PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL

PROGRAMA DE PÓS-GRADUAÇÃO EM ZOOLOGIA

DISSERTAÇÃO DE MESTRADO

Relações filogenéticas entre espécies do gênero *Lycalopex* (Mammalia, Canidae) inferidas com o uso de marcadores do DNA mitocondrial

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Porto Alegre - 2011

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PORTO ALEGRE – RS – BRASIL

2011

Agradecimentos

Ao meu orientador e amigo Duda pela oportunidade de ampliar meus conhecimentos, por acreditar em mim e por todos os ensinamentos e conselhos dados ao longo destes dois anos.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela bolsa de mestrado, sem a qual este estudo não seria possível.

Aos Professores da Pós-Graduação pelos ensinamentos e experiências passadas.

Aos colaboradores pelas amostras, em especial ao CENAP, ao Carlos Benhur e ao Manoel.

Aos queridos colegas de Pós-graduação, pelos momentos de discussão, descontração, por toda a ajuda, em especial à Stela, Paola, Tani e ao David (que me deu várias dicas computacionais).

A todos os queridos genômicos por me acolherem e me proporcionaram momentos de aprendizagem em especial ao Henrique (por toda assistência de bancada), ao Maneco (por todo incentivo e ajuda com o projeto) e ao Felipe (por toda a ajuda com as análises), sou muito grata.

Às queridas, únicas e insubstituíveis genômicas: Tali, Flá, Lisie, Carla, Rê, Aninha, Ale, Taia, Tati, Ane, Fê e a Laura pelos ótimos dias compartilhados no Laboratório e também fora dele, pelas conversas, conselhos e risadas, que tornaram os meus dias muito melhores, vocês são Mara. Em especial a Cris e a Miriam que me salvaram nas horas de maior sufoco com a dissertação, sou imensamente grata.

À Elenara que entrou junto comigo nesta jornada, agora também é minha colega de Genoma, e hoje eu considero uma grande amiga, por toda companhia, pelos conselhos e risadas.

À Cladi que sempre manteve o laboratório em ordem e por toda a ajuda dada, até pelas broncas, hoje não está mais trabalhando conosco, mas nos deixou muitos ensinamentos (principalmente o dos CupCakes).

À Priscila que gerou a minha primeira placa toda verde.

À Tahiana pela força e o incentivo final, sempre disposta a me ajudar.

As minhas velhas amigas Pri, Fê, Biba e a Cris pela amizade de longa data.

Ao meu querido cão amigo Bobby, que também foi minha fonte de inspiração para este trabalho, pela companhia, estando ao meu lado todo o tempo.

Ao meu namorado, William, por todo o carinho, apoio e compreensão principalmente nas horas difíceis.

Aos meus pais, meus exemplos, Paulo e Mariza, e meu irmão por toda a compreensão que mesmo sem entender muito bem o que eu faço me incentivaram e apoiaram sempre.

E claro, as raposinhas por existirem para que eu pudesse contar um pouco da sua história.

"É muito melhor arriscar coisas grandiosas, alcançar triunfos e glórias, mesmo expondo-se a derrota, do que formar fila com os pobres de espírito que nem gozam muito nem sofrem muito, porque vivem nessa penumbra cinzenta que não conhece vitória nem derrota."

Theodore Roosevelt

Dedico esta dissertação a todos os admiradores da vida, e dos processos evolutivos que estão representados nas suas mais diversas formas.

SUMÁRIO

RESUMO

A América do Sul possui a maior diversidade de canídeos (Mammalia, Carnivora, Canidae) do mundo, contendo representantes de seis gêneros e um total de 10 espécies. O registro fóssil indica que representantes da família Canidae teriam saído da América do Norte e conquistado a América do Sul durante o Grande Intercâmbio Americano, há cerca de 2,5 milhões de anos. Estima-se que tenham ocorrido desde uma única até quatro invasões independentes do continente sul-americano, sendo que o número exato é ainda motivo de controvérsias. Diversos estudos morfológicos e moleculares buscaram compreender as relações filogenéticas entre os canídeos, porém ainda há muitas incertezas, especialmente no que se refere ao clado de raposas da América do Sul formado pelo gênero *Lycalopex*, que conta com seis espécies atuais. Estudos recentes indicam que este gênero sofreu uma radiação muito rápida há aproximadamente um milhão de anos, o que explica a dificuldade histórica em resolver a filogenia destes canídeos. Em virtude disto, este estudo buscou reconstruir as relações filogenéticas e datar a divergência entre as espécies componentes deste gênero, através do uso de diferentes segmentos do DNA mitocondrial (mtDNA), perfazendo um total de 6000 pb. Foram utilizados diferentes métodos de reconstrução filogenética, e todas as análises apoiaram a mesma árvore. Múltiplos indivíduos de cada espécie foram incluídos, viabilizando a avaliação da monofilia de cada uma delas (incluindo *L. sechurae*, testado aqui pela primeira vez). Todas as espécies formaram grupos monofiléticos bem apoiados, corroborando seu reconhecimento como entidades taxonômicas. Uma única exceção a este padrão foi a presença de dois indivíduos de *L. vetulus* provenientes de São Paulo portando mtDNA de *L. gymnocercus*, indicando um potencial caso de expansão na distribuição desta última, ou hibridação entre estas espécies. As análises de datação molecular indicaram que o gênero iniciou sua radiação evolutiva há cerca de 1 milhão de anos, corroborando estudos anteriores que reportaram uma origem muito recente para este grupo de canídeos. A espécie mais basal foi *L. vetulus*, seguida de *L. sechurae*, e o grupo mais interno contém *L. culpaeus* e *L. fulvipes*, cuja divergência ocorreu há apenas cerca de 390 mil anos. A partir dos padrões filogenéticos inferidos, discutimos hipóteses sobre a biogeografia histórica do gênero, buscando compreender este rápido processo de diversificação endêmico da região neotropical.

ABSTRACT

South America harbors the greatest diversity of canids (Mammalia, Carnivora, Canidae) worldwide, containing representatives of six genera and a total of 10 species. The fossil record indicates that canid representatives have colonized South America from North America during the Great American Biotic Interchange, *ca.* 2.5 million years ago (Mya). Current hypotheses postulate between one and four independent canid invasions to South America, with the exact number being a recurrent topic for controversy. Several morphological and molecular studies have attempted to unravel the phylogenetic relationships among canids, but many uncertainties remain. This is particularly the case of the South American fox clade corresponding to genus *Lycalopex*, which comprises six extant species. Recent studies have indicated that this genus has undergone a very rapid radiation *ca.* one million years ago, which underlies the historical difficulty in resolving the phylogeny of these canids. In this context, the present study aimed to reconstruct the phylogenetic relationships among the species comprised in this genus, as well as to date their divergences. We used multiple segments of the mitochondrial DNA (mtDNA), encompassing a total of 6000 bp. Several different phylogenetic methods were employed, with all trees converging on the same inter-specific topology. We included multiple individuals from each species, allowing us the evaluation of the monophyly of each of them (including *L. sechurae*, tested here for the first time). All species formed well-supported monophyletic clusters, corroborating their recognition as taxonomic entities. The single exception to this pattern was the identification of two *L. vetulus* individuals sampled in São Paulo state, Brazil, which bore mtDNA sequences that clustered within the *L. gymnocercus* clade. This result could indicate that *L. gymnocercus* is expanding its range in to São Paulo state, or else that these two species may by hybridizing in the wild. Molecular dating analyses indicated that the genus began its radiation *ca.* 1 Mya, corroborating earlier studies which reported a very recent origin for this canid group. The most basal species was *L. vetulus*, followed by *L. sechurae*. The most internal cluster contains *L. culpaeus* and *L. fulvipes*, with our results indicating that they diverged from each other ca. 390,000 years ago. On the basis of the reconstructed phylogenetic patterns, we discuss hypotheses regarding the biogeography of this genus, aiming to understand the history of its rapid diversification process in the Neotropics.

