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ABORDAGENS FILOGENÉTICAS, FILOGEOGRÁFICAS E POPULACIONAIS EM CANÍDEOS SUL-AMERICANOS

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SUMÁRIO

RESUMO

O presente estudo foi realizado para investigar os padrões filogeográficos de sete espécies de canídeos sul-americanos. Um fragmento de 588pb da região controladora do DNA mitocondrial foi obtido para seis espécies do gênero *Lycalopex*, e usado para inferir as relações evolutivas entre estas espécies. A análise indicou *L. vetulus* como espécie basal, *L. fulvipes* como taxa monofilético, *L. culpaeus* e *L. griseus* como espécies muito próximas. Padrões intraespecificos da variação genética foram também abordados para o gênero *Lycalopex* e intensivamente investigados em *Cerdocyon thous*, neste através de um fragmento de 512pb da região controladora do DNA mitocondrial, três íntrons nucleares e dez *loci* de microssatélites.

L. fulvipes, *L. gymnocercus* e *Cerdocyon thous* mostraram partição geográfica entre a distribuição de seus haplótipos, indicando que barreiras históricas influenciaram a variabilidade genética atual. Os processos que geraram estes padrões e causaram a especiação do gênero *Lycalopex* provavelmente ocorreram no Pleistoceno, determinados pelas modificações na distribuição da vegetação e pelas oscilações nos níveis do mar.

Os três diferentes marcadores utilizados para a abordagem de *Cerdocyon thous* mostraram-se informativos e sua análise conjunta indicou que a maior parte do fluxo gênico é determinado pelos machos nesta espécie. Os marcadores nucleares inferiram alta variabilidade e ausência de isolamento entre as populações do cachorro-do-mato.

ABSTRACT

The present study was performed to investigate phylogeographic patterns of seven South American canid species. A 588bp fragment of mitochondrial DNA control region was obtained for the six species of *Lycalopex* genera, and used to infer evolutionary relationships between them. These analysis indicates *L. vetulus* as a basal species, *L. fulvipes* as a monophyletic taxa, and that *L. culpaeus* and *L. griseus* are closer species. Intraspecific patterns of genetic variation were also investigated for *Lycalopex* genera and were intensively investigated on *Cerdocyon thous*, in this latter by 512bp fragment of mitochondrial DNA control region, three nuclear introns and ten microsatellite *loci*.

L. gymnocercus, *L. fulvipes* and *Cerdocyon thous* shown geographic partition between haplotype distributions, inferring that historical barriers had influenced the actual genetic variability. The processes that generate these patterns and caused the *Lycalopex* speciation probably took place on Pleistocene, caused by the modifications on vegetation distribution and variation on sea levels.

Three different markers used for *Cerdocyon thous* approach were informative and their conjunct analysis indicates that gene flow can be male biased in this species. Nuclear markers inferred high variability and no geographic isolation between crab-eating fox populations.

Capítulo I
INTRODUÇÃO

1.1 Os Canídeos

A família Canidae, pertencente à Ordem Carnívora (subordem Caniformia, superfamília Canoidea) (Flyn e Nedbal, 1998) compreende 16 gêneros e 36 espécies atuais (Nowak, 1999).

De distribuição ampla, os canídeos são habitantes nativos de quase todos os continentes, com exceção de ilhas do Caribe, Madagascar, Taiwan, Filipinas, Borneo, Nova Guiné e Antártica. Na Austrália e Nova Zelândia existem populações selvagens destes animais, resultantes, porém, da introdução de linhagens primitivas do cão doméstico pelo homem. Sua distribuição abrange, assim, uma grande variedade de hábitats, desde os quentes desertos até os gelados campos árticos (Eisenberg, 1981; Wayne, 1996; Sillero-Zubiri *et al*., 2004).

Os representantes desta família são bem caracterizados por adaptações relacionadas ao hábito cursorial: os membros alongados e semi-rígidos terminam em patas digitígradas. As patas anteriores usualmente possuem cinco dígitos (sendo um deles reduzido) e as posteriores quatro, acompanhados por garras bem desenvolvidas, algo rombas e não retráteis.

A cabeça e o focinho são alongados e os músculos da bochecha são fortes, características ligadas à captura e contenção da presa. A dentição apresenta incisivos não especializados, longos e fortes caninos, pré-molares afiados e molares preensores, num total de geralmente 42 dentes (Stains, 1975; Emmons e Feer, 1990).

Nas espécies selvagens o tamanho está associado à disponibilidade de alimento, e vai desde menos de 1 Kg e aproximadamente 400cm de comprimento nas raposas dos gêneros *Fenecus* e *Vulpes* (nativas de zonas áridas do Oriente Médio) até, cerca de 60Kg e 1600mm para alguns lobos (*Canis lupus* - os maiores animais são encontrados no Alasca e Canadá) (Nowak, 1999; Sillero-Zubiri e Macdonald, 2004).

Muitos canídeos vivem em grupos e são exclusivamente carnívoros, capturando presas grandes em cooperação, apresentando uma organização social bastante complexa. Outros são solitários ou formam pares, predando

principalmente pequenos animais, sendo em vários casos onívoros. Algumas espécies mostram-se bastante oportunistas, variando sua dieta de acordo com a disponibilidade de alimento (Stains, 1975; Ginsberg e Macdonald, 1990; Wayne, 1996).

Quanto à reprodução, as fêmeas têm, em geral, uma gestação por ano que dura em média 63 dias, parindo grandes ninhadas que recebem cuidado dos pais e muitas vezes de outros membros do grupo (Emmons e Feer, 1990; Nowak, 1999).

Considerados ótimos dispersores, os canídeos são pouco limitados por barreiras topográficas ou de hábitat. Cada indivíduo ou grupo possui uma grande área de vida, exclusiva ou compartilhada em parte. Tal fato confere às populações a ocupação de grandes áreas geográficas, e assim, extensa distribuição a cada espécie (Wayne, 1996). Algumas delas habitam praticamente todo um continente, como a raposa vermelha (*Vulpes vulpes*), por exemplo, que está presente em todo o hemisfério Norte (Sillero-Zubiri e Macdonald, 2004).

As características ecológicas, morfológicas e comportamentais podem, entretanto, variar mesmo entre populações de uma mesma espécie de canídeo, como acontece em *Canis lupus* (lobo cinza). Nesta espécie, cujo peso vai de 15-60 Kg, as ninhadas podem ter de 1 a 11 filhotes, a área de vida difere em 50-100 vezes e os indivíduos podem viver solitários, em pares ou em matilhas (Bekoff *et al*., 1984).

Carismáticos e fascinantes, os canídeos sempre despertaram o interesse do homem, espécie com a qual tem sua história intimamente ligada. A origem do cão atual a partir do lobo, um dos poucos animais não herbívoros domesticados, pode não ter sido o único evento de domesticação na família (Clutton-Brock, 1977; Ostrander e Wayne, 2006). A associação entre humanos e canídeos na América pré-colombiana é documentada por registros fósseis de cães domésticos e pela existência de estoques ancestrais da espécie associados aos primeiros grupos humanos da região (Olsen, 1974). No antigo Egito, os chacais estavam entre os animais considerados divindades (Anúbis, o Deus da morte) e eram respeitados e mumificados (Souza, 1990).

A alta mobilidade e o oportunismo ecológico são características que, a despeito de conferirem sucesso à muitas espécies, atualmente aproximam os canídeos selvagens do homem e os colocam em conflito (Ginsberg e Macdonald, 1990). A modificação antrópica do ambiente tem alterado amplamente a distribuição de várias espécies da família: pelo menos sete delas aumentaram e nove diminuíram sua distribuição no último século (Sillero-Zubiri e Macdonald, 2004).

1.2 A família Canidae na América do Sul

De acordo com o registro fóssil, a origem dos canídeos se deu no Hemisfério Norte durante o Eoceno (Stains, 1975). Sua chegada a América do Sul, a partir da América do Norte, foi feita através do istmo do Panamá, formado durante o fim do Plioceno e início do Pleistoceno. Tal dispersão foi provavelmente provocada por mudanças ambientais nas áreas próximas ou adjacentes ao ponto de travessia e deve ter provocado um ou mais eventos de invasão (Langguth, 1975; Berta, 1987; Wayne *et al*., 1997).

Após este período o grupo sofreu uma radiação adaptativa que pode ter tido como centro de ocorrência a Argentina (Berta, 1987) ou as terras altas brasileiras (Langguth, 1975), dando origem à grande diversidade de espécies atuais.

Os canídeos sul-americanos, como os demais mamíferos da região, são caracterizados por alto endemismo. Doze espécies são nativas deste continente, sendo onze delas endêmicas: *Cerdocyon thous* (cachorro-do-mato); *Chrysocyon brachyurus* (lobo-guará); *Speothos venaticus* (cachorro-vinagre); *Atelocynus microtis* (cachorro-de-orelha-curta); *Lycalopex vetulus* (raposinha-do-cerrado); *Lycalopex gymnocercus* (cachorro-do-campo); *Lycalopex culpaeus* (zorro culpeo); *Lycalopex griseus* (chilla); *Lycalopex fulvipes* (raposa de Darwin); *Lycalopex sechurae* (zorro sechura); e *Dusicyon australis* (raposa das Ilhas Falkland, atualmente extinta). Apenas a raposa cinza (*Urocyon cinereoargenteus*) ocorre na América do Norte e Central e estende sua distribuição até o Norte da América do Sul (Fig.1).

Os representantes da família são encontrados em todos os hábitats do continente, dos desertos da costa do Oceano Pacífico (*L. sechurae*) até os campos abertos (*L. gymnocercus, L. culpaeus e L. griseus*). Enquanto na África e Ásia os canídeos evitam as florestas úmidas, uma espécie de canídeo neotropical é habitante da floresta Amazônica (*Atelocynus microtis*) e outro habita as florestas da costa atlântica e matas de galeria (*Cerdocyon thous*) (Ginsberg e Macdonald, 1990).

Em várias regiões do continente duas ou mais espécies sobrepõe suas áreas: para um total de 55 pares de espécies sul-americanas possíveis, 12 mostram algum grau de simpatria (Medel e Jaksic, 1988).

A viabilidade da existência em simpatria neste grupo está provavelmente relacionada ao grande oportunismo alimentar. Os estudos conduzidos revelaram que, a sobreposição de habitat é compensada pela diminuição na sobreposição da dieta (para *L. culpaeus e L. griseus*: Fuentes e Jaksik, 1979; Jaksic *et al*., 1983; para *C. thous, C. brachyurus e L. vetulus:* Juarez e Marinho-Filho, 2002).

Apesar da alta diversificação dos canídeos sul-americanos em relação aos outros continentes, esta é ainda a região com maior carência de dados acerca de suas espécies. Três delas (*A. microtis*; *L. vetulus* e *L. sechurae*) não tem seu status de conservação determinado devido à insuficiência de dados, enquanto *Speothos venaticus* é considerada vulnerável; *Chrysocyon brachyurus* ameaçada; *L. fulvipes* criticamente ameaçada; e *D. australis* foi recentemente extinta (1880) por ação humana (Ginsberg e Macdonald, 1990; Clutton-Brock 1977; IUCN, 2003; Sillero-Zubiri e Macdonald 2004).

1.2.1 Taxonomia dos canídeos sul-americanos

Segundo Berta (1987), a primeira referência a um canídeo na América do Sul foi feita por Kerr 1792, descrevendo *Canis australis* das ilhas Falkland (costa leste da Argentina). Desde então seguiram-se várias descrições, e diferentes esquemas taxonômicos foram propostos baseados em métodos diversos (ex. Thomas, 1914; Kraglievich, 1930; Cabrera, 1931; Osgood, 1934; Hough, 1948;

Thenius, 1954; Langguth, 1969; 1975; Clutton-Brock *et al*., 1976; Van Gelder, 1978; Berta, 1987; Wayne, 1993; Zunino *et al*., 1995).

Entre estes, Langguth (1975), baseado em dados ecológicos e morfológicos, arranjou os canídeos sul americanos em dois grupos: o grupo com estruturas diferenciadas e o grupo com padrões gerais de canídeos. Do primeiro fazem parte os gêneros *Chrysocyon, Cerdocyon, Speothos, Atelocynus* e *Lycalopex*. O segundo grupo é considerado como pertencente a um único gênero: *Canis*, composto por dois subgêneros, *Dusicyon e Pseudalopex*. O primeiro compreende apenas o recentemente extinto *C. (Dusicyon) australis* e o segundo possui quatro espécies *C.(P.) culpaeus, C.(P.) gymnocercus, C.(P.) griseus* e *C.(P.) sechurae* (a espécie *L. fulvipes* não foi considerada válida neste e em vários outros esquemas taxonômicos do grupo).

Através de características morfológicas e comportamentais, utilizando taxonomia numérica, Clutton-Brock *et al*. (1976) indicaram como monotípicos *Chrysocyon e Speothos*; e incluíram *Pseudalopex, Atelocynus, Cerdocyon e Lycalopex* no gênero politípico *Dusicyon*. Este autor ainda considerou a raposa de Darwin como subespécie de *L. griseus,* o que corrobora Langguth (1969).

Van Gelder (1978) propôs uma nova classificação utilizando o grau de hibridação entre os taxa. Segundo este autor, *Chrysocyon e Speothos* representariam gêneros monotípicos, ao passo que *Canis* possuiria oito subgêneros, dos quais seis são sul americanos*: Dusicyon, Pseudalopex, Lycalopex, Cerdocyon, Atelocynus e Vulpes (Urocyon*).

Berta (1987), baseada em análise cladística de dados morfológicos e no registro fóssil, reconheceu quatro grupos principais para os canídeos sul americanos: (1) *Urocyon*; (2) *Cerdocyon* - incluindo os gêneros atuais *Speothos, Atelocynus* e *Cerdocyon*, bem como outros gêneros extintos; (3) *Chrysocyon* e (4) *Dusicyon* – incluindo dois gêneros distintos, o atual *Pseudalopex* e o recentemente extinto *Dusicyon*. O gênero *Cerdocyon*, segundo a autora, é representado atualmente apenas por *C. thous*, já *Pseudalopex* possuiria cinco espécies atuais: *P. culpaeus*, *P. gymnocercus*, *P. griseus*, *P. sechurae* e *P. vetulus*.

Mais recentemente, Zunino (1995), analisando características da pelagem e medidas de crânio para as espécies de raposa da Argentina, propôs a utilização de *Lycalopex* como nome para o gênero que inclui *L. vetulus*, *L. culpaeus*, *L. sechurae*, e a união de *L. griseus* e *L. gymnocercus* em uma única espécie: *Lycalopex gymnocercus*.

Na mesma época, Yahnke *et al*. (1996), através de estudos moleculares, confirmaram a monofilia da raposa de Darwin em relação a *P. griseus*, elevando-a à categoria de espécie, utilizando a nomenclatura *Pseudalopex fulvipes*.

Dado o grande número de diferentes propostas, a classificação permanece controversa, especialmente no que se refere ao grupo dos animais popularmente chamados "*zorros*" (espanhol), do qual fazem parte *Cerdocyon thous* e o gênero *Lycalopex*, espécies abordadas neste estudo.

