	**43**	**40**	**19**	**17**	**39**	**33**

Table S2: Genbank accession numbers for *Leopardus* genus

Species \ Gene	*ATP7AE4*	*CHRNA1*	*GHR*	*PLP1I2*	*SILV*	*TCP1*
Leopardus colocolo	DQ082617.1	-	DQ082097.1	-	DQ082447.1	DQ082490.1
Leopardus geoffroyi	DQ082615.1	-	DQ082095.1	DQ082703.1	DQ082445.1	DQ082488.1
Leopardus guigna	DQ082616.1	DQ081838.1	DQ082096.1	DQ082704.1	DQ082446.1	DQ082489.1
Leopardus jacobita	DQ082614.1	DQ081836.1	DQ082094.1	DQ082702.1	DQ082444.1	DQ082487.1
Leopardus pardalis	DQ082612.1 and AY011434.1	-	DQ082092.1	-	DQ082442.1	DQ082485.1
Leopardus tigrinus	DQ082618.1	-	DQ082098.1	DQ082706.1		DQ082491.1
Leopardus wiedii	DQ082613.1	-	DQ082093.1	DQ082701.1	DQ082443.1	DQ082486.1
Puma concolor	-	-	DQ082082.1	DQ082692.1	-	DQ082475.1
Felis catus	-	-	DQ205829.1 and DQ082070.1	DQ082680.1	DQ082420.1	DQ082464.1

Table S3: Genbank accession numbers for genus *Lycalopex*.

Species \ Gene	*CHRNA1*	*CYP1A1*	*FES*	*GHR*	*VTN*
Lycalopex culpaeus	DQ239440.1	DQ239448.1	DQ239456.1	DQ239464.1	DQ239480.1
Lycalopex fulvipes	DQ239441.1	DQ239449.1	DQ239457.1	DQ239465.1	DQ239481.1
Lycalopex griseus	AY885319.1	AY885343.1	AY885366.1	AY885390.1	AY885414.1
Lycalopex gymnocercus	EF106987.1 and AY885320.1	AY885344.1	AY885367.1	AY885391.1	AY885415.1
Lycalopex sechurae	AY885321.1	AY885345.1	AY885368.1	AY885392.1	AY885416.1
Lycalopex vetulus	DQ239442.1 and EF106988.1	DQ239450.1	DQ239458.1	DQ239466.1	DQ239482.1
Cerdocyon thous	EF106982.1	AY885341.1	AY885364.1; EF107038.1; EF107041.1 and EF107040.1	AY885388.1	-

Table S4: List of the segments and primers used in this study.

Gene	Gene Name	Reference
ATP7AE3	*ATPase, Cu++ transporting, alpha polypeptide*, exon 3	This study. F: 5'aaaaatgcaactattatttatgacccta3' R: 5' taattcgctgaacaccttgc 3'
ATP7AE4	*ATPase, Cu++ transporting, alpha polypeptide*, exon 4	Eizirik et al. (2001)
BTK	*Bruton agammaglobulinemia tyrosine kinase*	Janecka et al. (2008)
CHRNA1	*Cholinergic receptor,nicotinic alpha polypeptide precursor 1*, intron 8	Lyons et al. (1997)
CYP1A1	*Cytochrome P-450*, intron 3 to exon 6	Venta et al. (1996)
FES	*Feline sarcoma protooncogene*, intron 14	Venta et al. (1996)
GHR	*Growth hormone receptor*, intron 9 to exon 10	Venta et al. (1996)
PLP 1	*Proteolipid protein-1*, intron 2	Murphy et al. (1999)
SILV	Melanocyte protein Pmel 17	Johnson et al. (2006)
TCP1	*T-complex protein 1, alpha subunit*	Lyons et al. (1997)
VTN	*Vitronectin*, intron 4	Jiang et al. (1998)

Table S5: Parameters employed for the *BEAST analyses.