APRESENTAÇÃO

A presente dissertação de mestrado, intitulada "Relações filogenéticas entre espécies do gênero *Lycalopex* (Mammalia, Canidae) inferidas com o uso de marcadores do DNA mitocondrial" foi desenvolvida como parte dos requisitos necessários para obtenção do título de Mestre junto ao programa de Pós-Graduação em Zoologia da Pontifícia Universidade Católica do Rio Grande do Sul.

Este trabalho teve como principais objetivos (i) investigar as relações filogenéticas entre as espécies do gênero *Lycalopex* (*L. gymnocercus, L. vetulus, L. sechurae, L. griseus, L. culpaeus* e *L. fulvipes*) através do uso de marcadores do DNA mitocondrial; e (ii) estimar os tempos de divergência entre as linhagens identificadas, contribuindo para reconstruir a história evolutiva e biogeográfica deste grupo.

Esta dissertação é apresentada no formato de artigo científico a ser submetido ao periódico *Molecular Phylogenetics and Evolution.*

Molecular phylogeny and dating of the recently diversified fox genus *Lycalopex* (Mammalia, Carnivora, Canidae) inferred from multiple mitochondrial DNA markers

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Abstract

The canid genus *Lycalopex* includes six fox species that are endemic to South America. Fossil and molecular evidence have indicated that this genus has undergone a rapid and recent radiation after its entering in South America during the Great American Biotic Interchange. Several recent studies have attempted to reconstruct the canid phylogeny, showing that this genus is monophyletic, but its intrageneric relationships have remained unresolved. In this study we have investigated the phylogenetic relationships among the species comprised by the genus *Lycalopex*, including tests of species-level monophyly, as well as estimates of divergence times using a relaxed molecular clock approach. To reconstruct the phylogeny, we used 6000 bp of concatenated mitochondrial DNA (mtDNA) segments, employing the methods of Maximum Likelihood, Maximum Parsimony, and Bayesian Inference. The three methods converged onto the same tree topology, most of whose nodes received considerably high support. All species were confirmed as monophyletic groups, some of which exhibited interesting patterns of intra-specific phylogenetic structure. With respect to inter-specific relationships, our results supported *L. vetulus* as the most basal species, having diverged from the remaining lineages *ca.* 1.2 Mya. The second species to diverge was the Pacific coast endemic *L. sechurae*, followed by the pampas fox *L. gymnocercus*. The most internal group comprised *L. griseus* and the sister-species *L. culpaeus* and *L. fulvipes*, likely representing a very recent radiation (*ca.* 430,000 years old) that took place in southern Argentina and Chile. The estimated relationships and divergence times allow for an improved inference of the biogeographic context of the speciation events that led to this rapid Neotropical radiation.

1. Introduction

The reconstruction of phylogenetic relationships within groups that have undergone rapid radiation is a major challenge in the process of inferring the tree of life on Earth. Several studies have shown how problematic it is to work with these groups, as exemplified by *Palinurus* (Decapoda) (Palero et al. 2009), *Serinus* (Passeriformes) (Arnaiz-Villena et al. 1999), triplefin blennies (Perciformes) (Carreras-Carbonell et al. 2005), *Thomomys* (Rodentia) (Belfiore et al. 2008), or South American deer (family Cervidae) (Duarte et al. 2008). The main underlying difficulty is related to the absence of sufficient time to accumulate enough phylogenetically informative characters on each branch, prior to the next round of cladogenesis.

Among mammals, carnivores (order Carnivora) seem to be often prone to exhibit a pattern of lineage rise and fall, where declining clades are replaced by new ones (Van Valkenburg 1999), thus providing an opportunity for rapid evolutionary radiation in the latter. Several recent examples of this pattern may be inferred from the fossil record and also from molecular phylogenies (e.g. Eizirik et al. 2010). Remarkable cases of recent radiations in the Carnivora may be seen in the families Ursidae (Waits et al. 1999; Yu et al. 2007), Felidae (Johnson et al. 2006), Canidae (Lindblad-Toh et al. 2005; Perini et al. 2010) and Phocidae (Higdon et al. 2007, Davis et al. 2004), among others.

Within the family Canidae, the South American genus *Lycalopex* seems to have experienced a particularly rapid and recent radiation, likely originating a mere 1.3 - 1.2 million years ago (Mya) (Perini et al. 2010; Tchaicka et al. in prep). Due to its present diversity, along with the occurrence of four other extant endemic canid species (*Cerdocyon thous, Chrysocyon brachyurus, Speothos venaticus* and *Atelocynus microtis*) South America is currently the possessor of the greatest diversity of Canidae worldwide. Fossil evidence indicates that this group entered South America coming from North America during the Great American Biotic Interchange (GABI), which happened *ca.* 2.5 Mya, after the closure of the Panamanian land bridge (Eisenberg & Redford 1999; Woodburne 2010). Their subsequent evolutionary success is possibly related with an apparently unoccupied ecological space in South America, which resulted in a rapid adaptive radiation (Van Valkenburg 1999). Previous phylogenetic studies have suggested that the immigration of canids to South America has occurred in different episodes (e.g. Wayne et al. 1997), possibly involving as many as two ancestral

fox lineages and two additional groups (*Chrysocyon* and *Speothos*). More recent studies, however, have supported topologies in which all South American genera form a monophyletic group (e.g. Lindblad-Toh et al. 2005), raising the possibility that a single immigrant species might have led to this endemic radiation (Eizirik, in press). A recent molecular dating analysis has estimated that the basal diversification of South American canids occurred ca. 4 Mya, preceding the GABI and suggesting that at least two independent lineages would have entered the continent (Perini et al. 2010). One such lineage would be the ancestor of the fox genera *Cerdocyon*, *Atelocynus* and *Lycalopex*, and the precursor of the rapid radiation that took place within the latter.

The genus *Lycalopex* is currently thought to comprise six extant species: pampas fox (*L. gymnocercus)*, hoary fox (*L. vetulus)*, chilla (*L. griseus)*, culpeo (*L. culpaeus)*, Darwin's fox (*L. fulvipes*) and sechuran fox (*L. sechurae*) (Wozencraft 2005). They are widespread in South America (Figure 1) and generally occur in grasslands, with the exception of Darwin's fox, which occurs in temperate rainforests in a restricted distribution including Chiloé Island and Nahuelbuta National Park in Chile (Yahnke et al. 1996, Vilà et al. 2004). The sechuran fox is the smallest species of the genus and is restricted to the Pacific coast of Peru and southwestern Ecuador. The hoary fox is associated with the Brazilian Cerrado biome, while the pampas fox occurs in southern Brazil, eastern Bolivia, western Paraguay and eastern Argentina (see Figure 1). The culpeo fox is the largest of these species, and occurs along the Andes from southern Colombia to southern Chile. Finally, the chilla fox presents considerable range overlap with the culpeo, and occurs on both sides of the Andes, from northern Chile to Tierra del Fuego (Eisenberg and Redford 1999).

Several authors proposed different classifications for the *Lycalopex* species: Cabrera (1958) included some of these species in the genus *Dusicyon*; Langguth (1975) classified them within *Canis*; both Berta (1987) and Wozencraft (1993) included them in *Pseudalopex*. Finally, these species were all classified within *Lycalopex* by Berta et al. (1987) and Zrzavý & Ricánková (2004), given the view that they formed a monophyletic assemblage and that this name represents the oldest genus in the cluster (described by Burmeister in 1854 for the hoary fox). Subsequently, the use of *Lycalopex* for this group has been supported by Wozencraft (2005), and we follow the same scheme here.

The difficulty in resolving the evolutionary relationships among these fox species has been remarkable, especially given the overall effort placed historically on

resolving the phylogeny of the Canidae. Canid phylogenetics has been a research focus for a relatively long time, including several studies that used different approaches and character sets, such as morphology (Berta, 1987; Tedford et al. 1995), allozymes (Wayne & O´Brien 1987), cytogenetics (Wayne et al,. 1987a,b, Nash et al. 2001), mitochondrial DNA (mtDNA) sequences (Wayne et al. 1997), and multi-locus nuclear DNA sequences (Bardeleben et al., 2005, Lindblad-Toh et al. 2005). In addition, recent analyses have combined large morphological and molecular data sets (Zrzavý & Ricánková 2004; Perini et al 2010), but still failed to resolve several nodes within the Canidae, including the relationships among *Lycalopex* species.