A nomenclatura mais amplamente utilizada até pouco tempo era a proposta por Wozencraft (1993). Esse autor, revisando diversos trabalhos, concordava com Berta (1987), descrevendo *Cerdocyon thous* como única espécie atual de seu gênero, e para o gênero *Pseudalopex* (nomenclatura genérica proposta por Burmeister, 1856): *P. vetulus, P. gymnocercus, P. griseus, P. culpaeus* e *P. sechurae*. Tal classificação foi também seguida no Capitulo III do presente estudo (pelo qual se iniciaram os trabalhos), sendo depois modificada para a proposição mais recente de Wozencraft (2005), que propõe *Lycalopex* (nomenclatura genérica mais antiga proposta por Burmeister, 1854) como nomenclatura para o gênero que inclui: *L. vetulus, L. gymnocercus, L. griseus, L. culpaeus, L. sechurae* e *L. fulvipes.*

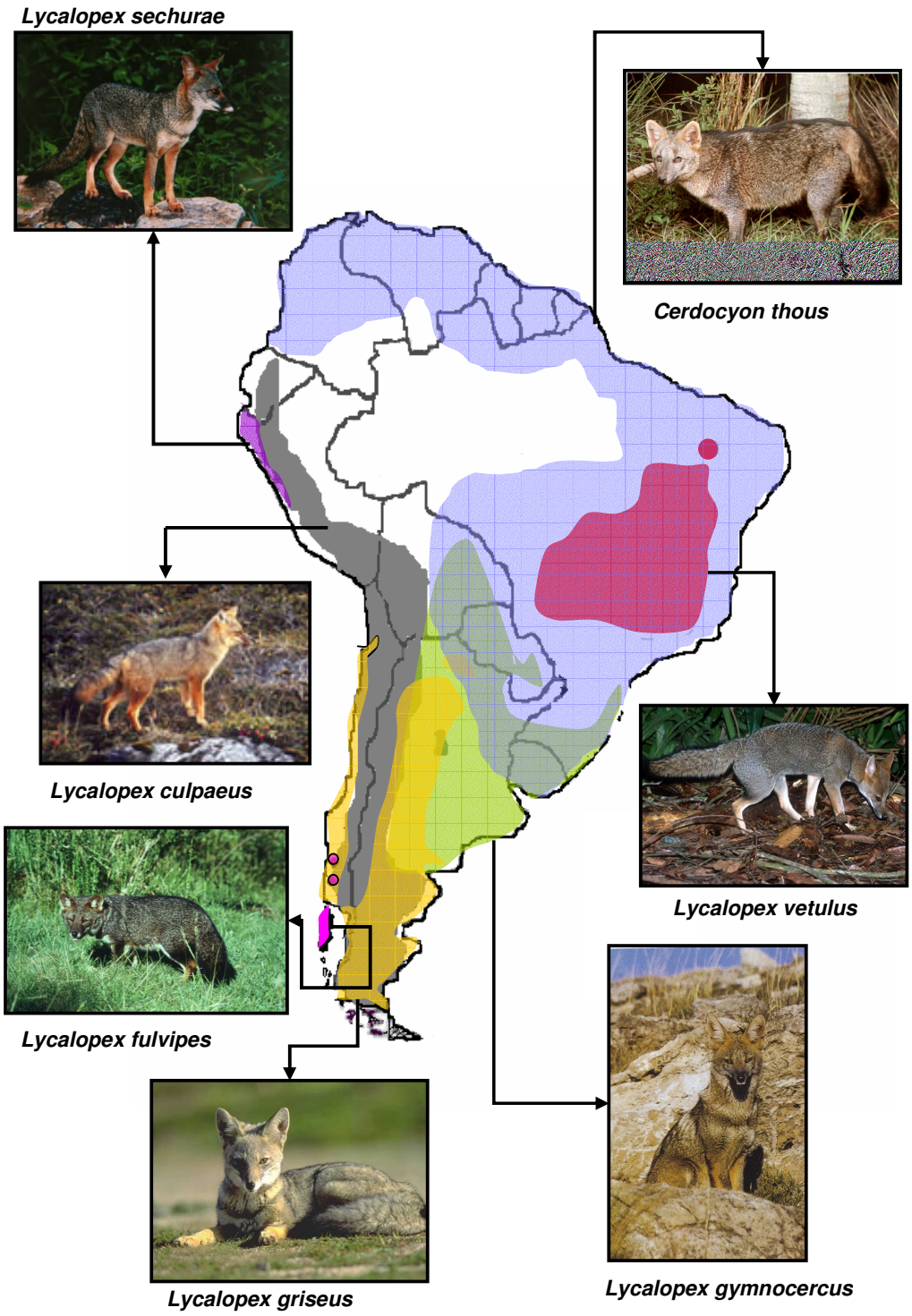

Figura 1. Distribuição geográfica aproximada das espécies de canídeos do gênero *Lycalopex* e de *Cerdocyon thous* (modificada a partir de Sillero-Zubiri *et al.* 2004; fotos de Jaime Jimenez, Daniel Anscencios, Adriano Gambarini, Marcelo Dolsan, Enrique Montané)

1.2.2. O gênero *Lycalopex*

As espécies atuais do gênero *Lycalopex* tiveram sua origem na América do Sul, a partir da radiação adaptativa ocorrida durante o Pleistoceno (Berta, 1987) e distribuem-se pela maior parte da região (Fig.1).

O maior canídeo do gênero é *L. culpaeus*, o zorro colorado ou zorro andino. A espécie apresenta um considerável dimorfismo sexual, sendo os machos em média 1.5 vezes maior que as fêmeas, os primeiros chegando a pesar 11 Kg. Possui as extremidades das patas claras, assim como a área ventral, e o restante do corpo castanho com as pontas da cauda e orelhas escuras (Crespo, 1975; Parera, 2002; Jimenez e Novaro, 2004). Habita as terras altas do oeste da América do Sul, ao longo dos Andes, desde a Colombia até a Terra do Fogo (Argentina), passando pelo Peru, Chile, Equador e Bolívia, sendo encontrado em ambientes de florestas até desertos (Langguth, 1975; Jiménez e Novaro, 2004).

Em algumas dessas áreas ocorre em simpatria com *L. griseus,* ou zorro gris, cuja distribuição abrange as planícies e montanhas aos dois lados dos Andes, desde os 17S no Chile ate 54S na Terra do Fogo, sendo encontrado a oeste até a costa chilena e a leste até aproximadamente a região central da Argentina. Esta raposa e bastante tolerante a variações de clima e apesar de habitar diversos ambientes prefere áreas abertas (González del Solar e Rau, 2004).

Lycalopex griseus é uma das menores raposas sul-americanas (aproximadamente 4Kg), possui orelhas grandes e coloração acinzentada, com uma linha dorsal e a ponta da cauda escuras (Duran *et al*., 1985).

Lycalopex fulvipes, conhecida popularmente como raposa de Darwin (o primeiro exemplar foi coletado por Charles Darwin em 1834) é uma espécie de distribuição muito restrita. Apenas duas populações, disjuntas, limitadas a áreas de floresta úmida, são registradas para estas raposas: uma na Ilha Chiloé e outra nas montanhas do Parque Nacional de Nahuelbuta, ambos na costa chilena. Os indivíduos dessa espécie são muito pequenos, apresentam corpo alongado e pernas curtas, com tonalidade entre cinza e negro (Yahnke *et al*., 1996; Vilà *et al.,* 2004; Jiménez e McMahon, 2004).

Lycalopex sechurae é um canídeo de coloração amarelo-acinzentada e região ventral mais clara, cabeça pequena com orelhas longas e um anel castanho ao redor dos olhos. É uma espécie de tamanho pequeno pesando em media 3,5 Kg. Existem registros de sua distribuição apenas na costa noroeste do Peru, chegando à fronteira com o Equador (Nowak, 1999; Eisenberg e Redford 1999; Asa e Cossíos; 2004).

Duas espécies do gênero *Lycalopex* habitam as terras brasileiras: *L. gymnocercus* e *L. vetulus* (ver Fig. 1).

Lycalopex gymnocercus é o canídeo popularmente conhecido como graxaim-de-campo, cachorro-do-campo ou *zorro pampeano*. Sua coloração cinza amarelada tem tendência ao marrom ferrugineo no alto da cabeça. O peito é claro, bem como as extremidades das orelhas e patas e a ponta da cauda apresenta-se escura (Crespo 1971; Silva, 1994).

É bastante similar, em termos de morfologia externa, a *Cerdocyon thous*, sendo na língua guarani denominado da mesma forma que o primeiro como *Aguará cha'i*. No entanto distingui-se do cachorro do mato pelas orelhas maiores e mais triangulares, o focinho mais anguloso, a cauda mais comprida e peluda. Seu peso varia de 4-6,5Kg, sendo os machos 10% mais pesados do que as fêmeas (Parera, 2002).

Habitante de áreas abertas, freqüentemente bordas de matas, campos e capoeiras, *L. gymnocercus* é encontrado desde o leste da Bolívia, Paraguai, sudeste e sul do Brasil até o Uruguai e a Argentina (até a Província de Rio Negro) (Crespo, 1971, 1975; Medel e Jaksic 1988).

Lycalopex vetulus, a raposinha-do-cerrado, e uma espécie endêmica do Brasil, associada ao Cerrado e áreas de transição como o Pantanal. Sua ocorrência é registrada nos estados de Minas Gerais, São Paulo, Goiás, Tocantins, Mato Grosso do Sul e Mato Grosso, Piauí e Bahia. De pequeno porte, *L. vetulus* tem em média 3,5Kg, apresenta coloração cinza-amarelada, com as patas e a região ventral mais claras (Courtenay *et al*., 2006).

Em geral, os representantes do gênero *Lycalopex* são considerados solitários, podendo em algumas espécies ser encontrados em pares durante a

época reprodutiva, e apresentam hábitos noturnos e crepusculares (Silva, 1994; Cimardi, 1996; Eisenberg e Redford, 1999; Parera, 2002; Courtenay *et al*., 2006).

Estudos acerca da dieta nestas espécies relatam que os indivíduos alimentam-se tanto de pequenos animais como vegetais (Parera, 2002; Jaksic *et al.,* 1980). Apresentam-se também bastante oportunistas, variando a composição de sua dieta de acordo com a época do ano e o tipo de ambiente. Entre os animais consumidos encontram-se aves, répteis, insetos e mamíferos, a maioria silvestres. Em áreas habitadas por humanos, apenas uma pequena parte dos animais incluídos na dieta são domésticos, e estes muitas vezes são encontrados já mortos (não predados), porém o estigma de ameaça aos rebanhos de ovinos tem tornado estas raposas alvo de combate por parte de produtores rurais (Crespo, 1971; Dotto, 1997; Nowak, 1999; Parera, 2002). Dentre as espécies deste grupo, considera-se que *L. culpaeus* possui a dieta com maior porcentagem de carne, e *L. vetulus* possui a dieta mais especializada, sendo composta predominantemente de insetos, especialmente térmitas e besouros (Courtenay *et al*., 2006; Jaksic *et al*., 1980; Pia *et al*., 2003).

Estes canídeos aproximam-se com facilidade do homem e habitam áreas antropicamente modificadas como pastagens. Uma das principais ameaças às espécies é o uso de sua pele na produção de casacos (Crespo, 1971; Cimardi, 1996; Nowak, 1999; Parera, 2002).

1.2.3 *Cerdocyon thous*

Cerdocyon thous é uma espécie de distribuição ampla, presente em todo o Brasil (com exceção da planície amazônica); sua ocorrência estende-se pelas Guianas, Venezuela, Colômbia, Leste da Bolívia, Norte da Argentina e Uruguai (Langguth, 1975; Medel e Jaksic, 1988; Eisenberg, 1989; Redford e Eisenberg, 1992; Anderson, 1997; Eisenberg e Redford, 1999).

Por ser habitante característico de florestas abertas, recebe a denominação popular de cachorro-do-mato, graxaim-do-mato ou *zorro de monte* (países de língua espanhola), porém é também comumente encontrado em áreas de savana e pradaria (Langguth, 1975; Berta, 1982; Medel e Jaksic, 1988; Ginsberg e Macdonald, 1990; Nowak, 1999; Parera, 2002).

Apesar de haver variação na coloração em determinadas épocas, pode-se definir sua pelagem como amarela-acinzentada. A linha dorsal do corpo é mais escura, formando uma faixa negra característica que se estende da cabeça até a cauda, sendo escuras também as extremidade dos membros e da cauda (Berta, 1982; Silva, 1994; Parera, 2002). É um canídeo de aproximadamente 80-120cm e peso em torno de 5-8Kg (Nowak, 1999) com uma dentição caracterizada pelos carniceiros pequenos em relação aos molares grandes (Berta, 1982).

A reprodução da espécie foi abordada apenas nos trabalhos de Brady (1978), realizado com animais de cativeiro, e de Macdonald e Courtenay (1996), com populações naturais da Ilha de Marajó (Pará/Brasil). O primeiro autor relata a ocorrência de duas crias anuais, com intervalo de oito meses, já no segundo trabalho é observada apenas uma cria anual. A época de maior número de nascimentos fica entre os meses de janeiro e fevereiro para os animais cativos e entre novembro e dezembro para as populações de Marajó. Brady (1978) descreve o período de gestação como de 59 dias (dado corroborado por Macdonald e Courtenay, 1996) com o nascimento de três a seis filhotes, que recebem cuidados de ambos os pais.

A maior parte dos estudos realizados nesta espécie abordam sua dieta e o consideram um generalista. Através de estudos em ambientes bastante diversificados, em síntese, os trabalhos relatam o consumo de pequenos mamíferos, répteis, anfíbios, insetos, crustáceos, aves, frutos diversos e ovos. Embora prefira pequenos vertebrados, o graxaim do mato ajusta sua ingesta consumindo mais vegetais ou insetos de acordo com a disponibilidade do alimento em cada época. Como oportunista, pode contribuir com a regulação de populações naturais (Montgomery e Lubin, 1978; Brady, 1979; Bisbal e Ojasti, 1980; Berta, 1982; Olmos, 1993; Motta-Junior *et al*., 1994; Macdonald e Courtenay, 1996; Facure e Monteiro-Filho, 1996; Facure e Giaretta, 1996).

Geralmente atribui-se o hábito solitário a *C. thous*, porém, vários dados sugerem que este canídeo viva em pares ou em grupos maiores compostos por unidades familiares (Montgomery e Lubin, 1978; Brady, 1979; Macdonald e Courtenay, 1996).

Os valores estimados para a área de vida de cada indivíduo da espécie vão de 0,3 Km^2 a 15 Km^2, considerando diversas regiões de estudo e diversas estações anuais (Brady, 1979; Sunquist *et al*., 1989; Macdonald e Courtenay, 1996; Michalski, 2000). A extensão das áreas utilizadas parece variar de acordo com o tipo de ambiente e com a estação do ano, da mesma forma como varia a tolerância entre indivíduos na sobreposição de áreas. Tais diferenças podem estar relacionadas à disponibilidade de alimento (Brady, 1979).

Quando em pares, os indivíduos usam áreas que se sobrepõem e ambos as marcam com urina, embora não cacem em cooperação (Brady, 1979; Biben, 1982). Em relação à dispersão dos indivíduos, os novos casais formados passam a habitar áreas adjacentes as de seu grupo familiar natal, retornando posteriormente de forma intermitente ao antigo grupo (Macdonald e Courtenay, 1996).

Durante o dia o graxaim do mato permanece em repouso, geralmente em moitas de vegetação, forrageando intensamente durante a noite (Berta, 1982). Procuram com muita freqüência áreas habitadas por humanos em busca de restos alimentares ou margens de estradas onde predam pequenos mamíferos. Tais hábitos acabam por torná-os muito suscetíveis a ações humanas. Vistos como predadores de animais domésticos, são perseguidos, e nas rodovias são freqüentemente atropelados (Silva, 1994; Cimardi, 1996; Becker e Dalponte, 1999).

1.3 Estudos genéticos em populações naturais

As informações genéticas obtidas pela análise de marcadores moleculares têm contribuído muito para o entendimento das relações evolutivas e ecológicas entre indivíduos, populações e espécies.

É ampla a utilização de polimorfismos de DNA para estudos filogeográficos. Estes investigam os princípios e processos que determinam as distribuições geográficas das linhagens genealógicas, especialmente dentro e entre espécies muito próximas (Riddle, 1996; Avise, 2000). O tempo e o espaço são considerados os eixos principais nos quais são mapeadas as genealogias de

interesse, através da integração de informações provenientes da genética de populações, etologia, demografia, biologia filogenética, paleontologia, geologia e biogeografia histórica, num esforço em conciliar diversas disciplinas micro e macroevolutivas (Avise, 2000).

Nestes estudos, os caracteres moleculares representam uma fonte rica de informação para a reconstrução de filogenias e diversas análises baseadas em coalescência, para as quais o campo teórico e analítico tem se desenvolvido consideravelmente (ex. Huelsenbeck e Ronquist 2001; Beerli e Felsenstein, 2001; Hey e Nielsen, 2004). As inferências obtidas através destes métodos podem, dentro de seus limites, indicar o tempo aproximado e a seqüência de eventos que deram origem a grupos de indivíduos e servir como diagnóstico final para definir o status taxonômico destes (Templeton, 2001; Frankland *et al*., 2003; Zhang e Hewitth, 2003).

Em escala populacional, o uso de dados genéticos têm crescido significativamente em estudos de estruturação (ex. Dalén *et al*., 2002; Dalén *et al*., 2005; Iyengar *et al*., 2005); paternidade e parentesco (ex. Vigilant *et al*., 2001, Seddon *et al*., 2005; Cutrera *et al*., 2005), dispersão de indivíduos (ex. Sacks *et al*., 2004; Geffen *et al*., 2004) entre outras características da dinâmica das populações.

A reconstrução destes padrões evolutivos, especialmente a nível infra-específico, tem sido de grande importância para a determinação de estratégias adequadas para a conservação de espécies (Eizirik, 1996). As subdivisões genéticas encontradas correspondem a uma fração significativa da biodiversidade, e a diversidade genética é componente fundamental da diversidade biológica existente em uma determinada região (Moritz, 1994; Moritz e Faith, 1998; Avise, 2000).

1.3.1 Os Marcadores Moleculares

Diversos fatores como tipo de herança e processos mutacionais determinam características próprias a cada marcador molecular, especialmente entre

segmentos de DNA nuclear e de organelas. Torna-se interessante, assim, a incorporação de diferentes marcadores nos estudos em populações naturais.

1.3.1.1 DNA Mitocondrial

O DNA mitocondrial (mtDNA) de animais consiste de um genoma haplóide, circular, de pequeno tamanho que está presente em centenas a milhares de cópias por célula (Ferreira, 2001).

Nos vertebrados, está organizado em sua forma mais simples, onde um total de 37 genes é contido em um segmento circular de 16 a 17 kb, tendo pouco ou nenhum espaço entre eles (Snustad e Simmons, 1997). Sua seqüência única (não repetitiva) conta com 13 genes codificadores de proteínas, 2 genes para rRNA, 22 genes para tRNA e uma região controladora que contém seqüências regulatórias para duplicação e inicio de transcrição (Graur e Li, 2000). Esta última é rica em bases A-T (Brown, 1985) e chamada, em vertebrados, de *D-Loop* (*Displacement Loop*) devido à formação de uma estrutura em fita tripla no início de sua replicação (Brown *et al*., 1986).

A região controladora é frequentemente usada em estudos de genética de populações dada sua alta variabilidade em seqüência de nucleotídeos, resultado de sua alta taxa de mutação, consideravelmente maior em todo o DNA mitocondrial do que em segmentos nucleares. Já os genes codificadores de proteínas, mais conservados, como o do citocromo-b, são utilizados para análise de filogenia acima do nível específico (Pereira, 2000; Graur e Li, 2000).