Parameter	***Leopardus***	***Lycalopex***
Chain length*	100.000.000	200.000.000
Spacing between data samples	10.000	20.000
Species tree prior	Species tree: Yule process	Species tree: Yule process
Population Size Model	Piecewise linear & constant root	Piecewise linear & constant root
Clock Model	Strict Clock	Strict Clock
Base frequencies	Empirical	Empirical
Substitution Model	**HKY** for *ATP7AE4*, *BTK*, *CYP1A1* and *PLP1I2*	**HKY** for *ATP7AE3*, *GHR*, *TCP1* and *VTN*
	HKY+I for *CHRNA1* and *SILV*	**HKY+I** for *CHRNA1*, *CYP1A1* and FES
	GTR for *GHR*	
	GTR+I for *TCP1*	

* The initial 10% of each MCMC run was discarded as burn-in.

Figure S1: Median-joining networks estimated for genus *Leopardus*. Each network was derived from a different gene segment, whose name is indicated on the top. Each haplotype is represented by a circle, whose area is proportional to its frequency. The colors represent different species, described in the legend. Haplotypes shared between two or more species are represented by circles with mixed colors. Bars placed on connecting lines indicate the number of nucleotide differences between haplotypes.

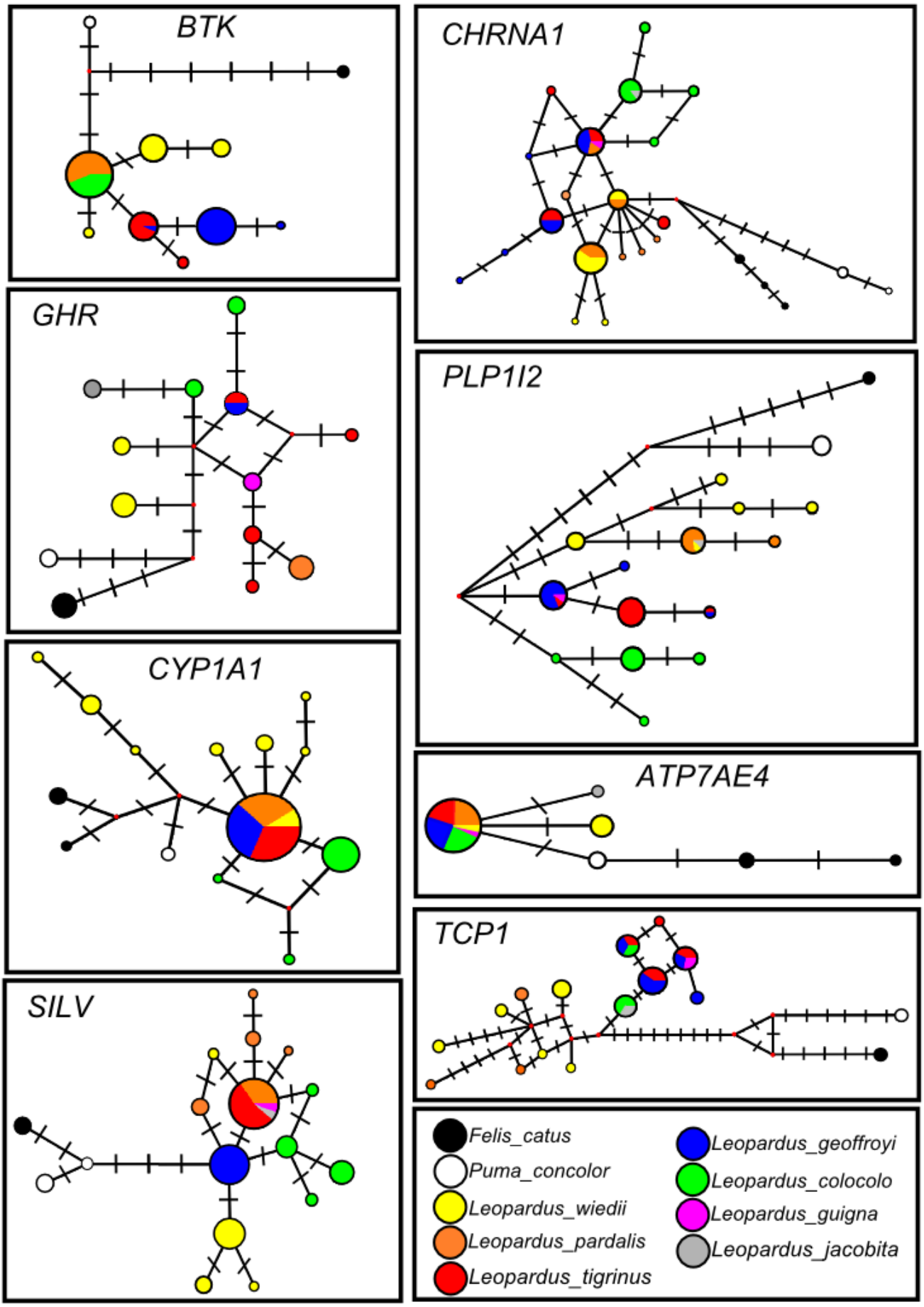


Figure S2: Median-joining networks estimated for genus *Lycalopex*. Gene names are indicated on the top of each box. Each haplotype is represented by a circle, whose area is proportional to its frequency. The colors represent different species, described in the legend. Haplotypes shared between two or more species are represented by circles with mixed colors. Bars placed on connecting lines indicate the number of nucleotide differences between haplotypes.