In most studies addressing phylogenetic questions in canids, only one individual per species was sampled, precluding an assessment of species-level monophyly. The main exceptions for the case of *Lycalopex* were the studies by Yahnke et al. (1996) and Vilà et al. (2004), which analyzed multiple individuals each of the chilla, culpeo and Darwin's foxes. Interestingly, these studies did not support the reciprocal monophyly of the chilla and culpeo, while that of Darwin's fox was recovered. In a more recent study based on mtDNA control region sequences, Tchaicka et al. (in prep), analyzed several individuals for each species except *L. sechurae*, and found support for their monophyly, although inter-specific relationships could not be robustly resolved.

Given the extreme difficulty in resolving the relationships within *Lycalopex*, even with the use of large nuclear data sets consisting of >15kb of DNA sequences (e.g. Lindblad-Toh et al. 2005), we have concluded that a first and important step would be to produce a robust phylogeny of the group based on the mitochondrial DNA. The mtDNA has been the marker of choice to resolve phylogenies underlying rapid and recent radiations, and has often been shown to produce better resolution in these cases when compared with other molecular markers (e.g. Yu et al., 2007, Davis et al. 2004, Delisle & Strobeck, 2002). This is an expected pattern, given the well-known features of the mtDNA such as maternal inheritance, absence of recombination and high substitution rates (Avise et al. 1987). As a consequence, mtDNA segments can be easily concatenated without the issues arising from genealogical discordance (e.g. differential lineage sorting) that seriously affect equivalent nuclear data sets. This allows the construction of large supermatrices that share the same genealogical history, and could contain sufficient phylogenetic information to resolve the sequence of divergence events that characterize a rapid and recent radiation. Although such mtDNA resolution cannot be claimed to necessarily be identical to the species genealogy, it can serve as a baseline

for further data collection and analysis (e.g. using species tree methods), and also lend itself to direct interpretation, including the estimation of divergence dates and the assessment of alternative biogeographic hypotheses. In this study, we have generated and analyzed a rather large mtDNA supermatrix including multiple individuals for each of the currently recognized *Lycalopex* species. Several phylogenetic analyses have converged onto a robustly supported topology for the genus, which has allowed an improved assessment of its evolutionary history.

2. Materials and Methods

2.1. Sample Collection

Biological samples (blood and tissue) were collected from 55 *Lycalopex* individuals and nine specimens from the closely related species: *Cerdocyon thous, Chrysocyon brachyurus* and *Speothos venaticus* (Table 1). Blood samples were collected from wild animals captured for ecological studies and also from captive individuals with known geographic origin, in both cases being preserved in a salt-saturated solution (100mM Tris, 100mM EDTA, 2% SDS). Tissue samples were obtained from road-killed specimens and maintained in 96% ethanol.

2.2. DNA extraction, amplification and sequencing

DNA extraction was conducted using a standard phenol/chloroform protocol (Sambrook et al. 1989), followed by verification of integrity and concentration on 1% agarose gels stained with GelRed (Biotium). We amplified via the Polymerase Chain Reaction (PCR) six different mitochondrial DNA segments (Table 2): (i) the 5' portion of the control region; (ii) the 5' portion of the *cytocrome oxidase c subunit I* (*COI*) gene; (iii) the complete *cytochrome b* (*cyt-b*) gene; and (iv-vi) three overlapping fragments (named '7mt', '8mt' and '9mt') proposed by Delisle & Strobeck (2002) as part of a strategy to amplify and sequence whole mitochondrial genomes of carnivore species. The contiguous segment produced when joining these three fragments includes complete or partial sequences of the genes *COIII*, *ND3*, *ND4L*, *ND4* and *ND5*, as well as tRNAs *Gly*, *Arg*, *His*, *Ser* and *Leu*.

Initial PCR reactions employed previously available primer sets that amplified medium to large fragments, except for the *cytochrome b* gene, for which we used a novel set developed here to span its entire coding region (Tables 2 and 3). Subsequent *cyt-b* reactions also used the primer sets reported by Irwin et al. (1991), which amplify the gene in two overlapping sub-fragments. In the case of segments 7mt and 8mt, we used initial *Lycalopex* sequences to design four additional primers for each of them (Table 3), which served as internal sequencing primers as well as to directly amplify sub-fragments spanning approximately 700bp each. For segment 9mt, we designed one internal primer (see Table 3), and also utilized primer ND5-DR1 (Trigo et al. 2008) for amplification and sequencing within this region. Since our *ND5* fragment was contained within the 9mt segment, and that the original primer set ND5-DF1/DR1 often amplified nuclear mtDNA copies (*numts*) in several of these canid species (not shown), we mostly used sequences derived from the 9mt segment to cover this gene.

PCR reactions were performed in a 20μL final volume containing 0.2u Taq Platinum (Invitrogen), 1x Buffer (Invitrogen), 0.2 μM each of the forward and reverse primers, 0.1 mM dNTPs and 1.5 mM $MgCl_2$ for all segments except for *COI* (in which case we changed the concentrations of dNTP and $MgCl_2$ to 0.2 mM and 2.5 mM, respectively). The thermocycling conditions followed those described by Tchaicka et al. (2007) *i.e.* a touchdown PCR that begins with 10 cycles (touchdown) decreasing the annealing temperature from 60°C to 51°C (45s per cycle), followed by 30 cycles with 50°C annealing temperature for 30s. In every case, the denaturing step was 45s at 94°C, and the extension step was 1.5 min at 72°C. PCR products were verified on a 1% agarose gel stained with GelRed, and subsequently purified using a protocol based on precipitation with ammonium acetate and isopropanol. We sequenced both strands of each purified PCR product using the DYEnamic ET Kit (GE Healthcare) and analyzed them in a MegaBACE 1000 automated sequencer (GE Healthcare).

2.3. Data Analyses

Sequence electropherograms were verified and manually corrected using the software FinchTV (Geospiza). Consensus sequences of forward and reverse strands, as well as contigs derived from multiple overlapping reads, were constructed using Phred/Phrap/Consed (Ewing et al. 1998, Ewing & Green 1998, Gordon et al. 1998). Resulting sequences were aligned using the ClustalW algorithm (Thompson et al.

1994), implemented in Mega 4.1 (Tamura et al. 2007). In the case of segment 7mt, only the 3' end (bound by primers 7mti-F3 and mtDNA7L) was incorporated into the final data set, as the remainder of the fragment could not be fully covered with high quality sequences for all taxa. Final alignments incorporated one sequence each of *Canis lupus* and *Canis latrans*, downloaded from GenBank (accession numbers AB499824.1 and DQ480510.1, respectively), to be used for calibration purposes in divergence dating analyses (see below). Alignments for each segment were checked by eye and edited if necessary with MEGA. In the case of the control region, we observed that a 40-bp long segment presented ambiguous alignment, and thus we excluded it from further analyses (see table 4).

Exploratory analyses assessing levels of diversity and phylogenetic information content within each segment were performed with MEGA and PAUP 4.0b10 (Swofford 2000). These included an assessment of the number of variable and phylogenetically-informative sites per segment, presence of potentially informative indels, and also preliminary phylogenetic analyses employing Maximum Parsimony (MP) and distance-based approaches, the latter using the Neighbor-Joining (NJ) algorithm (Saitou et al. 1987). Based on these initial analyses, we assessed whether there was any strongly supported phylogenetic conflict among segments, thus bearing upon the decision of concatenating them into a single supermatrix. Since no supported conflict was observed, we concatenated all segments and performed all subsequent analyses with this joint data set.

We initially assessed whether identical joint haplotypes of the sampled segments were present in the supermatrix, and removed any duplicates, so that a single representative of each sequence was used for phylogenetic inference. Final phylogenetic analyses were performed using three different optimality criteria: Maximum-likelihood (ML), Maximum Parsimony (MP) and Bayesian Inference (BI).