As altas taxas de substituição de bases apresentadas pela região controladora encontram-se acentuadas em duas de suas porções, chamadas segmentos hipervariáveis (HVS1 e HVS2 [*Hipervariable Segment*]). Estas porções têm extensões próximas a 350pb cada e estão separadas por uma região mais conservada de aproximadamente 200pb (Brown, 1985).

Outro fator importante no uso do mtDNA é sua herança matrilinear que permite a identificação de linhagens maternas que contribuíram para a formação de diferentes populações de uma espécie. Tal característica, aliada as baixas taxas de recombinação, rearranjos, transposições e inversões, possibilita a

obtenção de padrões filogenéticos sem a ambigüidade causada pela recombinação em genes nucleares (Brown, 1985; Snustad e Simmons, 1997; Avise, 2000; Ostrander e Wayne 2006).

Entretanto, apesar destas vantagens, pode haver certa limitação no emprego deste marcador, dado que sua análise representa apenas a história evolutiva das linhagens maternas das populações. Suas características estruturais, ainda, caracterizam os segmentos de mtDNA como um único conjunto de genes ligados, e sua análise diz respeito a apenas uma perspectiva na análise da variação genética total (Wayne, 1996). Devido a isto, o seu emprego combinado a locos nucleares nos estudos populacionais aumenta a confiabilidade dos resultados, podendo verificar, estender e aprofundar as inferências obtidas.

1.3.1.2 Marcadores nucleares

Polimorfismos nucleares, que constituem uma oportunidade quase ilimitada para estudos evolutivos, se encontram amplamente distribuídos pelo genoma de eucariotos. Neste, regiões não codificantes ou intergênicas, como íntrons e microssatélites, apresentam-se mais variáveis do que as regiões codificantes e tem assim um amplo emprego como marcadores moleculares.

Seqüências de íntrons são ferramentas muito poderosas para obtenção de dados de polimorfismos. Sua análise, no entanto, deve levar em conta fatores como recombinação, seleção, heterozigosidade, inserções, deleções, baixa divergência e politomias. Especialmente para estudos populacionais, sua utilização é ainda incipiente (ex: Antunes *et al*., 2002).

Os microssatélites são parte do grupo de *loci* chamados VNTRs (V*ariable Number of Tandem Repeats*). São compostos de uma seqüência simples de não mais que 6 pares de bases, repetidas em um número de 10 a 50 cópias, e sofrem uma elevada taxa de mutação (10^{-2} a 10^{-6} mutações por loco por geração) devido a eventos de *slippage* e recombinação desigual das moléculas de DNA (Scribner e Pearce, 2000). Por apresentarem altos níveis de variabilidade (Avise, 1994; Snustad e Simmons, 1997; Scribner e Pearce, 2000) e serem usualmente tidos como seletivamente neutros, têm altas chances de serem afetados por curtos

períodos de isolamento ou endocruzamento (Tautz, 1993; Scribner e Pearce, 2000), sendo assim marcadores moleculares úteis para investigar estes processos.

1.3.2 Alguns Dados Genéticos em Canídeos

Estudos moleculares e citogenéticos têm sido importantes ferramentas no entendimento dos padrões evolutivos na família Canidae.

Entre as espécies sul-americanas é encontrada uma grande semelhança cariotípica, o que indica uma ancestralidade recente (2n=74 com NF=76 para *L. gymnocercus, L. griseus, L. culpaeus, L. sechurae, A. microtis, S. venaticus e L.vetulus*; 2n= 76 e NF=78 para *C. brachyurus* e 2n= 74 NF=110 para *Cerdocyon thous*) (Wurster, 1969 *apud* Chiarelli, 1975; Brum-Zorrila e Langguth, 1980; Wayne *et al*., 1987; Wayne, 1993).

Wayne (1993) em revisão, incorporando dados aloenzimáticos de distâncias genéticas e de morfologia cromossômica em uma análise filogenética, reuniu os canídeos sul-americanos em um único ramo, indicando uma separação deste grupo em relação aos demais de 7-10 milhões de anos.

Geffen *et al*. (1996) utilizaram uma seqüência de 383pb do DNA mitocondrial (citocromo-b) para criar uma filogenia para as várias espécies de canídeos e relacioná-la à evolução do tamanho, organização social e outras características da história natural. O estudo indicou, entre outras inferências, que *Cerdocyon e Lycalopex* (referido como *Dusycion*) compunham um grupo monofilético.

Yahnke *et al*. (1996) analisando uma seqüência de 344pb da região controladora do DNA mitocondrial observaram que P. *fulvipes* representa uma linhagem distinta de *L. griseus* (que divergiu há cerca de 250,000 a 300,000 anos). Os haplótipos encontrados para este último e para *L. culpaeus* não definiram ramos diferentes na análise filogenética, indicando que as duas espécies são muito recentes e provavelmente possam produzir híbridos.

Wayne *et al*. (1997) sequênciaram 2001pb de genes mitocondriais codificadores de proteína em 23 espécies de canídeos a fim de entender as relações evolutivas dentro de Canidae. Os resultados indicaram como

monofiléticos os canídeos sul americanos, ocorrendo dentro deste grupo a formação de dois clados bem definidos: (1) o ramo ao qual pertencem *Speothos e Chrysocyon*, e (2) um grupo composto pelas demais espécies, sendo *Cerdocyon* a mais basal entre estas. O tempo de divergência entre as espécies, calculado por relógio molecular, indicou que a separação entre *Speothos e Chrysocyon* se deu antes da formação do istmo do Panamá, e talvez *Cerdocyon* tenha divergido também antes deste período. Assim, é possível que até quatro linhagens ancestrais de canídeos tenham invadido a América do Sul e que apenas a radiação de *Lycalopex* tenha acontecido neste continente.

Os dados desse trabalho foram aliados por Zrzavy e Ricancova (2004) à um total de 188 caracteres morfológicos, comportamentais e citogenéticos para analisar a filogenia de toda a família canidae. Neste estudo, o grupo das raposas sul americanas (*Atelocynus*, *Cerdocyon* e *Lycalopex*) foi definido como monofilético, sendo proximamente relacionado às espécies *L. griseus, L. gymnocercus* e *L. culpaeus*. *L. vetulus* e *L. sechurae* que definiriam uma linhagem comum.

Lindblad-Toh *et al*. (2006) ao concluírem o sequenciamento do genoma do cão doméstico, publicaram uma análise filogenética obtida através 15 Kb de seqüências nucleares, através da qual inferem a monofilia de todos os canídeos sul americanos atuais, sendo que: (1) *Chrysocyon brachyurus* e *Speothos venaticus* reúnem-se em uma linhagem basal; (2) *Lycalopex* corresponde a um grupo monofilético; (3) fazem parte de um dos ramos mais derivados *L. vetulus* e *L. sechurae* e do outro *L. gymnocercus* e *L. griseus*. Apesar da grande quantidade de dados empregados neste estudo, muitos dos ramos obtidos na análise filogenética não apresentam apoio considerável.

Apenas dois estudos genético-populacionais foram realizados com os canídeos da América do Sul. Ambos corresponderam a abordagens ecológicas, utilizando fezes para identificar espécies presentes em determinados habitats (*L. fulvipes* - Vilà *et al*., 2004) ou investigar composição de dieta (*C. thous* - Farrel *et al*., 2000).

Capítulo II
OBJETIVOS

O objetivo geral deste trabalho é fornecer subsídios ao conhecimento dos canídeos sul-americanos, contribuindo com dados genéticos ao entendimento da dinâmica das populações e história evolutiva das espécies. Para tal, tem como objetivos específicos:

- utilizar marcadores moleculares (regiões de introns e de microssatelites do DNA nuclear, fragmentos de DNA mitocondrial) para a análise filogeográfica e da estruturação populacional de *Cerdocyon thous*.

- obter inferências a respeito das relações filogenéticas e padrões intrapopulacionais de diversidade genética para as espécies de canídeos do gênero *Lycalopex*.

Capítulo III

1º ARTIGO

aceito para publicação na revista Molecular Ecology

Phylogeography and population history of the crab-eating fox (*Cerdocyon thous*)

Phylogeography and population history of the crab-eating fox

(*Cerdocyon thous*)

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ABSTRACT

The crab-eating fox is a medium-sized Neotropical canid with generalist habits and a broad distribution in South America. We have investigated its genetic diversity, population structure and demographic history across most of its geographic range by analyzing 512 base pairs (bp) of the mitochondrial DNA (mtDNA) control region, 615 bp of the mtDNA *cytochrome b* gene and 1,573 total nucleotides from three different nuclear fragments. Mitochondrial DNA data revealed a strong phylogeographic partition between Northeastern Brazil and other portions of the species' distribution, with complete separation between southern and northern components of the Atlantic Forest. We estimated that the two groups diverged from each other *ca.* 400,000 - 600,000 years ago, and have had contrasting population histories. A recent demographic expansion was inferred for the Southern group, while Northern populations seem to have had a longer history of large population size. Nuclear sequence data did not support this north-south pattern of subdivision, likely due at least in part to secondary male-mediated historical gene flow, inferred from multi-locus coalescent-based analyses. We compare the inferred phylogeographic patterns to those observed for other Neotropical vertebrates, and find evidence for a major north-south demographic discontinuity that seems to have marked the history of the Atlantic Forest biota.

INTRODUCTION

The study of comparative phylogeographic patterns often reveals important past evolutionary processes affecting regional faunas (Avise 2000). Most studies on phylogeography have employed mitochondrial DNA (mtDNA) sequences, which are amenable to sophisticated genealogical analyses but reflect only a portion of the total historical record of a sexual organismal pedigree. Thus, more complete conclusions can be obtained by adding nuclear sequences to phylogeographic studies (Hare 2001; Zhang & Hewitt 2003). Sequences of autosomal or X-linked intronic regions have an interesting informative potential for demographic analyses, due to their relatively rapid substitution rate (when compared to exons, or indirectly to protein polymorphisms) and longer retention of variants due to their diploid status and biparental inheritance. At the populational level, the examination of nuclear sequences is relatively recent and still limited to a few examples (e.g. Antunes *et al.* 2002; Gay *et al*. 2004; Spinks & Shaffer 2005).

So far few studies have addressed the evolutionary history of South American taxa from an intra-specific phylogeography perspective, so that major evolutionary patterns have yet to be uncovered and explored. A few studies have investigated biogeographic patterns in Neotropical small vertebrates, in some cases identifying a south-north discontinuity in the Atlantic Forest (Vanzolini [1988], Bates *et al.* [1998], and Costa *et al.* [2000] based on species distribution data; Lara & Patton [2000], Ditchfield [2000], and Costa [2003] based on intra-specific molecular data). This regional pattern has not yet been tested in larger mammals with higher dispersal capabilities, which might provide an interesting comparison to these small-bodied taxa.

The crab-eating fox (*Cerdocyon thous*) is a medium-sized canid (weight *ca.* 4-7 Kg), a versatile and fairly common species exhibiting a generalist diet and opportunistic hunting behavior, feeding on fruits, eggs, crabs, small mammals and insects. It is currently found in a wide variety of habitats including tropical and subtropical forest, open woodlands, savannas, and anthropic areas ranging from Colombia and Venezuela to Paraguay, northern Argentina, Uruguay, and

throughout Brazil except for the Amazon basin lowlands (Courtenay & Maffei 2004). (Fig. 1). Throughout this range, this fox is subject to constant persecution by ranchers over supposed depredation on sheep and other small livestock (Berta 1982; Ginsberg & Macdonald 1990), and are also heavily killed on roads, although the demographic impact of these sources of mortality is currently unknown.

Several studies have addressed aspects of intra-specific phylogeography and population history of various canids (Lehman & Wayne 1991; Wayne 1996; Girman *et al.* 2001; Dalén *et al.* 2005); however no large-scale study has yet been published on Neotropical members of the family. *C. thous* is the single living species of its genus; its fossil record suggests that it has evolved in North America in the late Miocene to early Pliocene, and later dispersed to South America in the Pleistocene (Langguth 1975; Berta 1987). Five subspecies of *C. thous* have been recognized on the basis of classical morphological studies (Cabrera 1931; Langguth 1969; Berta 1982): (I) *C. t. entrerianus* (southern Brazil, Bolivia, Uruguay, Paraguay, Argentina); (II) *C. t. azarae* (north-eastern and central Brazil); (III) *C. t. thous* (south-eastern Venezuela, Guyana, Surinam, French Guiana, northern Brazil; (IV) *C. t. aquilus* (northern Venezuela, Colombia); (V) *C. t. germanus* (Bogotá region, Colombia) (see Fig. 1). As it has been observed for other Neotropical carnivores that classical subspecies often do not reflect inferred patterns of historical population subdivision (e.g. Eizirik *et al.* 1998), it would be important to test such partitions in a molecular phylogeographic context.

In this study, we report patterns of genetic variation in *Cerdocyon thous*, based on the analysis of DNA sequences from two mitochondrial segments and three nuclear introns, obtained from wild-born individuals sampled throughout most of the species' geographic range. We draw inferences on the evolutionary history of this species, and discuss it in comparison with patterns observed for other Neotropical taxa. In particular, we use the combination of mitochondrial and nuclear data sets to test the following hypotheses: (i) there is a north-south phylogeographic break in the Atlantic Forest populations of this fox (as observed in smaller-bodied species) in spite of its higher mobility; (ii) historical partitions coincide with classically recognized subspecies; (iii) the observed partitions are

derived from events that occurred in the Early or Middle Pleistocene; and (iv) male-biased gene flow leads to detection of demographic partitions with mtDNA but not with nuclear markers.

MATERIALS AND METHODS

Sample Collection and Laboratory Techniques

Biological material was collected from 106 crab-eating fox individuals (Table 1, Fig.1) across a large area of the species' range. Blood samples (preserved in a salt saturated solution; 100mM Tris, 100mM EDTA, 2% SDS) were collected from captive individuals (of known origin) and wild animals captured for ecological studies. Other tissue samples (preserved in 95% ethanol) were obtained from road-killed individuals. Three samples each from *Pseudalopex gymnocercus* and *P. vetulus* were included in the protocol and used as outgroups in phylogenetic and network-based analyses.

Genomic DNA was extracted from samples using a standard phenol/chloroform protocol (Sambrook *et al.* 1989). Five different fragments were amplified by the Polymerase Chain Reaction (PCR; Saiki *et al.* 1985): (I) the 5' portion of the mtDNA control region, containing the first hypervariable segment (HVS-I), was amplified using primers MTLPRO2 (5'-CACTATCAGCACCCAAAGCTG) and CCR-DR1 (5'-CTGTGACCATTGACTGAATAGC) (or H16498 [Ward *et al.* 1991] as an alternative reverse primer); (II) the complete *cytochrome b* gene using primers CytB-DF1 (5' - TCTCACATGGAATTTAACCATGA - 3') and CytB-DR1 (5' – GAATTTCAGCTTTGGGTGCT – 3'); (III) the second intron of the *Proteolipid Protein 1* (*PLP1*) gene using primers described by Murphy *et al.* (1999); (IV) intron 14 of the *Feline Sarcoma Protooncogene* (*FES*) using primers described by Venta *et al.* (1996); and (V) intron 8 of the *Precursor 1 of Cholinergic Receptor Nicotinic Alpha Polypeptide* (*CHRNA1*) using primers described by Lyons *et al.* (1997).

PCR was performed in 20-µl reactions containing 2µl 10X buffer, 1.5 mM $MgCl_2$, 0.2 µM dNTPs, 0.2 µM each primer, 0.75 unit Taq polymerase and empirical template dilutions. Thermocycling conditions for control region, *cytochrome b* and *PLP1* DNA amplification began with 10 cycles (Touchdown) each including a 45s denaturing step at 94ºC, 45s annealing at 60-51ºC, and a 1.5 min extension at 72 ºC; this was followed by 30 cycles of 45s denaturing at 94ºC, 30s annealing at 50ºC and 1.5 min extension at 72 ºC. The PCR amplification for *CHRNA1* and *FES* began with 10 cycles (Touchdown) of which each had a 30s denaturing step at 94ºC, 30s annealing at 60-51ºC, and 1min extension at 72ºC, followed by 30-34 cycles of 30s denaturing at 94ºC, 30s annealing at 50ºC and 1min extension at 72ºC. Products were examined on a 1% agarose gel stained with ethidium bromide, purified with Shrimp Alkaline Phosphatase and Exonuclease I, and sequenced using ABI chemistry and an ABI-PRISM 3100 automated sequencer. Sequences were deposited in GenBank under accession numbers XXXX-XXXX.

Sequence analysis

Sequence electropherograms were visually inspected and edited using CHROMAS 1.45 (http://www.thecnelysium.com.au/chromas.html), and aligned using the CLUSTALW algorithm implemented in MEGA 3.0 (Kumar *et al*. 2004). Alignments were checked and edited by hand, and segments that could not be unambiguously aligned were excluded from all analyses. Initial sequence comparisons and measures of variability were performed using MEGA. To determine the appropriate model of nucleotide sequence evolution, we used the Akaike Information Criterion as implemented in MODELTEST 3.6 (Posada & Crandall 1998). Details of the sequence analyses will be described separately below for each data set (control region, *cytochrome b* and nuclear introns). For the two latter data sets only aspects that differ from the control region analyses will be specified.

mtDNA control region data set

The Tamura-Nei model (Tamura & Nei 1993) with a proportion of invariable sites and a gamma distribution of rate heterogeneity across sites (TN+G+I)

provided the best fit to this data set (I= 0.7456; alpha=1.158), and was applied in all subsequent model-based analyses. Phylogenetic relationships among haplotypes were inferred using PAUP*4.0b10 (Swofford 1998) for three of the different optimality criteria: (i) maximum parsimony (MP) with heuristic searches using 10 replicates of random taxon addition; (ii) maximum likelihood (ML) incorporating the TN+G+I model; and (iii) minimum evolution (ME) with a heuristic search starting from a neighbor-joining (NJ: Saitou & Nei 1987) tree, and employing three different types of distance: ML distance, TN+G+I distance, and p-distance. In each case 100 bootstrap replicates were used to evaluate nodal support. A separate phylogenetic analysis using Bayesian Inference (BI) was performed with MrBayes 3.0b4 (Huelsenbeck & Ronquist 2001), incorporating the GTR+G+I model. Two separate runs of the Markov chain Monte Carlo search were performed with 100,000 and 200,000 generations, respectively, sampling trees every 100 generations, and discarding the first 200 trees as burn-in.