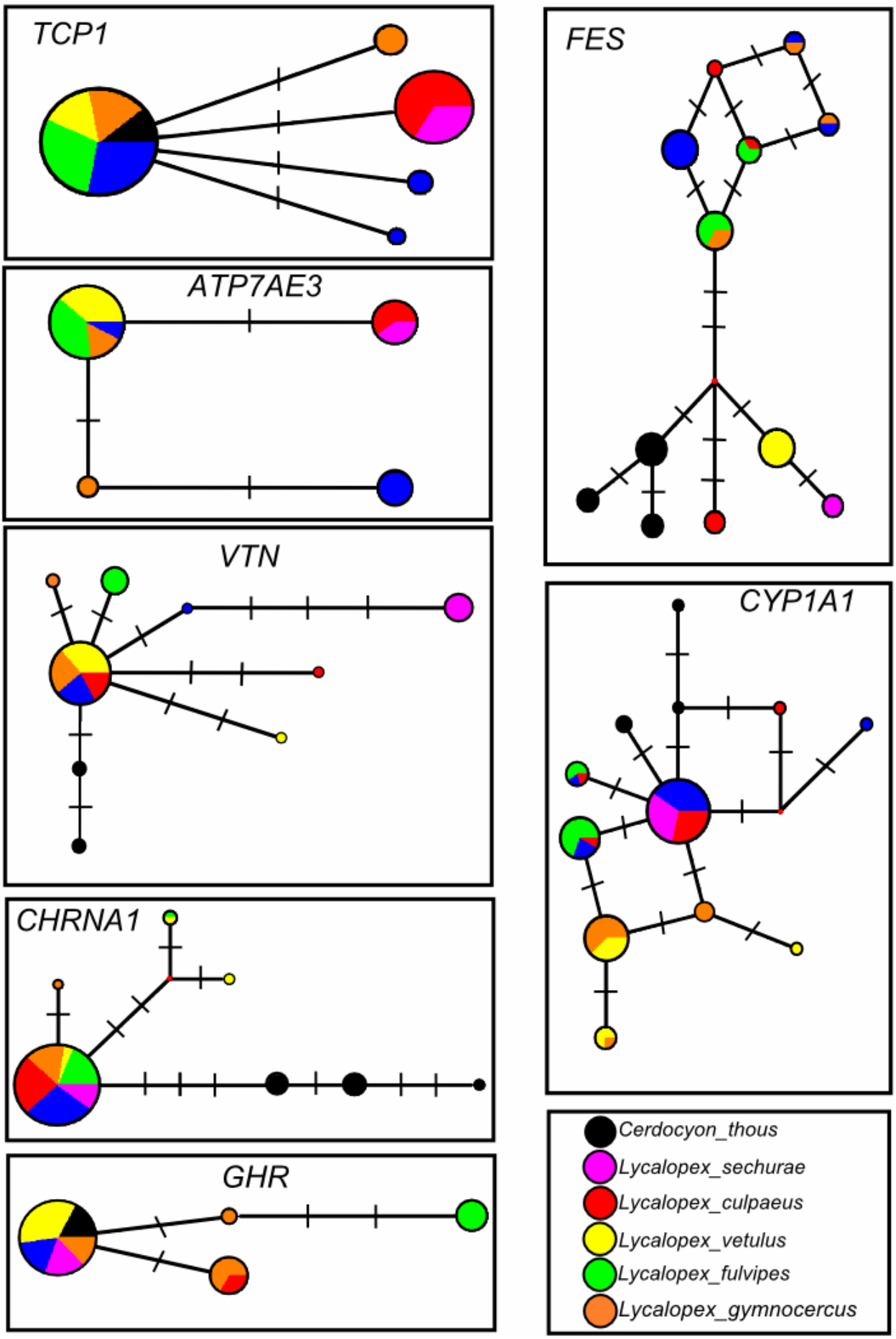
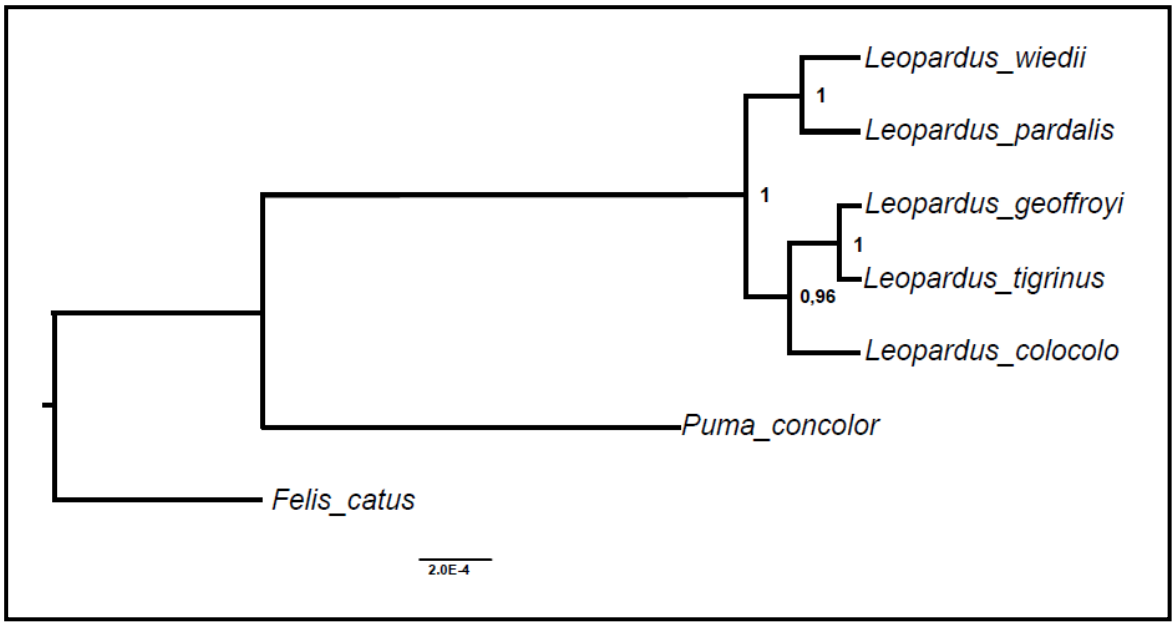


Figure S3: Species trees inferred with *BEAST for genus *Leopardus*. Only species represented by multiple individuals (*L. colocolo*, *L. geoffroyi*, *L. pardalis*, *L. tigrinus* and *L. wiedii*) were analyzed. The Bayesian posterior probability of each clade is indicated next to the defining node.



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