ML phylogenies were inferred with two different approaches, both of which employed the best-fit evolutionary model estimated using ModelTest3.7 (Posada and Crandall, 1998) under the Akaike Information Criterion (AIC). One of the approaches used the full data set and the software GARLI (Zwickl, 2006), which generated a starting tree with stepwise taxon addition, and subsequently performed branch-swapping with the nearest-neighbor interchange (NNI) and subtree pruning regrafting (SPR) algorithms. Nodal support was assessed with 1000 nonparametric bootstrap replications. The second ML approach was that implemented in PAUP*, and used a

pruned data set containing all of the ingroup and only *Cerdocyon thous* sequences as the outgroup (so as to speed up the computation, and given that this species is clearly the most immediate relative of the ingroup – see Results). In this case, we initially estimated a starting tree with NJ, and then conducted extensive branch-swapping with the TBR algorithm. We then verified that an identical result could be achieved with the less computationally-intensive NNI branch-swapping approach. Given this observation, we estimated branch support for the PAUP* ML run using 100 replications with NNI branch-swapping upon the starting NJ topology.

MP trees were also obtained with two different approaches. The first one used PAUP*, with a heuristic search employing 50 replicates of random taxon addition followed by tree-bisection reconnection (TBR) branch-swapping. To assess nodal support, we performed 100 bootstrap replications, each of which included 10 replicates of random taxon addition, TBR branch-swapping, and a maximum of 1000 trees kept per replicate. The second MP approach used the software TNT (Goloboff et al. 2008), which employed the new technology search methods of sectorial search and tree fusion, with 100 replicates, holding 10000 trees, and saving 100 trees per replication. Nodal support was assessed with 1000 bootstrap replications.

BI was performed with Beast 1.6.0 (Drummond and Rambaut 2007) with a partitioned dataset, in which every segment was treated as an independent partition, except for the five tRNA genes, that were concatenated into a single partition, and the *COI* and *COIII* genes, which were also joined into another partition (Table 4). Independent substitution and clock models were allowed for each partition, but their tree topology was constrained to be identical, as it is expected that all mtDNA segments should bear the same phylogenetic history. For each partition, we implemented the best-fit evolutionary model as estimated under the AIC with MrModelTest2.3 (Nylander 2004). We ran the Markov chain Monte Carlo (MCMC) process for 100 million generations, with data sampled every 10,000 steps, and excluded the first 10% of each run by considering it the burn-in phase.

We estimated divergence dates using the relaxed molecular clock approach implemented in Beast. We modeled the relaxed molecular clock using the uncorrelated lognormal option, allowing each partition to have its own rate. We used two calibration points, and the priors were set as follows: (i) divergence between *Canis* and the south American canids, using a uniform prior with a conservative minimum time of 5.3 Mya based on the first fossil appearance of *Canis* (McKenna and Bell 1997) and a maximum

time of 11.5 Mya (Eizirik et al. 2010); (ii) divergence between *Canis lupus* and *C. latrans,* using a uniform prior with a minimum, fossil-based age of 1 Mya (Kurtén and Anderson 1980) and a conservative maximum of 3 Mya.

3. Results

We analyzed a total of 6,000 bp of the mtDNA, including seven protein coding genes (portions of the *COI*, *COIII* and *ND5* genes, and the complete *ND3*, *ND4L*, *ND4* and *Cytb* genes), five tRNAs and the control region. These regions were sequenced for 17 *Lycalopex gymnocercus* individuals, eight *L. griseus*, seven *L. culpaeus*, six *L. fulvipes*, four *L. sechurae* and 13 *L. vetulus*, in addition to the following outgroups: three *Cerdocyon thous,* three *Chrysocyon brachyurus* and three *Speothos venaticus*. The full data set contained 1,671 variable sites, 1,399 of which were parsimony-informative (Table 4).

Our preliminary analyses did not reveal any supported incongruence among segments. In most cases, individual segments did not resolve the relationships among species with substantial or consistent support. Moreover, they did not always support the monophyly of all species (especially *L. gymnocercus* and *L. culpaeus*, which presented a deeper intra-specific phylogenetic structure – see below). Nevertheless, some features of the *Lycalopex* topology, such as a basal position for *L. vetulus*, were apparent with most of the individual-segment phylogenies (not shown).

Final analyses, based on the concatenated data set, led to robust support for species-level monophyly and consistent resolution of the *Lycalopex* inter-specific topology (Figures 2-4). The best-fit model of sequence evolution estimated for the concatenated data set was GTR+I+G, which was implemented in the ML analyses. The reconstruction performed with GARLI retrieved a single ML tree (lnL: -22131.06802), while PAUP* found two trees with identical scores (lnL: -15559.20359). Both approaches led to the same resolution of the *Lycalopex* topology, with considerably high bootstrap support for species-level and supra-specific nodes (Figure 2 and Table 5).

The MP reconstruction in PAUP* found 270 equally parsimonious trees (length: 2889 steps), while the TNT analysis retrieved five trees that were slightly longer (2908 steps). The strict consensus trees generated from both analyses were quite well resolved (i.e. almost all the differences among the original trees pertained to intra-specific tips), and highly congruent with each other. Bootstrap support was considerably high for most

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nodes, including the majority of those defining species-level monophyly as well inter-specific relationships (Figure 3).

For the Bayesian inference, the algorithm implemented in MrModeltest identified the following models as providing the best fit to each of the segments: HKI+G for *COI+COIII* and *ND3*; HKY+I for *ND4L*; HKI+I+G for *ND4*, Control Region and tRNAs; GTR+G for *ND5* and GTR+I for *Cytb* (see table 4). These models were implemented in the partitioned Beast run, which produced a well-supported phylogeny congruent with those retrieved by other methods (Figure 4).

Overall, *Lycalopex* was supported as a monophyletic group with high branch support with all methods. Each of the six species of the genus was also found to be monophyletic: *L. vetulus, L. sechurae* and *L. fulvipes* received 100% support with all methods, while *L. gymnocercus* and *L. griseus* varied between 99% and 100%. Interestingly, *L. culpaeus* received the least consistent support, varying between 75 and 98%. This was due to the relatively deep partition between two divergent *L. culpaeus* phylogeographic lineages (see below).

The inter-specific topology supported the hypothesis that *L. vetulus* is the most basal species in the genus (see Figures 2-4). Interestingly, our results indicated that the next divergence led to the little-known Pacific species *L. sechurae*, followed by *L. gymnocercus*. The most internal clade was composed by *L. griseus*, *L. fulvipes* and *L. culpaeus*, with the latter two being sister-species. Most of these nodes received high support, especially with the model-based methods ML and BI (see Figures 2-4 and Table 5).

The separation between genus *Lycalopex* and the closest outgroup *Cerdocyon thous* was estimated to have occurred *ca.* 3 Mya (Figure 5). The coalescence age (as estimated by the time to the most recent common ancestor - TMRCA) of genus *Lycalopex* as a whole was estimated to be *ca.* 1.2 Mya, when *L. vetulus* diverged from the other lineages. The next divergence (that of *L. sechurae*) occurred *ca.* 0.8 Mya, followed by a very rapid succession of speciation events between 0.53 Mya and 0.39 Mya, which led to the formation of *L. gymnocercus, L. griseus, L. culpaeus* and *L. fulvipes* (see Figure 5).

In addition to the reconstruction of inter-specific relationships, some patterns of within-species variation could also be observed. Species-level coalescence age was somewhat variable, ranging from 60,000 years ago for *L. fulvipes* to 390,000 years ago for *L. gymnocercus* (Figure 5, Table 6). The species presenting the deepest coalescence

(*L. gymnocercus* and *L. culpaeus*) were also found to exhibit considerable intra-specific phylogenetic structure. *L. gymnocercus* contains at least three well-supported clades, identified here as Lgy-I to Lgy-III (see Figure 2 and Table 5). There was no precise geographic signal in this structure, although it can be noted that clade Lgy-II was restricted to the northeastern portion of the species' range, by including haplotypes sampled in the mountainous grasslands of Rio Grande do Sul state, Brazil ('Campos de Cima da Serra' region), and the only currently available sample from Paraná state (Brazil), supposedly the northernmost limit for this species in Brazil. Interestingly two additional haplotypes allocated in this clade were sampled in individuals that were phenotypically identified as *L. vetulus* (bPve328 and bPve353), and wild-caught in São Paulo state, north of Paraná, where this species is not known to occur (see Figures 1 and 2).