Haplotype networks were generated using two different methods: (i) statistical parsimony as implemented in TCS 1.18 (Clement *et al.* 2000), with connections constrained by 95% confidence intervals; and (ii) the median-joining approach (Bandelt *et al.* 1999) implemented in Network4.1.0.8 (www.fluxus-engineering.com). A Nested Clade Analysis (NCA: Templeton *et al.* 1995) was performed on the basis of a TCS network, whose clades were nested by hand following the approach suggested by Templeton *et al*. (1987; 1995). The nested structure was analyzed with Geodis 2.0 (Posada *et al.* 2000), employing 10,000 permutations to test the significance of genealogy-geography associations, and using the latest inference key (http//:darwin.uvigo.es/download/geodisKeys_14jul04.pdf) to interpret the processes underlying significant results.

To investigate patterns of historical population structure, an Analysis of Molecular Variance (AMOVA; Excoffier *et al.* 1992) was performed with ARLEQUIN 2.0 (Schneider *et al.* 2000) under several variants of three different scenarios of hypothesized geographic subdivision: (i) each sampling locale treated as a distinct population; (ii) considering broad regional units called ecoregions

(adapted from Dinerstein *et al.* 1995 and www2.ibge.gov.br/downloads/mapa_murais/biomas_pdf.zip), representing major vegetational domains, with the Atlantic Forest subdivided into Northern and Southern portions (see Fig. 1); and (iii) using the major phylogeographic partition identified here (see Results) to define the two principal groups as population units. Significance of estimated Φ_{ST} values was tested using 10,000 permutations. We also applied the program SAMOVA (Dupanloup *et al.* 2002) to investigate the possibility of alternative patterns of population subdivision. This approach starts from individual sampling locales as populations and surveys all possible combinations forming two or more broader groups, attempting to identify the most likely position of inferred historical barriers. To test for the occurrence of isolation by distance, we assessed the correlation between genetic and geographic distances among the 32 sample-sites (mean genetic distance value for each unit), using a Mantel test (1967) performed in ARLEQUIN with 100,000 permutations.

For each population defined in the various schemes outlined above, DnaSP 4.0 (Rozas *et al.* 2003) was used to estimate gene diversity (h, the probability that two randomly chosen mtDNA lineages were different in the sample) and nucleotide diversity (π per nucleotide site, the probability that two randomly chosen homologous nucleotides are different in the sample). We used this value of π as an estimator of the populational parameter θ ($\theta=2N_{ef}\mu$, where N_{ef} is the historical effective number of females, and μ is the substitution rate per site per generation [see below]), referred to here as θ_π (Tajima 1996). We also estimated θ using coalescent-based approaches with LAMARC 2.0.2 (Kuhner *et al.* 1995; Beerli & Felsenstein 2001) and IM (Hey & Nielsen 2004). Details of these runs are presented below, in the context of divergence dating (IM) and multi-locus (LAMARC) analyses. Inferences regarding the occurrence of past events of population expansion or decline were based on Mismatch Distribution analyses (Rogers & Harpending 1992) and estimates of neutrality tests such as Tajima's D (Tajima 1989), Fu and Li's F* and D*, and Fu's F_S (Fu 1997) computed in DnaSP and ARLEQUIN. LAMARC was also used to infer historical changes in population size, as described below.

To date the coalescence time of *Cerdocyon* mtDNA lineages (and also to estimate the historical effective number of females, as outlined above), we obtained the substitution rate (μ) for the control region using data from grey wolves and coyotes, for whose divergence a fossil calibration was available (1 million years ago [MYA]; Vilá *et al.* 1999). Sequences from several individuals of these two species (GenBank accession numbers AY280940 to AY280930; AY280928 to AY 280926; AY280923 to AY280912; AY812741; AY812739 to AY812730; AY289995) were aligned with our data set, and only the overlapping region (274 bp) was employed in this analysis. The μ value was then estimated using the formula $d_a=2\mu T$ (Nei 1987), where T is the time to the most recent common ancestor and d_a is the genetic distance (TN model as implemented in MEGA) between species corrected for ancestral polymorphism ($d_a=d_{xy}-[d_x+d_y]/2$; Nei 1987). This substitution rate and the same formulae were then used to estimate the divergence time between major intra-specific clades in *C. thous* and also between *Cerdocyon* and *Pseudalopex*. In addition to reporting the point estimate for μ, we also calculated a conservative range for each of these divergence events, by applying the 95% confidence interval ($\pm$ 2 standard errors [SE]) to both the divergence estimation and rate calibration steps (Eizirik *et al*. 2001). For effective size estimations, a generation time of 2 years was assumed, based on studies of *Alopex lagopus* (Dalén *et al.* 2005) and *Canis latrans* (Vilà *et al.* 1999), which exhibit similar life histories (*e.g.* age at maturity, gestation time and longevity) relative to *Cerdocyon*.

Additional dating analyses included a linearized tree approach performed with MEGA, using the same estimate of μ described above, and a phylogeny based on our full (512 bp) control region data set. To test whether it was acceptable to extrapolate the estimate of μ to the entire segment, we performed comparisons focused on the Southern Clade, for which a historical expansion process was identified (see Results). In this case, we attempted to date the expansion event by estimating the internal coalescence of this group via the value of π (and its 95% confidence interval), using both the 274 bp and 512 bp segments.

In addition to phylogeny-based dating analyses, we estimated the timing of divergence between the two major *Cerdocyon* population units using the isolation-with-migration model developed by Nielsen & Wakeley (2001) and Hey & Nielsen (2004), and implemented in the program IM. This allowed us to incorporate population-level processes (*e.g.* lineage sorting, fluctuations in size) in the dating inference, likely leading to more realistic estimates of when divergence events took place. Multiple runs of IM were performed, aiming to promote and to verify convergence of parameter estimates. The two final runs consisted of (i) four Metropolis-coupled Markov chain Monte Carlo (MCMC) chains, each run for 10 million steps after a burn-in period of 300,000 steps; and (ii) 10 Metropolis-coupled MCMC chains, each run for 3 million steps after 100,000 steps of burn-in. The substitution rate and generation time were the same as in the analyses outlined above. In addition to estimating the divergence time between the two major mtDNA phylogroups (taking into consideration the observed geographic swap of some haplotypes between them, *i.e.* using geography as a defining criterion), we also used the IM results to directly infer the historical effective population size in each of these population units.

***Cytochrome b* data set**

Since we could not obtain samples of individuals collected north of the Amazon river, we generated a smaller additional data set (representing major geographic regions) of the mtDNA *cytochrome b* gene to allow a comparison with a single Venezuelan sample available in GenBank (Accession number AF266472; Farrel *et al.* 2000). We determined with MODELTEST that the HKY model of sequence evolution provides the best fit to our *cytochrome b* gene data. ME (NJ with HKY distance), MP (heuristic search, random addition of taxa), and ML (HKY model) trees were computed with PAUP. Nodal support was assessed with 1,000 bootstrap replicates.

Nuclear intron data set

Heterozygote sites in nuclear segments were identified when two different nucleotides were present at the same position in electropherograms of both

strands, with the weakest peak reaching at least 25% of the strongest signal. When two or more heterozygote sites were identified in the same segment, the gametic phase of the variants was determined computationally using PHASE 2.1 (Stephens *et al.* 2001; Stephens & Donnelly 2003). In the case of the X-linked segment *PLP1*, the obtained haplotypes were also verified using male individuals as known hemizygotes. Haplotypes were then used for multiple analyses, such as generating median-joining networks with NETWORK, which were rooted using sequences generated for *P. gymnocercus* and *P. vetulus*. For each locus, we estimated population diversity parameters such as θ, using DnaSP (θ_π) and LAMARC (see below for search details). ARLEQUIN was used to perform Fu´s and Tajima´s tests of neutrality, and to investigate population subdivision via AMOVA (ecoregions and the major phylogenetic groups inferred from mtDNA were used as units). Departure from a neutral model of evolution was also assessed with Fu and Li's test using DnaSP.

Multi-locus analyses

Our final set of analyses consisted of the coalescent-based estimation of relevant demographic parameters (effective sizes, migration rates, and population growth rates) including simultaneously the mtDNA control region and nuclear intron data sets. These analyses were performed with LAMARC, allowing for differing sample sizes for each segment, and normalizing the effective population size of each genomic region (autosomal or X-linked) so as to match the mtDNA value. This should allow for a direct comparison of inferred patterns between the mtDNA and nuclear data sets, so that inferences such as male-biased gene flow can be tested while minimizing the effect of the varying rates of drift occurring in these genomic partitions. Also, more accurate estimates of demographic parameters can be achieved by integrating the results obtained from multiple loci. In the case of analyses incorporating nuclear loci, we did not directly calculate N_e (as presented for the mtDNA control region data set) since no reliable estimates of their substitution rates were available. Rather, indirect comparisons of N_e were performed by directly analyzing estimates of the parameter θ for different data sets and geographic partitions.

Seven final LAMARC runs were performed, all of which included two populations (north and south), allowing for growth in both of them, as well as for bi-directional migration. LAMARC searches were initiated with a starting value of θ based on Waterson's (1975) formula, and applied the F84 model of sequence evolution with empirical base frequencies and transition/transversion ratios. Four runs used a Maximum-likelihood (ML) search approach with 10 initial chains (10,000 steps each) and 2 final chains (200,000 steps each). Samples were taken very 20 steps, and the initial 1000 genealogies were discarded as burn-in. Three other runs used a Bayesian search strategy: two of them included three replicates, each consisting of one initial chain (10,000 steps) and one final chain (200,000 steps), with samples taken every 20 steps and burn-in of 1,000 genealogies. The third Bayesian run used a single replicate of one long chain (800,000 steps), with samples taken every 40 steps and burn-in of 2,000 genealogies. Consistency of estimated parameters across different ML and Bayesian runs was used to assess convergence, and LAMARC profiles were used to calculate confidence (or credibility) intervals around point estimates.

RESULTS

mtDNA control region

A 512 base-pair (bp) sequence of the mtDNA control region (CR) was obtained for 106 crab-eating fox individuals. The segment contained 58 variable sites (41 of which were parsimony-informative), defining 35 different haplotypes (Table 2). All the observed polymorphisms were single base-pair substitutions and consisted of 58 transitions and one transversion (see online Supplementary Data). Between *Cerdocyon* and outgroups there were 88 variable sites of which 73 were parsimony-informative. High levels of gene diversity and nucleotide diversity were observed among individuals (Table 3). Both ecoregion-specific and shared sequences were identified in the crab-eating fox, however the majority of haplotypes (77%) was sample-site specific. The most common and widespread haplotype was shared by 37 individuals; it was only absent in the Eastern Amazonia and Northern Atlantic Forest ecoregions. In these two areas, all haplotypes were ecoregion or sample site-specific (Table 2).

Parsimony, minimum evolution, maximum likelihood and Bayesian analyses retrieved topologically equivalent trees that differed only at nodes with bootstrap values below 50%. ME trees generated using different distance methods were equivalent, and maximum likelihood distances were conservatively chosen, as their bootstrap values were the lowest. Two monophyletic groups of *Cerdocyon* mtDNA lineages were evident (Fig. 2). The first clade (bearing support values of 85,<50, 62 and 53% in MP, ME, ML and Bayesian analyses, respectively) contained haplotypes from the Cerrado, Pantanal, Southern Atlantic Forest and Eastern Amazonia ecoregions, encompassing most of the sampled area, and including almost all samples from southern locales (Table 2, Fig. 1). The shape of its internal phylogeny, with short branches and little robust structure, is suggestive of a recent population expansion (Avise 2000). Only two Northern individuals (bCth196 [haplotype C20] and bCth202 [C15], both from the Caatinga region) were present in this cluster. The second clade (supported by values of 99, 94, 99 and 100% in MP, ME, ML and Bayesian analyses, respectively) included all the sequences obtained from the Northern Atlantic Forest, almost all samples from the Caatinga, and some samples from Eastern Amazonia, with overwhelming presence in the northern portion of the surveyed area (Fig. 1). The only exceptions were one individual from the Pantanal ecoregion (bCth51 [C30]) and one from the Cerrado (bCth177 [C23]). We found no evidence for phylogeographic subdivision within either clade (Fig. 2).

The haplotype networks produced with TCS and NETWORK were nearly identical, and only the former is shown here (Fig. 3) in the context of a Nested Clade Analysis (NCA). To minimize the ambiguities and facilitate the NCA we removed from this data set one haplotype (C23; see Table 2), whose incomplete sequence led to the creation of ambiguous links. The network corroborated the existence of two main clusters, Northern and Southern, with the presence of Eastern Amazonian haplotypes in both groups (often in basal positions). These two clades were separated by over 12 mutational steps (which is the 95% confidence threshold for the statistical parsimony approach with our data, thus

creating two discrete clusters in the TCS analysis). In the NETWORK analysis (not shown) these clusters were connected by haplotypes C31 and C14, which is identical to the TCS result using a 90% threshold.

The networks are indicative of a relatively recent population expansion in the Southern Clade, in which several localized lineages are connected by short branches to the most common, widespread haplotype (Fig. 3). The null hypothesis of no association between haplotypes and geographic location was rejected for some clades positioned at all levels of the nested diagram (Fig. 3). Inference of historical processes based on the NCA interpretation key suggested several plausible scenarios of factors affecting population history in this species (see online Supplementary Data and Discussion).

The AMOVA results indicated that most of the genetic variability in *C. thous* can be explained by a single north *vs.* south partition (Table 4). For these two geographic groups, suggested by the phylogenetic and network analyses, the estimated Φ_{ST} values were $\geq$ 0.68. High fixation indices were also observed in some other scenarios comprising three to five major groups, especially when the Caatinga samples were treated as a separate unit (see Table 4 and Fig. 1). When we calculated the Φ_{ST} considering each of the 32 sample-sites as a separate population, the estimated Φ_{ST} was 0.52. However, if the two major groups (Southern and Northern) were analyzed separately (in each case including Eastern Amazonia, as it does not bear specific affiliation with either one), the AMOVA results indicated that most of the diversity (>83%) occurs within sample-sites, and not among local populations. The same observation resulted from ecoregion-based analyses, strongly indicating that in every case the fixation indices had been due to the main partition between Southern and Northern groups (Table 4). Moreover, AMOVA analyses at the regional level indicated that several pairwise comparisons among sites yielded non-significant values. Results from the Mantel test showed a significant correlation between genetic and geographic distances among the 32 sample-sites (r=0.54; determination of Y by X=30%;

p=0.000; Fig. 4), indicating that isolation by distance also plays a role in the genetic structure of this species.

Since a deep phylogeographic partition was identified between Southern and Northern groups, all subsequent analyses of population history were conducted separately for each group, as well as for the total sample. Pairwise mismatch distribution analyses of mtDNA CR sequences revealed a shape that was approximately unimodal for the Southern Clade (Fig. 5), compatible with a recent expansion scenario (perhaps followed by a more complex population history, such as some level of subdivision). All neutrality tests performed with mtDNA CR sequences from the Southern clade corroborated this inference, by rejecting the null model assuming constant size (Tajima's D: -1.78; Fu's F_s: -9.16; F*: -2.373; D*: -2.337; significance: F_s: $p<0.02$; all others: $p<0.05$). Conversely, mtDNA CR analyses of both the Northern clade and the total sample revealed a multimodal pattern of mismatch distribution (not shown) and neutrality tests that were non-significant except for Fu's F_s value in the Northern group (F_s: -6.52; p=0.003). Coalescent-based analyses performed with LAMARC supported the inference of historical population growth in both major demographic units (g values > 61 in all runs). However, confidence intervals were broad and overlapped zero in some of the Bayesian runs, thus warranting cautious interpretation. Maximum likelihood estimates, on the other hand, were highly positive (g > 383), with confidence intervals that did not overlap zero.

The point estimate for the mtDNA CR substitution rate was $\mu=3.68 \times 10^{-8}$ substitutions/site/year (considering only the shared segment with wolf and coyote sequences). Taking into account the 95% CI around the wolf-coyote divergence (see Methods), we obtained low and high limits for μ (2.02×10^{-8} and 5.34×10^{-8}, respectively), which were used for every divergence event in combination with the variance observed for d_a at each node. This approach allowed us to estimate a conservative interval for the time of each divergence event, accounting for the variance at both the calibration and divergence estimation procedures. The point estimate for the divergence between the Southern and Northern clades of *Cerdocyon* was 578,082 years before the present (ybp), and the overall CI ranged

from 1,640,000 ybp to 177,358 ybp. The age of the divergence between *Cerdocyon* and *Pseudalopex* was estimated at 908,216 ybp (CI: 2,337,500 - 368,867 ybp). The linearized tree method produced congruent results: 623,237 ybp for the south-north split in *Cerdocyon*, and 1,231,320 ybp for *Cerdocyon vs. Pseudalopex* divergence.

Using the coalescent-based approach implemented in the program IM and the same input parameters as above, we obtained in the two final runs of the program (see online Supplementary Data) mean divergence dates of 369,957 and 421,975 ybp for the two main *Cerdocyon* mtDNA clades (overall 95% area interval across both runs: 57,856 – 1,029,193 ybp). The estimated surface of the posterior distribution of this parameter was not smoothly unimodal in any of the IM runs, suggesting that this result should be taken with caution. However, the inferred values (and conservative intervals) are consistent with the phylogeny-based results presented above (and younger than those, as would be expected after taking population processes more realistically into account), corroborating the conclusion that this population split took place in Middle Pleistocene.

The age of the demographic expansion inferred for the Southern clade was calculated based on the estimate of π, which is expected to approximate the coalescence time for the base of this group. Using only the 274 bp segment applied for the dating analyses mentioned above, this expansion was dated at 123,641 ybp (CI = 247,524 - 70,888 ybp). Extrapolating the substitution rate to our full segment (512 bp), the estimate for this node was 108,625 ybp (CI = 237,623 - 59,925 ybp).

We estimated the female effective population size (N_{ef}) from the mtDNA CR data set using the formula $\theta=2N_{ef}\mu$, with the point estimate of the substitution rate mentioned above, and θ calculated from the nucleotide diversity (θ_{π}) or from the coalescent-based approaches implemented in LAMARC and IM (see Methods for details, and Tables 3 and 5 for several estimated values of θ). The estimates of N_{ef} generated from θ_{π} were 54,421 and 142,857 individuals for the Southern and Northern clades, respectively. The observed difference between these values is

statistically significant, since a conservative estimate of their 95% CI (*i.e.* $\pm$ 2SE; see Table 3) indicates that their boundaries are clearly non-overlapping (*i.e.* $p << 0.05$). Estimates of N_{ef} produced with IM (in this case considering geography and not phylogeny as the criterion for group affiliation) were 133,105 (95% area interval: 70,266 – 239,361) and 273,256 (143,007 – 374,179) individuals for the Southern and Northern groups, respectively (results were concordant between the two final runs, only one of which is reported here). LAMARC analyses of the CR data set (also considering geography to define groups) led to estimates of N_{ef} of 339,546 (196,875 – 595,146) and 1,708,174 (505,003 – 5,370,428) individuals for the Southern and Northern groups, respectively (see Table 5 for more details). Overall, there was a clear trend in all analyses for the female historical effective size to be considerably higher in the Northern *versus* Southern group, even though in some cases the confidence (or credibility) intervals were very broad and overlapped.

Cytochrome b gene

A 615 bp segment of the *cytochrome b* gene was sequenced for six *Cerdocyon thous* individuals representing most of the sampled geographic region (Fig. 1). Each individual was found to have a different haplotype. They were compared to a sequence from a Venezuelan *C. thous* available in GenBank, consisting of a 101-bp subset of the same segment. Fifteen sites were variable, 10 of which showed parsimony-informative variation (Table 6).

The two-clade phylogenetic pattern inferred for the mtDNA control region was corroborated by the *cytochrome b* data set (Fig. 6), with increased bootstrap support values for this partition (>85% for all methods) when the Venezuelan sample was excluded from the analysis. This observation stems from the fact that the available Venezuelan sequence had been sequenced for a shorter segment (see Methods) that spanned only three of the included polymorphic sites, none of which was diagnostic for either the Northern or Southern clade (Table 6). Thus, the position of this Venezuelan sample relative to the two clades could not be established based on the available data: the NJ tree grouped it with the Southern Clade (Fig. 6), while ML placed it in the Northern Clade (not shown) and MP

created a polytomy relative to the other *Cerdocyon* sequences (not shown). All analyses showed low support for such connections, due to the absence of informative shared sites.

Nuclear intron sequences

Nuclear introns were amplified in a sub-sample of *C. thous* individuals representing all different ecoregions in the study area. Twenty-eight *Cerdocyon thous* individuals (representing 56 sampled chromosomes) were sequenced for a 423 bp fragment of the *FES* gene and for a 333 bp segment of the *CHRNA1* gene. A 817-bp segment of the X-linked *PLP1* locus was sequenced for 37 individuals (representing a total of 45 sampled chromosomes). Several polymorphic sites were identified in each of the introns: five in *CHRNA1* (including two transversions), three in *FES*, and four in *PLP1*. The inclusion of *Pseudalopex* outgroups (*P. vetulus* only, in the case of *FES*) in these data sets increased the number of variable sites to 10, 5 and 9 for *CHRNA1*, *FES* and *PLP1*, respectively. In addition, two indels were identified when *C. thous* and outgroups were compared: one in *CHRNA1* (2 bp) and one in *PLP1* (4 bp). No sequence sharing was observed between different species.

For each intron, polymorphic sites were computationally assigned to haplotypes (Table 7) with 95% to 100% phase probability estimation. Estimates of gene and nucleotide diversity were moderate to high for each intron (Table 3). Rejection of the assumption of neutrality was observed for Tajima´s D in *PLP1* (D= -1.56; p= 0.02) and *FES* (D=–1.47; p=0.02), and for Fu's F test in *FES* (F=-3.25; p=0.0017). Of the six haplotypes identified for *CHRNA1*, two were observed only in the Northern Atlantic Forest ecoregion, while two others were Caatinga-specific (Fig. 7). *FES* sequences comprised four different haplotypes of which one exhibited high frequency and was present in all ecoregions. The others were found only in the Southern Atlantic Forest. In the *PLP1* locus, four different haplotypes were identified, two of which were ecoregion-specific (Northern and Southern Atlantic Forest, respectively) (see Fig. 7 and Table 7). In spite of the occurrence of these private haplotypes in the sample, no indication of significant phylogeographic partitioning was identified, as the AMOVA analysis resulted in

non-significant values of Φ_{ST} among ecoregions, as well as between the two major groups inferred from mtDNA.

Multi-locus analyses

Demographic parameters were estimated with LAMARC for the combined nuclear introns, as well as for the complete data set including nuclear and mtDNA (control region) segments (see Table 5). This allowed for the comparison of nuclear *versus* mitochondrial patterns of diversity (normalizing for relative effective population size and mutation rate), so that demographic processes could be evaluated more directly. Results from multiple ML and Bayesian runs were mostly consistent, exhibiting largely concordant confidence intervals. Means across multiple runs were used to arrive at final estimates. The trend described above indicating higher diversity (and thus larger historical N_e) in the Northern group than in the Southern one was supported by the nuclear data set and the combined inference, even though confidence intervals (CIs) overlapped. Migration rates (expressed in Table 5 as the number of migrants per generation) were consistently higher from South to North than in the reverse direction, although broad CIs precluded the rejection of equality. Interestingly, there was a consistent trend of lower migration rates inferred from the mtDNA data set when compared to the nuclear segments, even after correction for lower effective size and higher mutation rates. Multi-locus estimates of population growth produced results that were similar to those presented above for the mtDNA CR data set. There was indication of historical growth for both the Southern and Northern populations (using nuclear alone or nuclear+CR data sets), with positive estimates of parameter “g”, although CIs did overlap zero in most runs. Several runs led to higher estimates of “g” in the Southern *versus* Northern group, supporting stronger growth in the former.

DISCUSSION

We observed high levels of genetic diversity in the crab-eating fox using both mtDNA and nuclear sequences. Genetic diversity in the mtDNA control region (CR) was similar to that observed in other widespread canids such as the gray wolf (*Canis lupus*; π = 0.026; Vilà *et al.* 1999) and the African wild dog (*Lycaon pictus*, π = 0.014; Girman *et al.* 2001), while higher than that reported for the arctic fox (*Alopex lagopus*; π=0.009 - Dalén *et al.* 2005) and lower than the values inferred for coyotes (*Canis latrans*; π= 0.046; Vilà *et al.* 1999). Nucleotide diversity was also rather high in the nuclear introns (see Table 3), however these values could not be compared to other canids due to lack of polymorphism data from additional species. Nevertheless, the number of polymorphic sites (*CHRNA1*= 5, *FES*=3 and *PLP1*=4) and different haplotypes (*CHRNA1*= 6, *FES*=4 and *PLP1*=4) found in *C. thous* is quite high if we consider sequence sizes and the small number of individuals analyzed. This result and the primary observation of no shared haplotypes between *Cerdocyon* and its close relatives indicate that these three segments are likely useful tools for canid phylogeographic studies.

The most apparent pattern observed with the mtDNA CR data was the deep partition between two phylogeographic groups, separated in a roughly south-north direction (Figs. 1, 2 and 3). These two distinct phylogenetic clades were supported by the AMOVA and SAMOVA results (Φ_{CT} =0.70 and 0.74 respectively), the NCA-based inference (allopatric fragmentation), and also corroborated by the *cytochrome b* phylogeny. Interestingly, this partition was not observed in the networks generated with the nuclear sequences, which also yielded non-significant Φ_{ST} values. Phylogeographic structure is expected to be less pronounced at diploid nuclear loci compared with mtDNA for three possible reasons: (i) autosomal segments have an effective population size four times larger than mtDNA, thus undergoing four times less genetic drift; (ii) they have slower mutation rates, thus accumulating fewer differences over time even in the absence of gene flow; and (iii) they are inherited from both parents, so that male-biased gene flow would erode the signature of a matrilineal partition (Hare 2001; Antunes *et al.* 2002; Zhang & Hewitt 2003). All three factors may play a role in the pattern

observed in *Cerdocyon*, and none could be completely ruled out by our results. However, an interesting inference from the comparison of coalescent-based nuclear *versus* mtDNA estimates of migration rates (Table 5) is that there is indeed a trend suggestive of male-biased gene flow detectable in our data. The estimated number of migrants per generation remained considerably smaller in mtDNA-based relative to the nuclear-based inference, even after correction for uneven effective sizes and mutation rates in the two genomes. This result supports the inference that persistent male-mediated gene flow is a relevant (or perhaps the main) factor leading to lack of geographic structure in the nuclear markers analyzed here (Table 8). Male-biased dispersal and female philopatry have been reported in field studies of small or medium-bodied canids (e.g. *Vulpes macrotica mutica* [Koopman *et al*. 2000]; *Vulpes velox* [Kamier *et al.* 2004]). The only study performed so far with *C. thous* suggested that both sexes are somewhat philopatric, but did not rule out the possibility that males disperse on average farther than females (Macdonald & Courtenay 1996).

The θ values estimated from the mtDNA control region and nuclear sequences (Tables 3 and 5) indicate that past diversity was quite large in this species, leading to the inference of large historical (as well as current) effective population sizes. Direct estimates of N_e were performed only for the mtDNA CR data set, due to feasibility of calculating a substitution rate for this segment. As a whole, estimates of N_e using multiple methods were broadly consistent and quite high, ranging from *ca.* 400,000 individuals estimated from θ_π ([Northern N_{ef} + Southern N_{ef}] X 2; considering that N_{ef} is the effective number of females and assuming a 1:1 sex ratio) to a few million individuals based on the LAMARC results. Although these values are indeed high, they are not incompatible with plausible estimates of census sizes for *C. thous*, even taking into account that the latter are likely larger than effective sizes. Estimates of current population sizes are unavailable for this species as a whole, but can be inferred by extrapolation from local demographic data sets. Density estimates range from 0.55 to 4 individuals/km^2 (Courtenay & Maffei 2004), which by an approximate extrapolation would imply total current population sizes of *ca.* 2.8 million to 20 million individuals for the entire range (treated as a simplified continuous landscape for this

exercise). These values of current census size are therefore compatible with the large effective population sizes estimated from our genetic data, even hypothesizing an N_e/N ratio of 0.5 or less.

The inferred scenario of two main phylogeographic groups for *C. thous* is somewhat different from the intra-specific subdivision compiled by Cabrera (1931), which lists three subspecies for our study area. He proposed that one subspecies, *C. t. entrerianus* occupies Argentina, Paraguay and the southern states of Brazil, with its northern limit corresponding approximately to the geographical position of our SP1, SP2 and MS sample sites (see Fig. 1). This area coincides in part with our Southern mtDNA phylogeographic group; however our data suggest a more northerly boundary for this assemblage (see Fig. 1). The second of Cabrera's subspecies, *C. t. azarae*, would occur in Central and Northeastern Brazil, from the northern boundary of *C. t. entrerianius* to the southern edge of Eastern Amazonia (with our PI and TO sample-sites as a northwestern limit). Our observed mtDNA partition shows a more restricted group in the *C. t. azarae* area, identified here as the Northern clade. The third subspecies listed by Cabrera was *C. t. thous*, occurring from Southeastern Amazonia (our sample-sites in MA and PA) to the Guyanas; our data do not identify a third group in this area, but rather an admixture zone between the two major phylogeographic clades. In spite of these discrepancies, it is possible to reconcile our phylogeographic results with two of Cabrera's subspecies (*C. t. entrerianus* and *C. t. azarae*), which might remain applicable in some contexts given appropriate adjustments in their geographic range, as long as affirmed by corroboratory nuclear data in future studies.

The two major clades inferred from mtDNA data appear to have different ages and/or contrasting population histories (Table 8). The nucleotide diversity estimated for the Northern Clade is significantly higher than that of the Southern Clade (see Table 3), and thus a larger effective population size may be inferred for the former. Likewise, all other estimates of N_e and/or θ were higher for the Northern group than the Southern one (see Table 5). A high estimate of the effective population size may be a result of two distinct situations: (i) a large population (*i.e.* census size), caused by a broad geographic range and/or by high

density; or (ii) a stable population persisting at moderate to large sizes for long periods of time, since the calculated N_e is determined by the harmonic mean of the population size throughout its history. The Northern Clade is almost completely restricted to Northeastern Brazil, occupying an area about three times smaller than that inhabited by the Southern Clade. Although there is no solid data on crab-eating fox density in the wild (see above for a range of possible values), there is no reason to assume that densities are considerably higher in the Brazilian Northeast than elsewhere in the species' range. On the contrary, current densities are likely lower in this region since most of it is occupied by the harsh semi-arid Caatinga biome. Densities in the Brazilian Northeast would have to be 7-12 times higher than in the remainder of the range to explain the observed pattern based on current demography alone. Since this is extremely unlikely, we view the latter hypothesis as more probable, since it is corroborated by the deeper and more structured pattern of the Northern clade's internal phylogeny (Fig. 2), the higher levels of divergence observed in the haplotype network for this group (Fig. 3), and the estimates of historical demographic parameters. This conclusion is also supported by the NCA inference of isolation by distance and restricted gene flow in the North, suggesting a more complex demographic history in this area, in contrast to a pattern of recent population expansion inferred for the Southern clade (see Fig. 3 and online Supplementary Data).

Reinforcing these results, the nuclear intron data revealed more private haplotypes for Northern Clade ecoregions: three for the Northern Atlantic Forest and two for the Caatinga (considering the three segments altogether – see Fig. 7). The only other ecoregion exhibiting private haplotypes was the Southern Atlantic Forest, which spans a much broader geographic area than the ones mentioned above. The Cerrado and Pantanal regions did not bear private alleles, perhaps reflecting their intermediate geographic position leading to poor isolation with either edge of the sampled distribution. The genetic distinctiveness of the Caatinga was also suggested by some of the SAMOVA results that supported a separation of sample-site CE (see Fig. 1) from the remaining areas, producing a three-group scenario that yielded a slightly higher Φ_{ST} value (0.75) than that observed for the two-group pattern indicated by most analyses (see Table 4). In

fact, the palynological record from the latest Pleistocene indicates that there was a dense forest covering the Caatinga region (De Oliveira *et al.* 2005), whose fluctuations may have caused the isolation of fragments in some periods. On the other hand, the inferred forest or savanna vegetation that covered the region seems to have been widespread and stable over broad regions, possibly supporting a large population of *C. thous* and thus playing a role in the maintenance of larger effective sizes in this region.

The Southern clade, on the other hand, consists of a group of closely related haplotypes exhibiting low levels of divergence. The patterns observed in the phylogenetic trees and haplotype networks are consistent with a recent population expansion for *C. thous* in this broad region. This inference is supported by the mtDNA control region mismatch distribution revealing a prominent (albeit not completely smooth) peak, suggestive of a population expansion event (Fig. 5; Rogers & Harpending 1992). The significantly negative neutrality tests also support this expansion scenario.

Within each major clade, all analyses suggested the existence of very little genetic structuring among populations. This observation, along with the low values of molecular divergence among ecoregions (8.8% and 9.6%), are expected since *C. thous* individuals are extremely flexible, and adaptable to a variety of natural or even suburban habitats. On a regional scale, the results from the AMOVA using sample-sites as populations (separate analysis for Northern and Southern areas – see Table 4) indicate a significant but weak genetic differentiation among units. This pattern seems to be at least in part due to isolation by distance, as can be inferred from the significant correlation between genetic and geographical distances (Fig. 4). The NCA corroborated these results by suggesting some restriction to gene flow between populations of the Northern Clade (clade 4-2). In the Southern group this NCA inference was achieved only for one 1-step clade (clade 1-2), as few newly arisen haplotypes were present and C15 was frequent and widespread. This scenario of high gene flow with some isolation by distance would be predicted for habitat generalists with a continuous distribution such as the crab-eating fox, and has been similarly observed for coyotes and wolves

(Sacks *et al.* 2004; Geffen *et al.* 2004). In this context, the dramatic genetic partition identified between the two major *C. thous* clades becomes quite striking, implying that a strong disruptive process must have produced intense historical isolation between these areas.