Also with respect to intra-specific structure, there was a clear phylogeographic pattern in *L. culpaeus*, with samples collected in Argentina and Chile forming one well-supported cluster (Lcu-I) and those collected in Peru forming another (Lcu-II) (Figure 2). There was also a possible phylogeographic pattern in *L. griseus*, with one well-supported clade (Lgr-I) containing samples from western Argentina (see Figure 1 and Table 1), and another (Lgr-II) including samples from central-eastern Argentina and Chile. Finally, *L. vetulus* also contained one sub-clade (Lve-I) that was rather well-supported (see Table 5) and geographically restricted, as its contained haplotypes were found only in samples collected in the northeastern Brazilian states of Maranhão and Piauí.

4. Discussion and Conclusions

All phylogenetic methods retrieved the same inter-specific tree topology with considerably high support, indicating that our data set was very consistent. This is the first time that a consistent resolution of the relationships among these six fox species is achieved. The monophyly of the genus was highly supported, which is consistent with previous studies (e.g. Zrzavý and Ricáncová 2004; Lindblad-Toh et al. 2005; Prevosti 2010). Within the genus, the taxonomic status of several *Lycalopex* species has been controversial for many years (e.g. Langguth 1969 and 1975; Zunino et al. 1995; Mendel et al. 1990; Yahnke et al. 1996). Our results strongly supported for the monophyly of each species, corroborating the view that this genus includes six extant species, as

proposed by Berta (1985), Zrzavý and Ricáncová (2004) and Tchaicka et al. (in prep). It may be noted that this is the first time that the monophyly of the sechuran fox is demonstrated with a molecular data set, as previous studies did not include multiple individuals of this species.

The position of *L. vetulus* as the most basal species of the group was strongly supported, and corroborates the studies of Lindblad-Toh et al. (2005) and Tchaicka et al. (in prep.). *L. sechurae* was found to be the second most basal lineage in the genus, which is a novel finding. The position of *L. gymnocercus* and the internal clade formed by *L. griseus* as sister group to *L. culpaeus* + *L. fulvipes* were variably retrieved in previous studies, with no consistent resolution observed in the literature. A common arrangement is the placement of *L. culpaeus* as a sister-group to *L. griseus*, as observed by Tchaicka et al. (in prep) and in the "total evidence" analyses reported by Prevosti et al. (2010), albeit with low support for this clade. Yahnke et al. (1996), using multiple individuals each from *L. fulvipes*, *L. culpaeus* and *L. griseus*, found this same cluster, but *L. culpaeus* and *L. griseus* were not reciprocally monophyletic. Our finding that *L. culpaeus* is the sister-group of *L. fulvipes* was also reported by Vilà et al. (2004), although in their analyses *L. culpaeus* was not completely monophyletic, with some individuals clustering with *L. fulvipes* and others with *L. griseus*. This is therefore the first study in which these species are retrieved as monophyletic entities, and their phylogenetic relationships clarified.

Our results indicate that the speciation of *Lycalopex* began during the Pleistocene *ca.* 1.2 Mya, in agreement with the time frame inferred by Tchaicka et al. (in prep) (1.2 Mya) and Perini et al. (2010) (1.3 Mya). In less than 1 million years, all six species were formed (see Figure 5), which helps explain the difficulty in resolving their evolutionary relationships. The speciation of the most internal cluster (*L. griseus* (*L. fulvipes* + *L. culpaeus*)) was particularly recent (*ca.* 0.43 Mya), and corroborates the estimate reported by Yahnke et al. (1996), who dated the divergence among these species as *ca.* 0.27 to 0.66 Mya.

The resolution of the mtDNA phylogeny of this Pleistocene radiation allows some attempts to interpret its biogeographic history. It is often considered that Pleistocene climatic changes have had important impacts on the phylogeographic structure of many mammals (e.g. Avise et al., 1998), due to the glacial cycles that likely affected plant communities, habitat composition and, as a consequence, the geographic distribution of mammalian species (MacFadden, 2006). During this time, South America went through

cycles that included times when vast regions were covered by savanna, open-country environments, which permitted the expansion of their associated fauna (Stebbins, 1974; Webb, 1977; Cartelle 1999). Such periods may also have induced the specialization of the locomotor systems of vertebrates adapted to this kind of environment, which permitted them to disperse more easily (Hildebrand, 1976), possibly allowing range expansions into new regions. In contrast, there were periods when grasslands contracted and forests expanded (Webb 1978; Vivo and Camignotto, 2004), which may have induced isolation among populations of open-habitat species, possibly fostering allopatric speciation. Such a system, when applied cyclically to medium-sized carnivores that are mostly adapted to open habitat formations, may have led to periods of range expansion followed by geographic isolation, thus inducing repeated episodes of speciation.

The position of the hoary fox as the most basal species of *Lycalopex* indicates that the emergence of this genus may have occurred in central South America, which is dominated by savanna formations such as the Brazilian Cerrado. This view agrees with that of Langguth (1975), who proposed central Brazil as the center of radiation for *Lycalopex*, but is in contrast to that of Berta (1987), who proposed that their first center of speciation was Argentina. The latter view was based on fossils attributed to *L. gymnocercus* and *L. vetulus* that date from the Uquian (Late Pliocene – Pleistocene) and Lujanian (Late Pleistocene), respectively. Both areas could have played important roles in the sequence of speciations that produced the present *Lycalopex* diversity, with the first event occurring in central South America, and the final set of quick divergences likely occurring in Argentina and/or Chile. In contrast to these classical views, an intriguing result was the basal position of *L. sechurae*, which was the second lineage to diverge in this genus (see Figure 2). This poorly known species occurs in open habitats near the Pacific coast of Peru and Ecuador, and may have become isolated after a trans-Andean colonization process. The current geographic distributions of the remaining species (see Figure 1), along with our reconstructed topology, suggest that their ancestors remained east of the Andes, and that the next round of speciation (leading to the separation of *L. gymnocercus* from the others) may have occurred in Argentina. Given the present distribution of *L. griseus* (and the paucity of fossils from this group as a whole), it is difficult to infer whether its divergence took place in Argentina or Chile, but it is possible that Andes-associated environments have also acted as barriers in this case. Finally, the event separating *L. culpaeus* from *L. fulvipes* likely occurred west of

the Andes, and may have been a case of parapatric speciation, with adaptive divergence driving the differentiation between the two species. Such hypotheses can be assessed in the future with expanded molecular, morphological and ecological data, so as to characterize in more detail the evolutionary history of this group.

In addition to resolving the mtDNA phylogeny among the extant *Lycalopex* species, our data set also revealed some interesting cases of intra-specific phylogeographic structure in this group (see Figure 2). Tchaicka et al. (in prep) had already observed two well-supported clades of pampas foxes, which were mostly (but not completely) restricted to the southern and northern grassland regions of Rio Grande do Sul (RS) state, Brazil. In our study we also observed a similar pattern, but found a more complex phylogeographic structure for this species (Figure 2). Individuals from cluster Lgy-I were sampled in Argentina and also in both the southern and northern regions of RS state, as well as in the adjacent Brazilian state of Santa Catarina (SC). Its internal phylogenetic pattern suggests that further structure may exist here (as the Argentinean sample was divergent from a Brazilian sub-cluster), and should be investigated with additional sampling.