The location of this genetic discontinuity agrees with patterns observed in other species, suggesting the occurrence of a shared, large-scale historical fragmentation event in that region. Our south-north partition coincides with latitudinal breaks (distributional or phylogeographic) displayed by other Atlantic Forest vertebrates such as reptiles, lowland birds and small mammals including the bat species *Carollia perspicillata* (Vanzolini 1988; Bates *et al.* 1998; Costa *et al.* 2000; Lara & Patton 2000, Ditchfield 2000). The study performed by Costa (2003) on small non-volant mammals strongly indicated that the Atlantic Forest is a composite area (with the breakpoint concordant with our results). Data obtained from marsupials and rodents showed that haplotypes from the easternmost Amazonian localities were often more closely related to those from the northern Atlantic Forest than to other Amazonian localities. For some species, a similar relationship was observed between the southern Atlantic Forest and western Amazonia, with the Caatinga and Cerrado appearing as past and present connection areas. Molecular dates for the inferred biogeographic divergences varied considerably across species and were often very old, pre-dating the Pleistocene (Costa 2003). It is plausible to postulate that cycles of vicariance shaped by ecological or physical barriers fragmented forest habitats at various times (affecting different taxa differently), so that their biotic elements might be of different ages (Cracraft 1988). These observations suggest that the apparent unity of present-day biomes may be misleading, and that a complex history may underlie the formation and biogeographic interactions of these ecosystems.

These South American phylogeographic patterns had so far not been investigated in larger terrestrial mammals possessing higher dispersal capabilities, such as carnivores. The deep genetic discontinuity observed in the crab-eating fox mtDNA data argues for the existence of a common process affecting multiple species. The divergence time estimated between the two major *C. thous* clades

(*ca.* 400,000 - 600.000 years) agrees with a Middle Pleistocene age, and was similar to that found for the bat *C. perspicillata* (1 mya) by Ditchfield (2000). Despite the generalist ecology and behavior of the crab-eating fox, environmental changes during the Pleistocene might have limited its range, perhaps due to strong vegetational shifts (*e.g.* increased aridity in connecting areas). The Brazilian Atlantic Forest was probably fragmented into distinct patches isolated by open grassland, with one of these splits separating the Northeastern and Southeastern areas of Brazil (Câmara 1988). Additional phylogeographic studies of diverse species occurring in this region should help test the occurrence of such a pervasive vicariant process in the Pleistocene.

An alternative historical scenario can be postulated for *Cerdocyon thous*. Since its current range excludes the core Amazon River basin and there is no record indicating occupation of this region in the past (Berta 1987), it is unlikely that the Southern Clade originated from Western Amazonia (as inferred for other species – Costa [2003]). However, this Southern group could represent a separate, more recent invasion from eastern Amazonia, using the Cerrado and the Paraná River basin habitats as dispersal corridors. This younger origin relative to the Northern clade is supported by historical demography inferences presented here. If the two major clades of *C. thous* indeed represent separate colonization processes that crossed the Amazon river, the original (source) population of this species might be present in northern South America (*e.g.* in Venezuela and the Guyanas). In this study, the samples from the Southeastern Amazon region exhibited the highest levels of mtDNA CR diversity, bearing haplotypes belonging to both major clades. This observation could be interpreted as evidence for current genetic interchange between formerly isolated areas, but is also compatible with the possibility of this being a more ancient area of distribution for this species. In any event, the observed patterns require the existence of a period of south-north demographic isolation in *C. thous*, particularly affecting Atlantic Forest populations.

The finding of a major phylogeographic discontinuity in *C. thous* in the Brazilian Atlantic Forest, which seems to agree with patterns observed in other species, highlights the complex history of this critically threatened biodiversity

hotspot, and argues for the urgency to develop efficient conservation plans for its biota. In particular, extreme present-day fragmentation of the Northeastern Brazil coastal forest currently threatens the persistence of several populations, whose uniqueness relative to other areas is still poorly assessed. Given the pattern inferred here of a history of isolation and differentiation of this region, it is critical to plan and enforce adequate conservation actions and research projects specifically targeting these areas.

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Figure Legends

Figure 1. Map showing the current geographic distribution of *Cerdocyon thous* (modified from Courtenay & Maffey 2004) and approximate sample collection sites (polygons) identified by ecoregion (*i.e.* major vegetation domains; the Southern Atlantic Forest region includes marginal portions of adjacent biomes [Chaco and southern grasslands]). Polygons are colored to indicate the presence of the two major mtDNA clades in each sample collection site (black: Southern Clade only; white: Northern Clade only; gray with black border: both clades present). The Venezuelan sample retrieved from GenBank is indicated by a "+" symbol. The thick dashed line indicates a region of Eastern Brazil where both mtDNA clades were completely separated geographically. The thin dotted lines indicate the limits of classical subspecies compiled by Cabrera (1931), identified by numbers: 1. *C. t. entrerianus;* 2. *C. t. azarae*; *3. C. t. thous*; *4. C. t. aquilus*; *5. C. t. germanus*.

Fig. 2. Maximum likelihood tree of *Cerdocyon thous* mitochondrial DNA haplotypes identified in this study, based on 512 bp of the control region. Labels are haplotype identification numbers (see Table 2). Values above branches indicate support for each node based on maximum parsimony/minimum evolution/maximum likelihood/Bayesian inference. Asterisks indicate bootstrap support below 50%. Polygons on the right indicate ecoregions where haplotypes are present (coded as in Fig. 1).

Fig. 3. Networks of *Cerdocyon thous* mtDNA control region (512bp) haplotypes generated with TCS using a 95% threshold for parsimony-based connections. The two major mtDNA clades (Northern and Southern) were treated as separate networks by TCS under these settings. Squares indicate haplotypes likely to be ancestral in each network (highest outgroup probability). The area of circles and squares is roughly proportional to the frequency of each haplotype. Dotted polygons demarcate clades for which significant results were observed in the Nested Clade Analysis (see online Supplementary Data).

Fig. 4. Graph depicting the correlation between genetic and geographic distances calculated for *C. thous* mtDNA control region sequences. Each sample-site was treated as a unit, and used to compute mean pairwise genetic distances (Kimura 2-parameter).

Fig. 5. Graph depicting the result of the Mismatch Distribution Analysis performed for the Southern Clade of *Cerdocyon thous*, based on mtDNA control region sequences. The main observed peak is at 3.4 differences between sequences.

Fig. 6. Minimum evolution trees of *Cerdocyon thous* mtDNA *cytochrome b* sequences (615 bp). Labels are individual identification numbers followed by ecoregion symbols (coded as in Fig. 1). Values above or below branches indicate nodal bootstrap support in maximum parsimony/minimum evolution/maximum likelihood trees. Asterisks indicate bootstrap support <50%. (a) Analysis including the Venezuelan sequence from GenBank (see text) (b) Analysis excluding the Venezuelan sequence.

Fig. 7. Median-joining networks generated for three nuclear introns: (a) *FES*, (b) *CHRNA1*, and (c) *PLP1*. The area of each circle indicates haplotype frequency in the total sample, with the frequency in each ecoregion represented by shading patterns. The arrow indicates the haplotype to which the root (outgroup) is connected in each network.

Table 1. Samples analyzed in the present study.

ECOREGION	*GEOGRAPHIC ORIGIN (SAMPLE SITE)*	*SAMPLES*	*INSTITUTION / CONTACT*
Southern Atlantic Forest	P.N. Iguaçu, Paraná State, S Brazil (PR)	bCth08[1], bCth59[1 2 3 4 5], bCth60[1],bCth61[1], bCth63[1 3 4 5], bCth64[1], bCth65[1], bCth66[1], bCth67[1], bCth68[1], bCth71[1], bCth74[1], bCth77[1]	UNIOESTE/José Flávio Cândido Jr. and Instituto Pró-Carnívoros
	Mato do Grosso do Sul State, SW Brazil (MS)	bCth11[1], bCth164[1], bCth166[1 3 4 5], bCth172[1 3 4 5],bCth174[1]	Instituto Pró-Carnívoros/ Dênis Sana
	Paraguay (PY1)	bCth91[1 3 4 5],	Guillermo D`Elia
	Paraguay (PY2)	bCth155[1]	L. Tchaicka
	Santa Catarina State, S Brazil (SC1)	bCth153[1 5],bCth154[1 3 4 5]	L. Tchaicka
	Santa Catarina State, S Brazil (SC2)	bCth178[1 3 4 5], bCth180[1], bCth182[1], bCth212[1], bCth213[1]	Sérgio Althoff, José F. Stholz and Zoológico de Pomerode
	Santa Catarina State, S Brazil (SC3)	bCth210[1],bCth211[1]	CENAP-IBAMA
	Rio de Janeiro State, E Brazil (RJ)	bCth214[1 3 4 5]	Zoológico de Pomerode
	São Paulo State, E Brazil (SP1)	bCth218[1], bCth219[1 3 4 5], bCth220[1], bCth301[1]	Instituto Pró-Carnívoros / C. Prada
	São Paulo State, E Brazil (SP2)	bCth305[1 4 5]	Eduardo Nakano
	Rio Grande do Sul State, S Brazil (RS1)	bCth15[1 3 4 5]	Margareth Mattevi
	Rio Grande do Sul State, S Brazil (RS2)	bCth106[1 5], bCth112[1 2 3 4 5], bCth116[1], bCth118[1], bCth126[1 5]	Alex Bager
	Rio Grande do Sul State, S Brazil (RS3)	bCth13[1] , bCth90[1]	Instituto Pró-Carnívoros, Mariana Faria-Corrêa
	Rio Grande do Sul State, S Brazil (RS4)	bCth01[1], bCth02[1], bCth12[1], bCth41[1] , bCth83[1], bCth98[1 3 4 5], bCth100[1], bCth142[15]	Mariana Faria-Corrêa, Instituto Pró-Carnívoros
	Rio Grande do Sul State, S Brazil (RS5)	bCth26[1], bCth27[1], bCth28[1], bCth31[15], bCth33[15], bCth34[15]	Instituto Pró-Carnívoros, T. C. Trigo, A. P. Brandt and F. Michalski
	Rio Grande do Sul State, S Brazil (RS6)	bCth20[1 5], bCth21[1] bCth23[1], bCth35[1] bCth39 [1 3 4 5], bCth40[1]	T. C. Trigo, A. P. Brandt and F. Michalski
Cerrado	Minas Gerais State, E Brazil (MG1)	bCth198[1], bCth199[1], bCth200[1]	Fabrício Horta
	Minas Gerais State, E Brazil (MG2)	bCth336[1 3 4 5]	Instituto Pró-Carnívoros / F. Rodrigues
	Goiás State, Central Brazil (GO1)	bCth203[1], bCth204[1], bCth205[1 3 4 5], bCth206[1], bCth207[1], bCth208[1 3 4 5], bCth209[1]	Zoológico de Goiânia/ Roberto Portela; CENAP-IBAMA
	Goiás State, Central Brazil (GO2)	bCth05[1]	Margarete Mattevi
	Mato Grosso State, SW Brazil (MT1)	bCth159[1], bCth161[1 3 4 5], bCth163[1]	R. Jorge, J. Dalponte, Instituto Pró-Carnívoros
	Tocantins State, Central Brazil (TO)	bCth177[1]	L. Tchaicka
Caatinga	PN Serra da Capivara, Piaui State, NE Brazil (PI)	bCth201[1 5], bCth202[1]	FUNDHAM/ Vanderson C.Vaz and Paulo César D´Andrea
	Ceará State, NE Brazil (CE)	bCth192 [1 2 3 4 5], bCth193[1], bCth194[1 3 4 5], bCth195[1], bCth196[1], bCth197[1]	Zoológico de Fortaleza/ Luiz C. Diniz

Table 1. (continued)

Eastern Amazonia	Pará State, N Brazil (PA)	bCth221[1], bCth223[1 3]	Tadeu de Oliveira
	Maranhão State, N Brazil (MA1)	bCth222[1]	Tadeu de Oliveira
	Maranhão State, N Brazil (MA2)	bCth230[1]	Tadeu de Oliveira
	Maranhão State, N Brazil (MA3)	bCth224[1 4], bCth225[1], bCth226[1], bCth227[1 4], bCth228[1 2 3 4 5]	Tadeu de Oliveira
Pantanal	Mato Grosso State, SW Brazil (MT2)	bCth48[1], bCth49[1], bCth50[1], bCth51[1 3 4 5], bCth56[1 2 3 4 5]	L. Tchaicka
Northern Atlantic Forest	Paraíba State, NE Brazil (PB)	bCth309[1 3 4]	F. Rodrigues / Instituto Pró-Carnívoros
	Bahia State, NE Brazil (BA)	bCth186[1 3 4 5], bCth187[1 3 5]	Zoológico de Salvador/Cláudio V. Lyra
	Pernambuco State, NE Brazil (PE)	bCth184[1 2 3 5], bCth185[1 3 4 5]	Zoológico do Recife/ Poly-Ana Celina
Outgroups	*Pseudalopex gymnocercus*	bPgy18[1 2 3 5] bPgy19[1], bPgy37[1]	Instituto Pró-Carnívoros
	Pseudalopex vetulus	bPve02[1], bPve03[1 5], bPve08[1 2 3 4]	L. Tchaicka, C. Prada

[1] samples typed for the mtDNA control region
[2] samples typed for the *cytochrome b* gene
[3] samples typed for the *CHRNA1* intron
[4] samples typed for the *FES* intron
[5] samples typed for the *PLP1* intron

Table 2. List of individuals that bear each mitochondrial DNA control region haplotype. Also indicated are the absolute frequency in the sample (Fr) and geographic distribution of haplotypes.
[a] Absolute frequency on the total sample.

Haplotype	Individuals	Fr[a]	Ecoregion
C1	bCth49	1	Pantanal
C2	bCth12,20,21,39,67,83,98,100,142,210,211	11	Southern Atlantic Forest
C3	bCth221,227,230	3	Eastern Amazonia
C4	bCth226	1	Eastern Amazonia
C5	bCth118	1	Southern Atlantic Forest
C6	bCth209	1	Cerrado
C7	bCth198,200	2	Cerrado
C8	bCth08,27,174,199,219,336	6	Southern Atlantic Forest,Cerrado
C9	bCth13,23,34,106,112,126,212,213,178	9	Southern Atlantic Forest
C10	bCth02,61,63,172	4	Southern Atlantic Forest
C11	bCth91,155	2	Southern Atlantic Forest
C12	bCth48	1	Pantanal
C13	bCth15	1	Southern Atlantic Forest
C14	bCth01	1	Southern Atlantic Forest
C15	bCth05,11,26,28,31,33,35,40,41,56,59,60,64,65,66,68,71,74,77,90, 116,153,154,159,161,164,166,180,182,202,203,204,207,214,218,220, 301	37	Southern Atlantic Forest, Pantanal,Cerrado, Caatinga
C16	bCth206,208	2	Cerrado
C17	bCth163	1	Cerrado
C18	bCth50	1	Pantanal
C19	bCth205,223	2	Eastern Amazonia, Cerrado
C20	bCth196	1	Caatinga
C21	bCth305	1	Southern Atlantic Forest
C22	bCth184	1	Northern Atlantic Forest
C23	bCth177	1	Cerrado
C24	bCth195	1	Caatinga
C25	bCth222	1	Eastern Amazonia
C26	bCth224	1	Eastern Amazonia
C27	bCth225	1	Eastern Amazonia
C28	bCth309	1	Northern Atlantic Forest
C29	bCth185	1	Northern Atlantic Forest
C30	bCth51	1	Pantanal
C31	bCth228	1	Eastern Amazonia
C32	bCth197	1	Caatinga
C33	bCth192,193,194	3	Caatinga
C34	bCth201	1	Caatinga
C35	bCth186,187	2	Northern Atlantic Forest

Table 3. Nucleotide and gene diversity observed in the *Cerdocyon thous* mtDNA control region (specified separately for different ecoregions and also for the two major phylogeographic clades) and three nuclear intron segments.

Locus	Group	N [a]	Nucleotide diversity (SE)	Gene diversity (SE)
mtDNA control region	Southern Atlantic Forest	62	0.009 (±0.001)	0.73 (±0.046)
	Northern Atlantic Forest	5	0.006 (±0.001)	0.90 (±0.160)
	Caatinga	8	0.028 (±0.005)	0.89 (±0.110)
	Eastern Amazonia	9	0.038 (±0.005)	0.91 (±0.092)
	Pantanal	5	0.021 (±0.025)	1 (± 0.120)
	Cerrado	16	0.012 (±0.005)	0.75 (±0.107)
	Southern Clade	88	0.008 (±0.001)	0.76 (±0.041)
	Northern Clade	17	0.021 (±0.001)	0.97 (±0.032)
	Total sample	106	0.019 (±0.002)	0.83 (±0.032)
CHRNA1	Total sample	56	0.00061(±0.0002)	0.17 (±0.067)
FES	Total sample	56	0.00041(±0.0001)	0.13 (±0.061)
PLP1	Total sample	45	0.00090(±0.0003)	0.20 (±0.060)

[a] Number of individuals indicated for the mtDNA control region; number of chromosomes indicated for nuclear introns.

Table 4. Support for population groupings estimated using Φ_{ST} values calculated for mtDNA control region haplotypes.

Scheme	Included Groups	Φ_{ST}*
Two groups suggested by the phylogenetic analysis (PI sample-site grouped with the Southern region)	(Eastern Amazonia without MA2 + Northern Atlantic Forest + Caatinga without PI +TO) (Cerrado without TO + Pantanal + Southern Atlantic Forest + MA2 + PI)	0.70
Two groups suggested by the phylogenetic analysis (PI sample-site grouped with the Northern region)	(Eastern Amazonia without MA2 + Northern Atlantic Forest + Caatinga +TO) (Cerrado - without TO + Pantanal + Southern Atlantic Forest + MA2)	0.68
Two geographic groups: Eastern Amazonia grouped with the Southern region	(Caatinga + Northern Atlantic Forest) (Eastern Amazonia + Southern Atlantic Forest + Cerrado + Pantanal)	0.68
Two geographic groups: Eastern Amazonia grouped with the Northern region	(Eastern Amazonia + Caatinga + Northern Atlantic Forest) (Southern Atlantic Forest + Cerrado + Pantanal)	0.61
Two groups suggested by SAMOVA based on ecoregions	(Northern Atlantic Forest) (Caatinga + Eastern Amazonia + Cerrado + Pantanal + Southern Atlantic Forest)	0,66
Three groups suggested by SAMOVA based on ecoregions	(Eastern Amazonia) (Northern Atlantic Forest + Caatinga) (Southern Atlantic Forest + Cerrado + Pantanal)	0.45
Four groups suggested by SAMOVA based on ecoregions	(Northern Atlantic Forest) (Caatinga) (Eastern Amazonia) (Cerrado + Pantanal + Southern Atlantic Forest)	0.66
Five groups suggested by SAMOVA based on ecoregions	(Northern Atlantic Forest) (Caatinga) (Eastern Amazonia) (Pantanal) (Cerrado + Southern Atlantic Forest)	0.63
Six ecoregions treated as individual units	See Fig. 1	0.54
Ecoregions treated as units (Northern region only)	(Caatinga) (Northern Atlantic Forest) (Eastern Amazonia)	0.10
Ecoregions treated as units (Southern region only)	(Cerrado)(Southern Atlantic Forest)(Eastern Amazonia)(Pantanal)	0.09
Two groups suggested by SAMOVA based on individual sample-sites	(CE + Northern Atlantic Forest + TO+ MA1) (PI + Eastern Amazonia without MA1+ Cerrado without TO + Pantanal + Southern Atlantic Forest)	0.74
Three groups suggested by SAMOVA based on individual sample-sites	(CE) (Northern Atlantic Forest + TO+ MA1) (PI + Eastern Amazonia without MA1 + Cerrado without TO + Pantanal + Southern Atlantic Forest)	0.75
32 sample-sites as separate units	See Fig. 1	0.52
Sample-sites treated as units (Southern region only)	(PR)(SC1)(SC2)(SC3)(MG1)(MG2)(MT1)(MT2)(PY1)(PY2)(RJ) (SP1)(SP2)(GO1)(GO2)(MS)(PA)(MA1)(MA2)(MA3)(RS1)(RS2) (RS3)(RS4)(RS5)(RS6)	0.16
Sample-sites treated as units (Northern region only)	(BA)(PB)(PE)(PI)(TO)(CE)(MA1)(MA2)(MA3)(PA)	0.14

* Φ_{CT} are reported in SAMOVA results

Table 5. Demographic parameters inferred for the two main geographic groups of *Cerdocyon thous* using the coalescent-based approaches implemented in LAMARC. Values are means calculated from multiple runs using maximum likelihood and Bayesian search strategies (see Methods). 95% confidence intervals (also means from multiple runs) are shown in parentheses.

	mtDNA-CR	Nuclear mean [b]	Overall [c]
θ - South	0.025 (0.015 – 0.044)	0.008 (0.002 – 0.804)	0.014 (0.012 – 0.035)
θ - North	0.126 (0.037 – 0.395)	0.032 (0.037 – 1.115)	0.031 (0.020 – 0.128)
Nm (South-North) [a]	1.544 (0.100 – 12.473)	6.383 (2.146 – 270.373)	1.556 (0.505 – 25.777)
Nm (North-South) [a]	0.394 (0.055 – 1.670)	1.223 (0.046 – 212.685)	0.761 (0.248 – 2.521)

[a] The number of migrants per generation (Nm) was calculated from the estimated migration rate parameter "M" in LAMARC (as suggested in the program documentation), incorporating the mean θ of the recipient population to correct for variation in the mutation rate among segments.

[b] The nuclear mean is the average estimate from the three nuclear intron segments (*CHRNA1*, *FES* and *PLP1*) analyzed here.

[c] Joint inference from the mtDNA control region and nuclear intron data sets.

Table 6. Mitochondrial DNA *cytochrome b* haplotypes identified from crab-eating fox samples (ecoregion representation indicated on the right), along with a Venezuelan sequence (VEN) available from GenBank. The top three lines comprise samples from the Northern phylogeographic group identified here, while the bottom three are samples from the Southern group (see text). Only variable sites are shown. Site numbers (vertical notation) refer to the aligned position in our 615 bp data set.

Sample	Variable Site 1 2 2 2 2 3 4 4 4 4 5 5 5 5 2 9 5 6 6 8 9 0 2 7 9 0 1 2 6 7 6 6 5 8 9 4 6 7 6 0 5 0 0 9	Ecoregion
bCth184	TTCTCTCCACCCTAG	Northern Atlantic Forest
bCth192	.C....T......G.	Caatinga
bCth228	.C....T..T...G.	Eastern Amazonia
VEN	??????...??????	Venezuela (GenBank)
bCth112	C.TCTC....TT..A	Southern Atlantic Forest
bCth59	C.TCTC.TG..T...	Southern Atlantic Forest
bCth56	C.TCTC..G..TG..	Pantanal

Table 7. Haplotypes identified in three nuclear introns (*CHRNA1*, *FES* and *PLP1*) sequenced for *Cerdocyon thous* individuals.

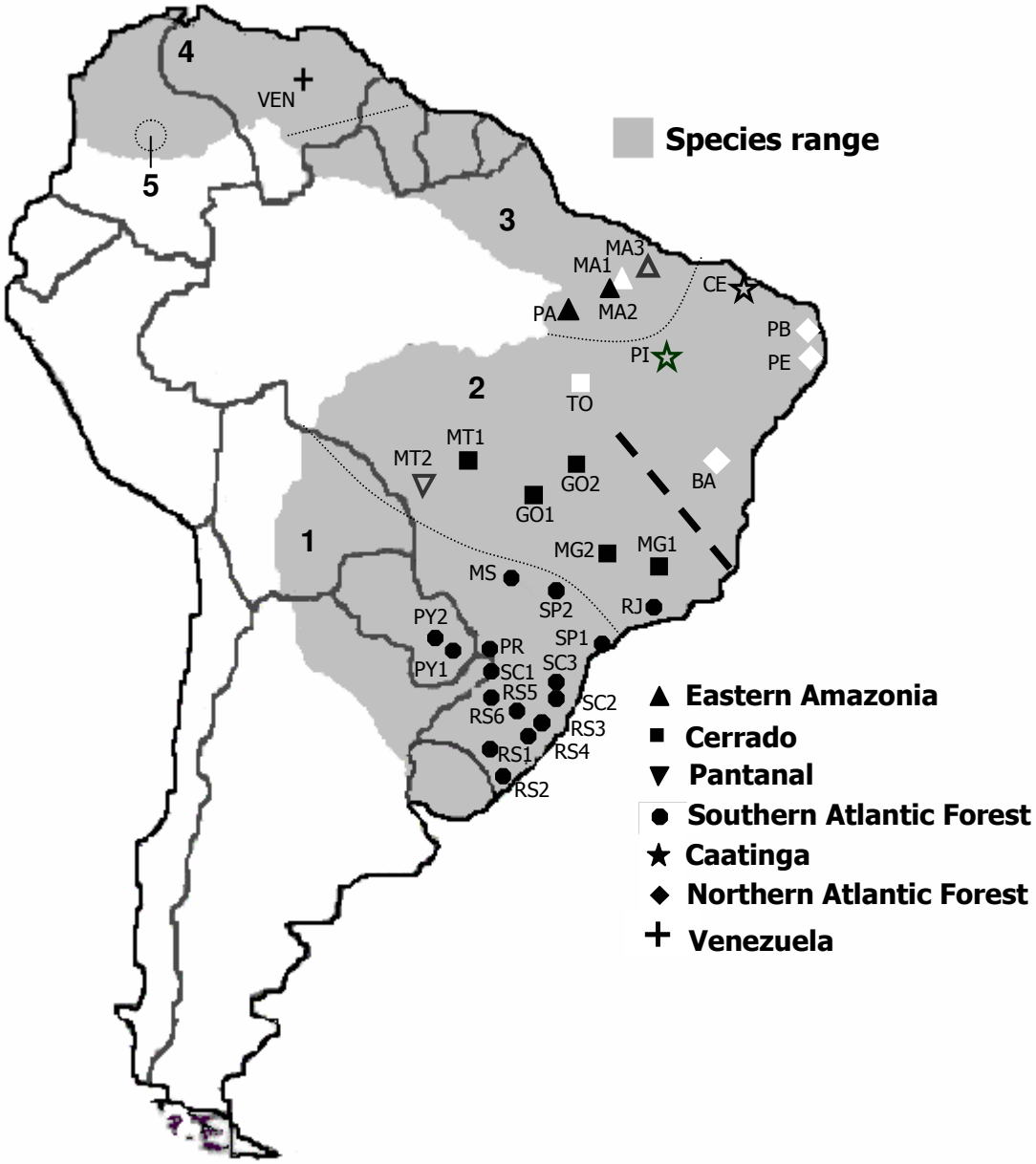
Haplotype	Variable sites [a]	Samples	Fr [b]
CHRNA1			
CH1	AGGCG	bCth15,39,51,59,56,63,91,98,112,336,154,172,178,184,185,186, 187, 205,208,214,219,223,228	40
CH2	...CC	bCth63,154,161,166,184,187,194,208,305	11
CH3	...TC	bCth305	1
CH4	..A..	bCth186	1
CH5	.C...	bCth192	1
CH6	GC...	bCth192,194	2
FES			
F1	CTC	bCth15,39,51,56,59,63,91,98,112,154,161,166,172,178,185,186,192,194, 205,208,214,219,224,227,228,305,309,336	51
F2	..T	bCth178	1
F3	.C.	bCth39,219,305	3
F4	T..	bCth98	1
PLP1			
P1	GCCT	bCth142	1
P2	.T..	bCth59,142,172,194	4
P3	...C	bCth15,20,31,33,34,39,51,56,63,86,91,98,336,106,112,126,153,154,161, 166,172,178,184,185,186,192,201,205,208,214,219,228,305	39
P4	A.TC	bCth187	1

[a] Variable sites correspond to the following nucleotide positions for each gene: *CHRNA1*: 29,143,210,220,276; *FES*: 82,160,230; *PLP1*: 336,425,473,610.
[b] Absolute frequency in the total sample of chromosomes (see Fig. 7).

Table 8. Summary of inferences on *C. thous* population history obtained from various analytical approaches performed in this study.

Analytical method	mtDNA	nDNA	Combined mtDNA+nDNA[a]
Phylogenetic and network-based analyses	Two well defined clades, population split of North vs. South took place in the Middle Pleistocene; population expansion of Southern clade *ca.* 100,000 years ago.	No phylogeographic partitioning.	N. P.
AMOVA, SAMOVA, Mantel test, NCA	North vs. south partition, isolation by distance.	No genetic structure.	N. P.
Mismatch distribution / Neutrality tests	Recent expansion in the South; longer time of stable population in the North.	No conclusive evidence of expansion.	N. P.
Diversity indices / Effective population size (N_e)	High molecular diversity; higher diversity in the Northern group, implying larger historical female N_e in this region.	High molecular diversity.	N. P.
Coalescent-based estimate of N_e (LAMARC and IM)	High molecular diversity; estimate of larger female historical N_e in the North than in the South.	Higher diversity (implying larger N_e) in the Northern region.	Higher diversity (implying larger N_e) in the Northern region.
Coalescent-based dating of South-North population divergence (IM)	Population split of North vs. south took place in Middle Pleistocene,	N. P.	N. P.
Coalescent-based estimation of population growth (g parameter - LAMARC)	Suggestive of population expansion in both North and South.	Suggestive of population expansion in both North and South.	Suggestive of population expansion in both North and South.
Estimate of migration rates (LAMARC)	Higher migration from South to North.	Higher migration from South to North; higher migration than estimated by the mtDNA data set, supporting male-biased gene flow.	Higher migration from South to North.