Cluster Lgy-II was found to be restricted to northern RS, PR and São Paulo (SP) states, possibly representing a lineage endemic to the altitude grasslands that were once surrounded by Atlantic Forest. The presence in this cluster of two individuals morphologically identified as *L. vetulus* is quite remarkable, and could be explained by two alternative hypotheses: (i) *L. gymnocercus* actually occurs in SP state (which would imply a revision of their currently accepted geographic distribution), and the individuals were misidentified upon sample collection; or (ii) these individuals could be hybrids between the two species, thus bearing *L. vetulus* morphology and an introgressed *L. gymnocercus* mtDNA haplotype. Both of these hypotheses warrant in-depth investigation, and should be the focus of more extensive sampling efforts targeting these foxes in SP state. It may be noted that both of these explanations may contain an underlying anthropogenic effect. In the former, the presence of pampas foxes in SP state may quite recent, and derive from an ongoing invasion of human-induced open habitats (e.g. pastures, grasslands) that were formerly covered by Atlantic Forest and thus likely inadequate for this species. Likewise, the hybridization hypothesis may also imply an anthropogenic process, as *L. gymnocercus* and *L. vetulus* are both open-habitat species that were likely isolated from each other by a broad swath of Atlantic Forest. Given the extreme deforestation process that has affected SP state and adjacent regions in the last

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few centuries, we can postulate that there could now be continuous open habitat joining their historical ranges, which may allow contact and possibly hybridization between them. If affirmed by additional analyses, the anthropogenic impact under either of these scenarios would raise important conservation concerns regarding these species in Brazil.

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Table 1: Samples analyzed in this study. Identification numbers correspond to those depicted in Figures 2-5. Superscript numbers indicated for each geographic origin refer to localities indicated in Figure 1. Asterisks indicate approximate coordinates based on the municipality of origin for each sample.

Species	Sample Identification Number	Geographic Origin	Geographic Coordinate			Institution / Contact
			X	Y	Zone	
Lycalopex culpaeus	bPcu01	La Negra – Argentina[1]	379811	5605006	19H	C. B. Kasper, M. F. Rodrigues
	bPcu02	Embalse Alicura – Argentina[2]	328469	5490460	19G	C. B. Kasper, M. F. Rodrigues
	bPcu03, bPcu04, bPcu05, bPcu06	Marcona – Peru[3]	498204*	8336155*	18L*	M. Roca, M. Cardeña, L. Oliveira
	bPcu07	Parque nacional Nahuelbuta - Chile[4]	701031*	5813918*	18H*	W. Johnson
Lycalopex fulvipes	bPfu01, bPfu02, bPfu03, bPfu04, bPfu05, bPfu06	Parque Nacional Nahuelbuta - Chile[4]	701031*	5813918*	18H*	W. Johnson
Lycalopex griseus	bPgr01	La Tranca - Argentina[5]	672430	6417311	19H	C. B. Kasper, M. F. Rodrigues
	bPgr02	Chos Malal - Argentina[6]	405228	5782986	19H	C. B. Kasper, M. F. Rodrigues
	bPgr03, bPgr04, bPgr05, bPgr06	Parque nacional Nahuelbuta - Chile[4]	701031*	5813918*	18H*	W. Johnson
	bPgr07, bPgr08	Benito Juarez - Argentina[7]	243769	5850621	21H	C. B. Kasper, M. F. Rodrigues
Lycalopex gymnocercus	bPgy50	Porto Alegre - Rio Grande do Sul (RS) - Brazil[8]	478419*	6672799*	22J*	J. Koeneman
	bPgy52	Camaquã (RS) – Brazil[9]	438974	6597533	22J	C. B. Kasper, M. F. Rodrigues
	bPgy53	Capão do Leão (RS) - Brazil[10]	344550	6471076	22J	C. B. Kasper, M. F. Rodrigues
	bPgy55	Bom Jesus (RS) - Brazil[11]	527799	6867148	22J	C. B. Kasper
	bPgy58	Bom Jesus (RS) - Brazil[11]	528423	6867429	22J	C. B. Kasper
	bPgy61	Campo Belo do Sul - Santa Catarina (SC) - Brazil[12]	518871	6917319	22J	C. B. Kasper, M. Piccoli
	bPgy62	Rio Grande (RS) - Brazil[13]	366426	6439298	22H	C. B. Kasper
	bPgy63	Arroio Grande (RS) - Brazil[14]	315508	6442605	22H	C. B. Kasper
	bPgy64	Jaguarão-Pelotas (RS) – Brazil				C. B. Kasper, M. F.Rodrigues
	bPgy66	Arroio Grande (RS) - Brazil[14]	303696*	6431792*	22H*	C. B. Kasper

Species	Sample	Locality				Collector
	bPgy67	Arapoti - Paraná (PA) – Brazil[15]	618549*	7330515*	22J*	M. H. N. Capão da Imbuia
	bPgy72	Anita Garibaldi - Santa Catarina (SC) - Brazil[16]	487433*	6936920*	22J*	Fundação Zoobotânica do RS
	bPgy73	Bom Jesus (RS) - Brazil[11]	528364	6867423	22J	C. B. Kasper
	bPgy77	Bom Jesus (RS) - Brazil[11]	527754	6868189	22J	C. B. Kasper
	bPgy80	Bom Jesus (RS) - Brazil[11]	528430	6867106	22J	C. B. Kasper
	bPgy83	Alegrete (RS) – Brazil[17]	534115	6689997	21J	C. B. Kasper, M. F. Rodrigues
	bPgy84	Azul - Argentina[18]	239503	5897935	21H	C. B. Kasper, M. F. Rodrigues
Lycalopex sechurae	bPse-01	Peru[19]	502363	9557449	17M	F. Angulo, L. Oliveira
	bPse02, bPse03	Peru[19]	519591	9575604	17M	F. Angulo, L. Oliveira
	bPse04	Peru[19]				F. Angulo, L. Oliveira
	bPve10	Goias (Go) - Brazil[20]	732337	8246068	22L	F. Grazziotin, A. Garda
	bPve13	Balsas - Maranhão (MA) - Brazil[21]	383846*	9168080*	23M*	C. B. Kasper
	bPve14	Benedito Leite - Maranhão (MA) - Brazil[22]	547833*	9202279*	23M*	C. B. Kasper
	bPve15	Loreto - Maranhão (MA) - Brazil[23]	483432*	9217044*	23M*	C. B. Kasper
	bPve16	Piaui (PI) – Brazil	605803*	9154815*	23M*	C. B. Kasper
Lycalopex vetulus	bPve307, bPve309, bPve310	Nova Xavantina - Mato Grosso (MT) - Brazil[24]	335290*	8360817*	22L*	CENAP/ ICMBio
	bPve18	Brazil				CENAP/ ICMBio
	bPve322	Campo Grande - Mato Grosso do Sul (MS) - Brasil[25]	739627*	7714545*	21K*	CENAP/ ICMBio
	bPve327	Ribeirão Preto - São Paulo (SP) - Brazil[26]	207439*	7654825*	23K*	CENAP/ ICMBio
	bPve328	São José do Rio Preto - São Paulo (SP) - Brazil[27]	668253*	7699139*	22K*	CENAP/ ICMBio
	bPve353	Piracicaba - São Paulo (SP) - Brazil[28]	229509*	7485446*	23K*	CENAP/ ICMBio

Table 2: Mitochondrial DNA segments amplified and sequenced in this study.

Segment	PCR/sequencing Primers	Reference
Cytocrome oxidase c subunit I (*COI*)	LCO1490 / HCO2198	Folmer et al (1994)
Control region (D-Loop)	MTLPRO2 / CCR-DR1	Tchaicka et al. (2007)
Cytochrome b (*cyt-b*)	Cytb-DF1 / Cytb-DR1	This study
	L14724/ H15494 L15162/ H15915	Irwin et al. (1991)
ND5	ND5-DF1 / ND5-DR1	Trigo et al. (2008)
7mt	mtDNA7H / mtDNA7L	Delisle & Strobeck (2002)
8mt	mtDNA8H / mtDNA8L	Delisle & Strobeck (2002)
9mt	mtDNA9H / mtDNA9L	Delisle & Strobeck (2002)

Table 3. PCR/sequencing primers generated in this study.

Segment	Primer	Primer (5' – 3')
Cytb	Cytb-DF1	TCTCACATGGAATTTAACCATGA
	Cytb-DR1	GAATTTCAGCTTTGGGTGCT
7mt	7mti-R1	CAAGTAATAGATACTCCGGAGGCTAG
	7mti-F2	ACCATACCCCTATCGTACAAAAAG
	7mti-R2	CATGGGGTCAAAACCACATT
	7mti-F3	CCGCTGCATGATATTGACA
8mt	8mti-R1	CTACTAGGAGTGGGAGGGATCCT
	8mti-F2	ACCACTATTAGCACTTACAACATGACT
	8mti-R2	AGTACGGCTATGGATTCGTTC
	8mti-F3	GTAGCGGTTCTTATTCAAACACC
9mt	9mti-F2	GCAAATACAGCTGCCCTACAAGC

Table 4: Data set features. N: Number of sequenced individuals; L: Length of the sequenced segment; V: variable sites; PI: parsimony-informative sites; EM: evolutionary model estimated for each partition.

Partition	N	L (bp)	Full data set		Ingroup only		EM
			V	PI	V	PI	
COI&III	59	715*	180	154	53	44	HKY+G
ND3	38	347	104	77	29	24	HKY+G
ND4L	60	297	82	71	27	21	HKY+I
ND4	65	1372	417	360	165	124	HKY+I+G
ND5	66	1197	369	319	146	112	GTR+G
Cytb	64	1139	316	255	135	96	GTR+I
CR	63	596**	153	129	103	87	HKY+I+G
tRNAs	63	337	50	34	17	12	HKY+I+G
Concatenated	56	6000	1671	1399	675	520	GTR+I+G

*COI spanned 676 bp and COIII spanned 39 bp.

**After exclusion of 40 bp presenting ambiguous alignment.

Table 5: Support values obtained with different phylogenetic methods for nodes marked with letters A – M in Figures 2 – 4.

	MP		ML		
Node	PAUP	TNT	PAUP	Garli	Beast
A	100	99	99	100	100
B	91	83	96	97	100
C	100	99	99	100	100
D	100	99	99	99	100
E	100	100	100	100	100
F	70	69	87	83	100
G	100	99	100	100	100
H	94	87	93	97	99
I	96	90	90	94	100
J	100	99	100	100	99
K	55	55	76	86	100
L	81	81	75	92	98
M	86	81	83	83	100

Table 6: Estimation of the times of origin (time to the most recent common ancestor – TMRCA) for genus *Lycalopex*, as well as each of the analyzed species.

	TMRCA						
	Lycalopex	L. vetulus	L. sechurae	L. gymnocercus	L. griseus	L. culpaeus	L. fulvipes
Mean	1.17	0.17	0.09	0.39	0.21	0.35	0.06
95% HPD lower	0.88	0.12	0.05	0.29	0.14	0.25	0.03
95% HPD upper	1.52	0.23	0.13	0.51	0.29	0.47	0.10

Figure Legends:

Figure 1. Maps depicting the currently recognized ranges for *Lycalopex* species (following Patterson et al. 2007). A) Sampling locations are indicated by the following symbols: ■ = *L. culpaeus;* ● = *L. gymnocercus;* ▲ = *L. vetulus*; △ = indicate the locales of origin of *L. vetulus* individuals whose mtDNA lineages clustered within the *L. gymnocercus* clade (see Results and Figure 3); B) Sampling locations are indicated by the following symbols: ■ = *L. sechurae*; ● = *L. griseus*; ▲ = *L. fulvipes*; * indicates two areas of distribution of *L. fulvipes*. The numbers are related with the geographic origin listed in Table 1. The diameter of each symbol is proportional to the sample size at each location. Maps were drawn using the software DIVA-GIS version 7.3.0 (Hijmans et al. 2005).

Figure 2. Maximum likelihood phylogram of genus *Lycalopex* estimated with GARLI. Bootstrap values shown above and below branches were calculated with GARLI and PAUP, respectively. Support values for lettered nodes are given in Table 5. Sample identification numbers for *Lycalopex* species correspond to those listed in Table 1. Outgroup species are identified by the following sample codes: 'bSve' for *Speothos venaticus* individuals, 'bCbr' for *Chrysocyon brachyurus*, and 'bCth' for *Cerdocyon thous*. Colored bars indicate species-level clades, whose names are indicated on the right. Supported intra-specific mtDNA clades are also indicated (e.g. Lgy-I within *L. gymnocercus*). Individuals phenotypically identified as *L. vetulus* but bearing *L. gymnocercus* mtDNA haplotypes (see Results) are indicated by purple circles.

Figure 3. Maximum parsimony phylogeny of genus *Lycalopex*. Strict consensus of 270 equally parsimonious trees (length: 2889) retrieved with PAUP*. Values above and below branches represent bootstrap support computed with PAUP and TNT, respectively. Support values for lettered nodes are given in Table 5. See Figure 2 and Table 1 for sample identification codes.

Figure 4. Bayesian phylogeny of South American foxes of genus *Lycalopex*, generated with Beast 1.6.0. Values above branches indicate the Bayesian posterior probability (expressed as percentages) of the clade defined by the adjacent node. The asterisk indicates a posterior probability below 0.50. Support values for lettered nodes are given in Table 5. Species-level branches are colored as in Figure 2.

Figure 5. Bayesian chronogram for South American foxes. Values above branches indicate the age of the adjacent node, while those below branches are the respective 95% credibility interval (based on the 95% Highest Posterior Density [HPD] range). Letters indicate nodes whose age is listed in the inset box. Species-level branches are colored as in Figure 2.
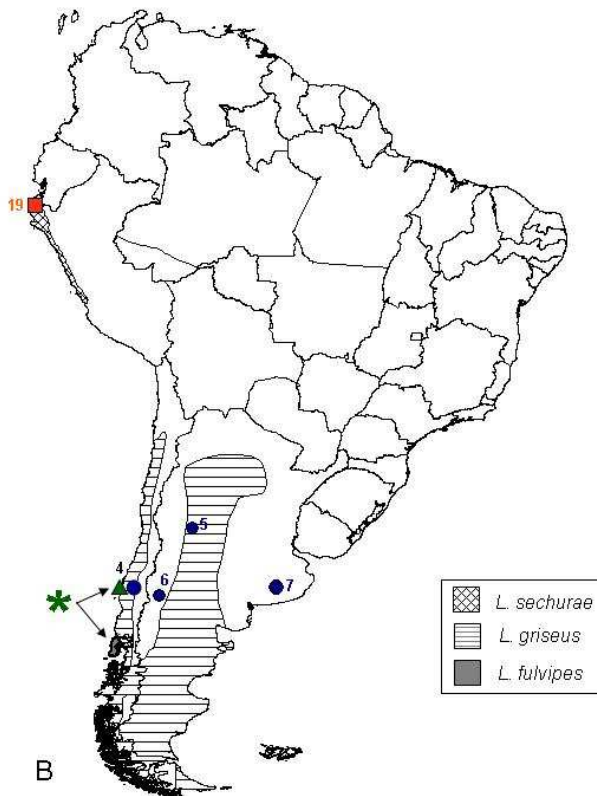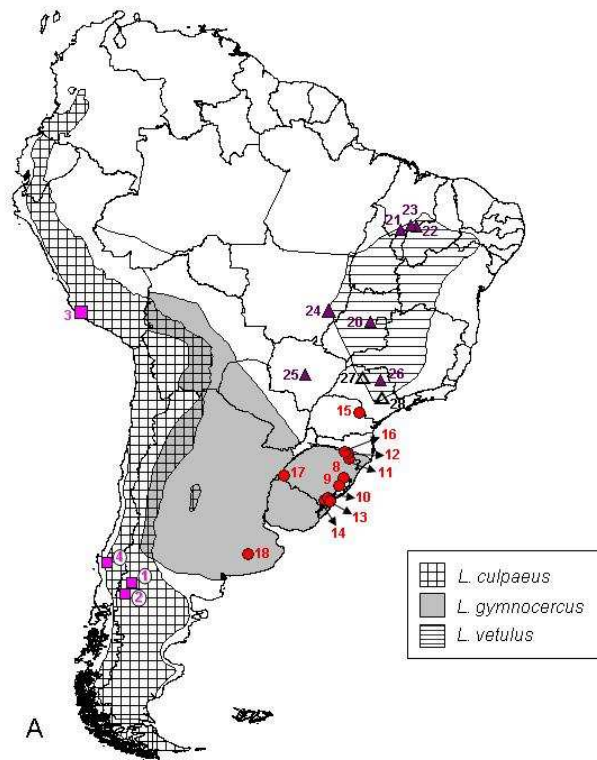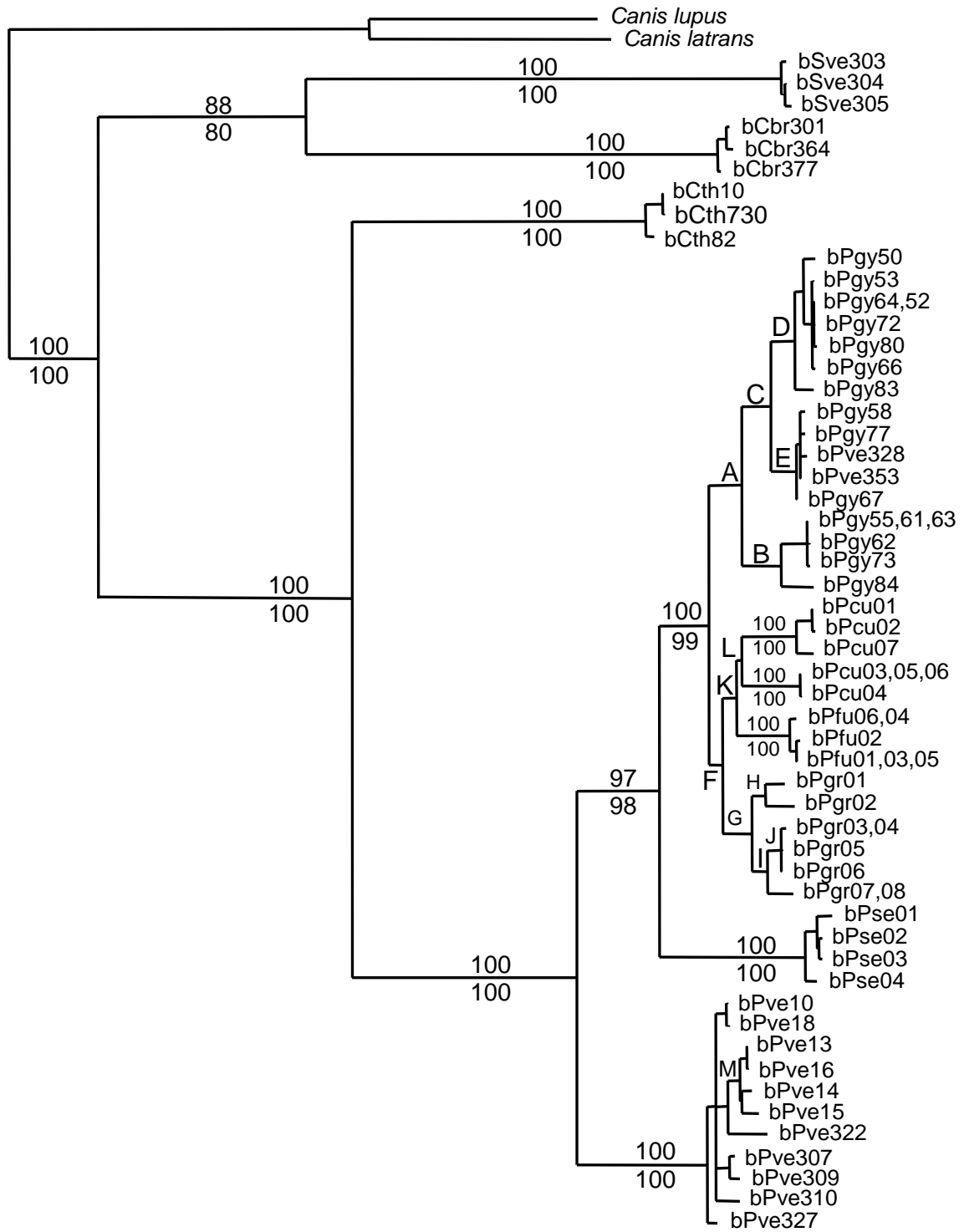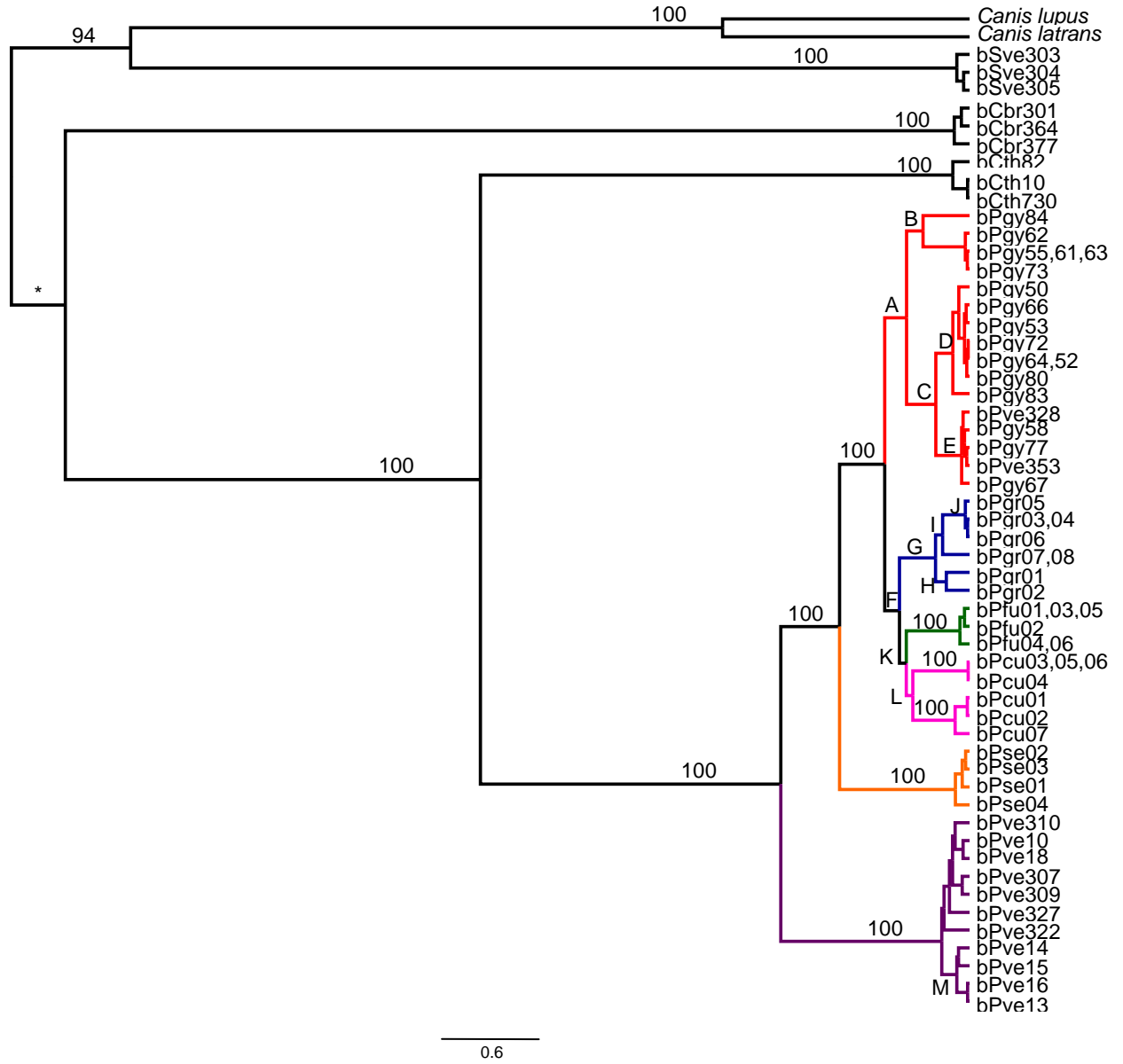
Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.