[a] N.P.: Analysis not performed with the combined mtDNA+nDNA data set.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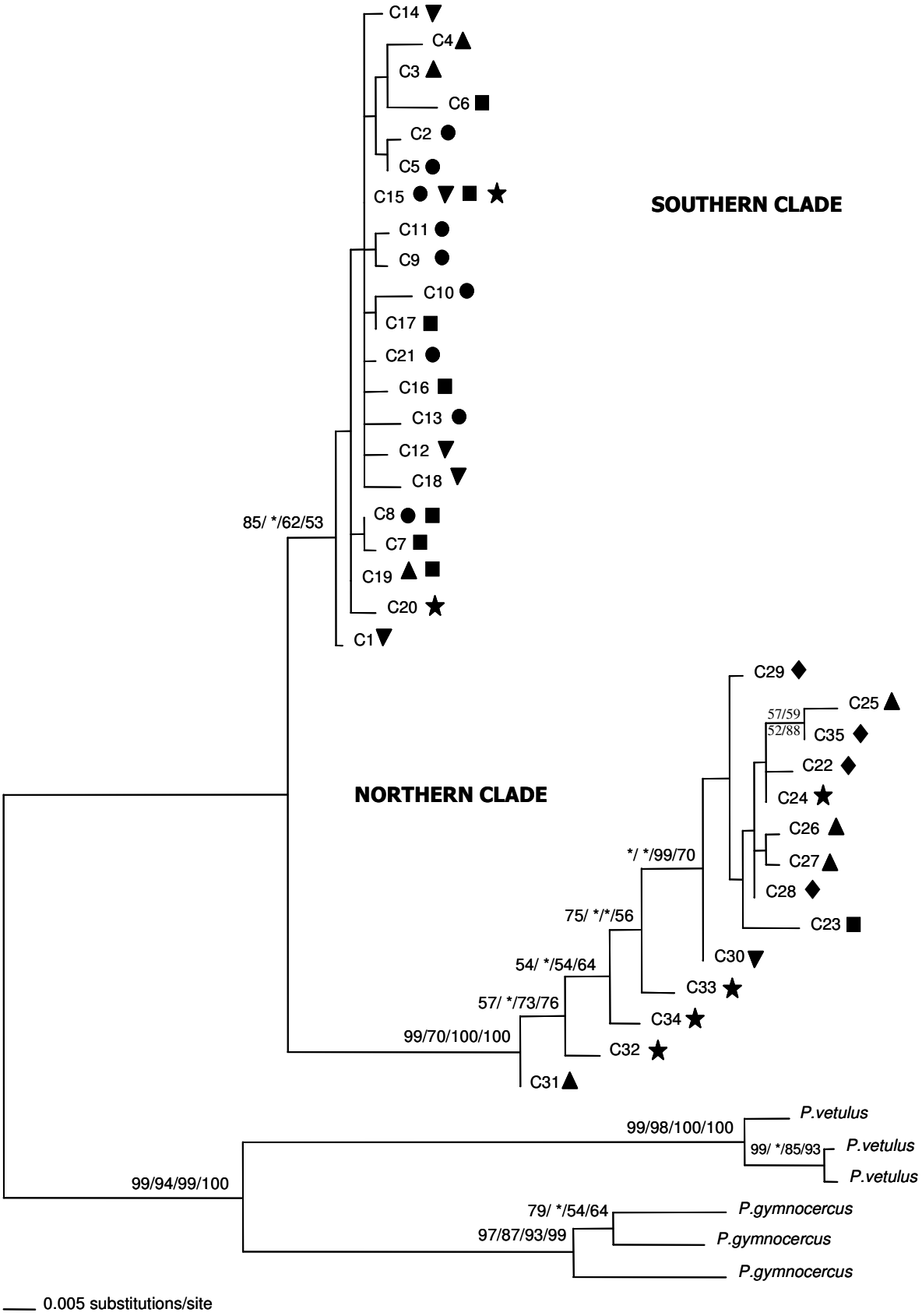
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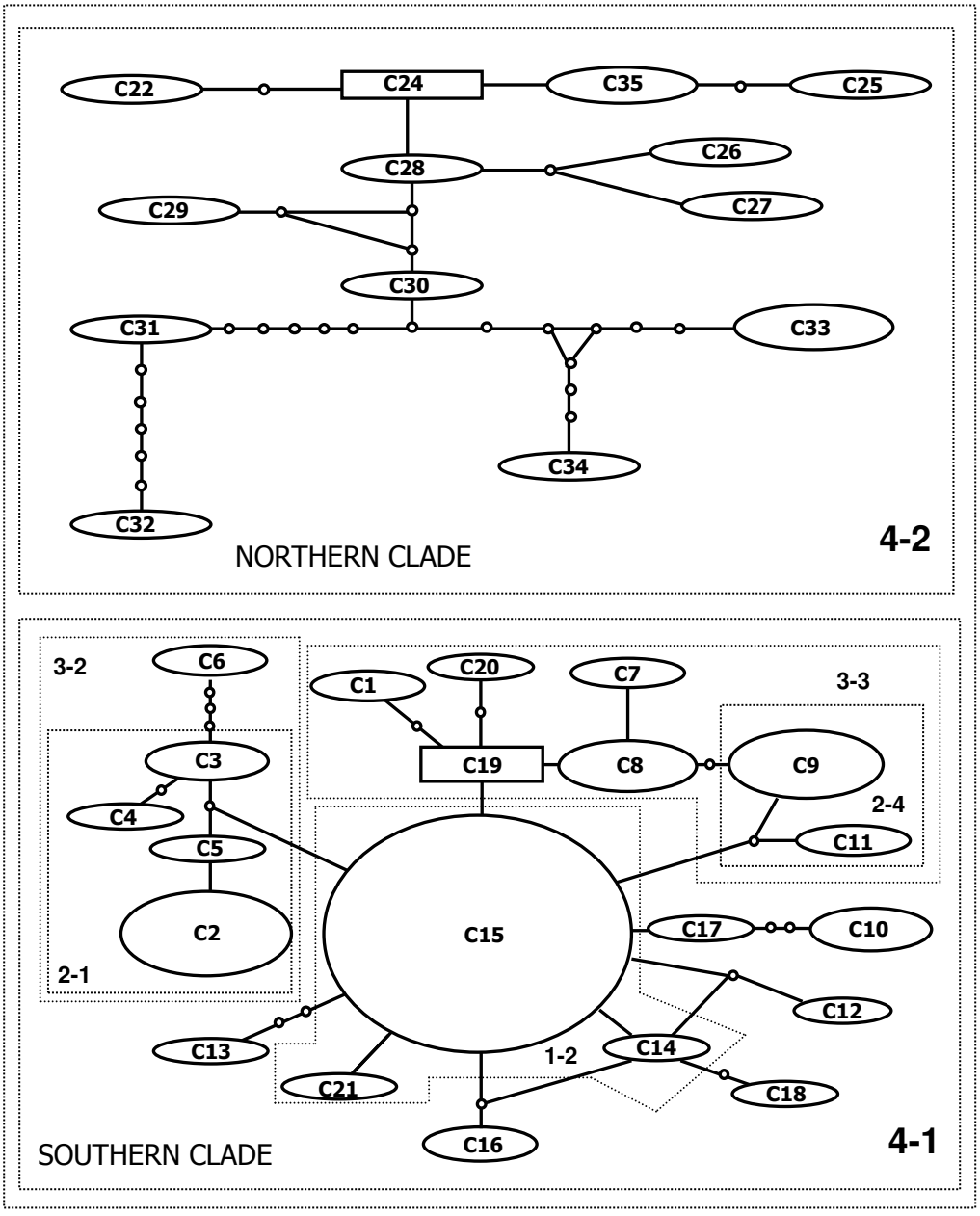
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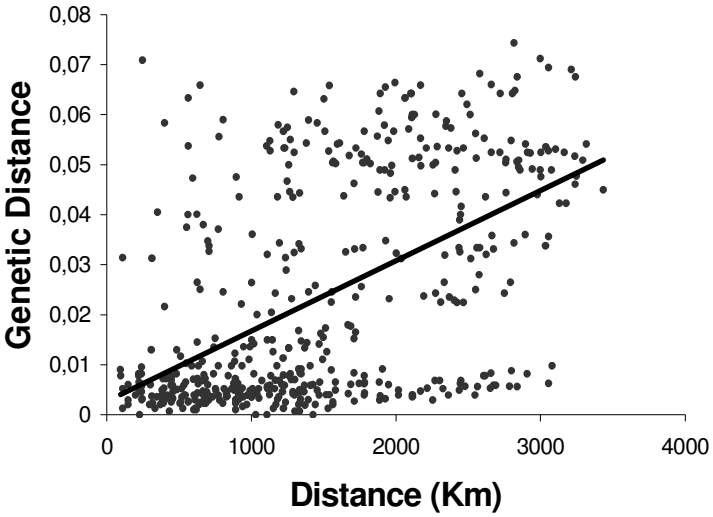
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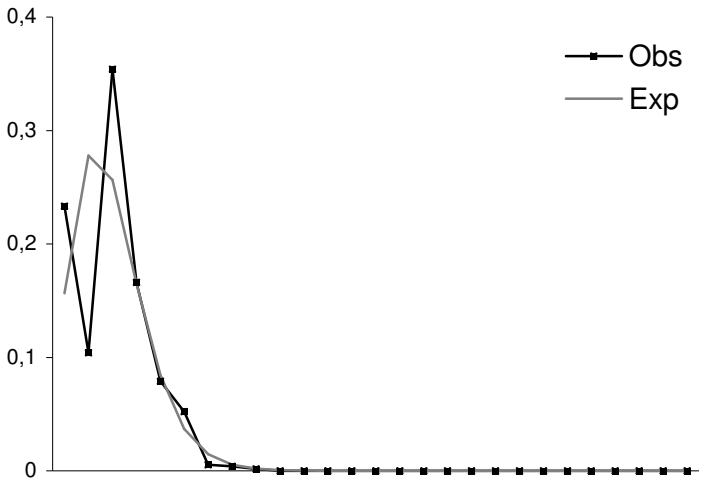
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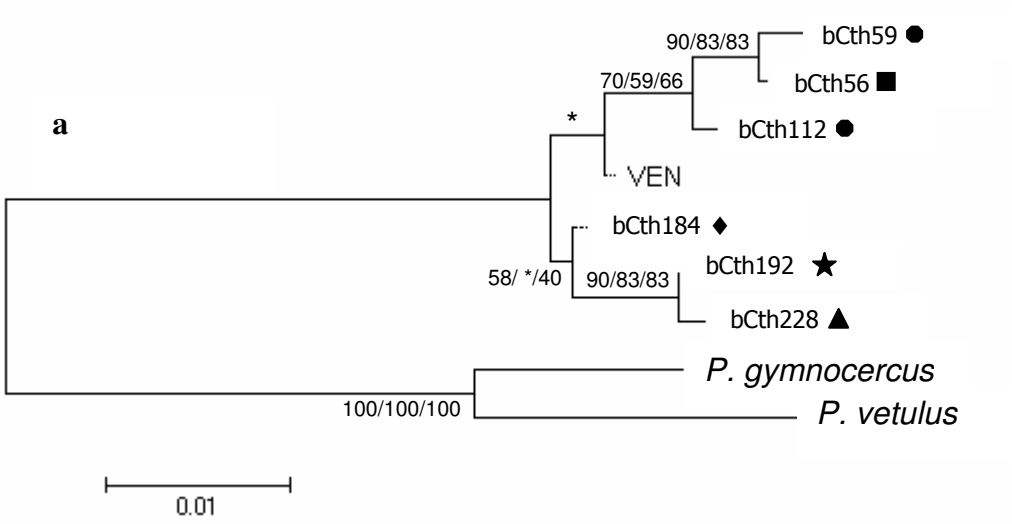
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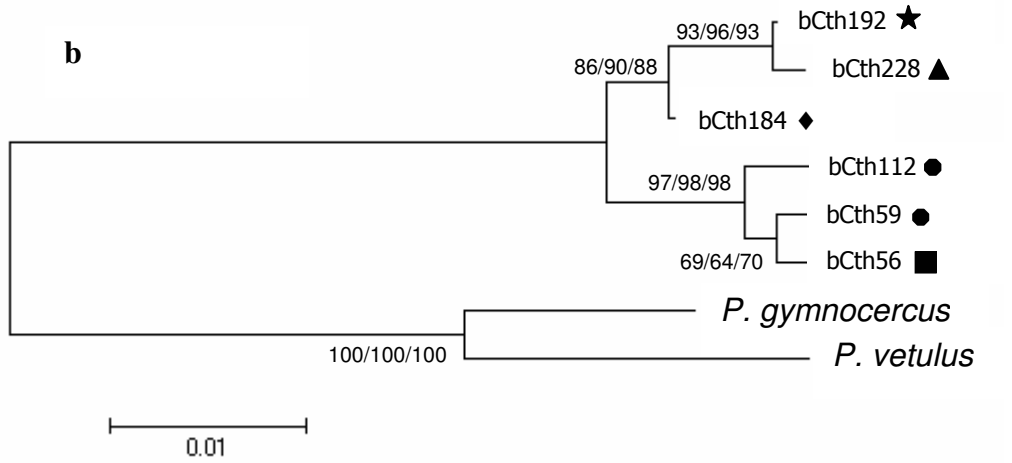
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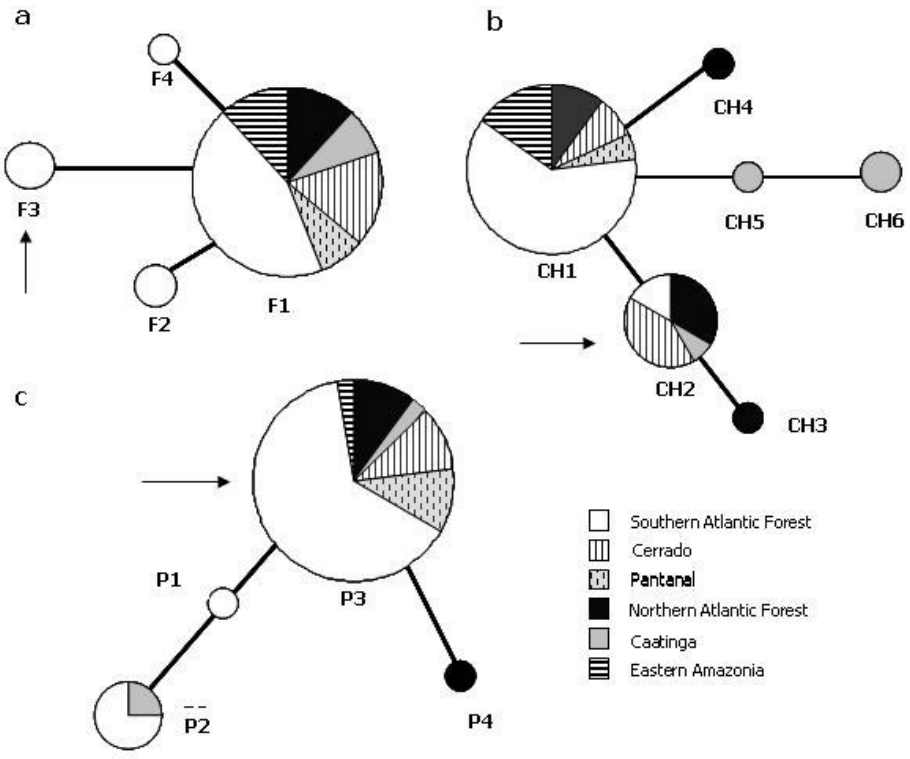
P. vetulus
0.01


a
F4
F3
F2
F1
b
CH4
CH5
CH6
CH1
CH2
CH3
c
P1
P3
P2
P4
Southern Atlantic Forest
Cerrado
Pantanal
Northern Atlantic Forest
Caatinga
Eastern Amazonia

Author Information Box

This project is part of Ligia Tchaicka's Ph.D. dissertation at the Graduate Program in Genetics and Molecular Biology of Federal University of Rio Grande do Sul (UFRGS), Brazil, where she is co-advised by Drs. Thales R.O. Freitas and Eduardo Eizirik. Her project addresses phylogeographic and population genetic aspects of South American canids, with emphasis on the genera *Cerdocyon* and *Pseudalopex*. Dr. Freitas is an evolutionary geneticist interested in diverse mammalian systems, with emphasis on Neotropical fossorial rodents. Dr. Eizirik is an evolutionary and conservation biologist focusing most of his research on Neotropical carnivores. Dr. Candido is a mammalogist interested in species occurring in Southern Brazil. Tadeu G. de Oliveira is a conservation biologist working on various species of Neotropical carnivores throughout Brazil, especially in the North of the country.

Online Supplementary Data for "Phylogeography and population history of the crab-eating fox (*Cerdocyon thous*)", by L. Tchaicka *et al.* (ME 06-034)

Contents:

1. Supplementary Table1. Mitochondrial DNA control region haplotypes identified from crab-eating fox samples (ecoregion representation indicated on the right). Only variable sites are shown. Site numbers (vertical notation) refer to the aligned position in our 512 bp data set.

	Nucleotide position 111111111111111111112222222222233333333444445 222233556890111122222333333445612244446799000011345588800 467834370554124573478912345618403014569061527892733783498 9	Fr[a]	Ecoregion
C1	CTCTCCACGATACTGTCTACCCAACTCAAATTTCTGTTCCCTCCACTACCGACCTTGT	1	PANTANAL
C2	...C.......G...........G.............C.....	11	SOUTH ATLANTIC FOREST
C3	...C.......G.C.................C....C.....	3	AMAZON
C4	...C.......G.C.................C....C...CT?????????????????	1	AMAZON
C5	...C..?....G...........G........C....C.....	1	SOUTH ATLANTIC FOREST
C6	...C......CG.C............T..G..C....C...........T.....	1	CERRADO
C7	...C..............T............C...C.....	2	CERRADO
C8	...C.............................C...C.....	6	SOUTH ATLANTIC FOREST AND CERRADO
C9	...C.......G.........T..........C...C.....	9	SOUTH ATLANTIC FOREST
C10	.C.C.......G........T...........C...C.T.....	4	SOUTH ATLANTIC FOREST
C11	...C..G....G.........T..........C.....	2	SOUTH ATLANTIC FOREST
C12	...C.....G.G.....................C...........G.....	1	PANTANAL
C13	...C.......G....T.........TG....C.....	1	SOUTH ATLANTIC FOREST
C14	...C.......G.....................C??....T.????????????????	1	SOUTH ATLANTIC FOREST
C15	...C.......G.....................C.....	37	SOUTH ATLANTIC FOREST, PANTANAL, CERRADO AND CAATINGA
C16	T..C.......G.....................C..............T.....	2	CERRADO
C17	...C.......G........T...........C.....	1	AMAZON
C18	...C.......G.....................C..A.......T..C.....	1	PANTANAL
C19	...C.............................C.....	2	AMAZON AND CERRADO
C20	...C.....................TC......C.....	1	NORTHEAST ATLANTIC FOREST
C21	...C....A..G.....................C.....	1	SOUTH ATLANTIC FOREST
C22	....TT.T..C...A.T.G.T.......G.C.CTCT....T..T.T?GT..GT..CAC	1	NORTHEAST ATLANTIC FOREST
C23	....TT.T......A.T.G.T.........C..TCT....TCT?????????????????	1	CERRADO
C24	??????GT..C...A.T.G.T.........C.CTCT....T..T.TCGT..GT..CAC	1	CAATINGA
C25	....TTGT..C...ACT.G.T.........C.CTCT....TCT?????????????????	1	AMAZON
C26	....TTGT........T.G.T....C....C.CTCT....T..T.TCGT..GT..CAC	1	AMAZON
C27	....TTGT......A.T.G.T.G..C....C.CTCT....T..T.TCGT..GT..CAC	1	AMAZON
C28	....TTGT......A.T.G.T.........C.CTCT....T..T.TCGT..GT..CAC	1	NORTHEAST ATLANTIC FOREST
C29	....TTGT....T.A.T.G.T.........C..TCT.C..T..T.TCGT..GT..???	1	NORTHEAST ATLANTIC FOREST
C30	....TTGT....T.A.T.G.TT.........C.?TCT...?T.??.T??...GT?.CAC	1	PANTANAL
C31	....TT.T..C...A.T....T.........C.CTCT...........G...GT..CAC	1	AMAZON
C32	..T?TT.T..C...A.TC...T.........C.CT.T.......T.T.G.T.GT..CAC	1	CAATINGA
C33	....TTGT......A.TC..TT.........CC.TCT..T....T.TCG.T.GT..CAC	3	CAATINGA
C34	....TTGT......A.TC...T.........C..TCT....T..T...G.TAGT..???	1	CAATINGA
C35	....TTGT..C...ACT.G.T.........C.CTCT....T..T.TCGT..GTTCCAC	2	NORTHEAST ATLANTIC FOREST

[a] Absolute frequence on the total sample

2. Supplementary Table 2. Phylogeographic interpretations derived from the Nested Clade

Clade	Chain of inference	Inference
Clade 1-2	1-2-3-4	Restricted gene flow with isolation by distance
Clade 2-1	1-2-3-4-9	Allopatric fragmentation
Clade 2-4	1-19-20-2-11-12	Range Expansion/Continuous range expansion
Clade 3-2	1-19-20-2-3-4-9-10	Allopatric fragmentation
Clade 3-3	1-2-3-4-9	Allopatric fragmentation
Clade 4-1	1-2-11	Range Expansion/Continuous range expansion
Clade 4-2	1-2-11-17-4	Restricted gene flow with isolation by distance
Total Cladogram	1-2-11-17-4-9	Allopatric fragmentation

Distance analysis of the *Cerdocyon thous* mtDNA control region (see Fig.3).

3. Summary of output files from the coalescent-based analyses performed with the Isolation-with-migration model implemented in the program IM.

Final Run 1:

```
Command line string :  -i controlgeoim.u -o Cerd6.out -b300000 -l10000000 -k4 -n4
-m110 -m210 -t20 -q110 -u 2 -a 20 -p5

MARGINAL HISTOGRAMS
---------------------------------
Summaries
                 q1       q2            qA             t             m1             m2
Minbin         3.7598   8.0648        0.0287        0.3700         0.0050         0.0050
Maxbin        57.1999  57.3721       57.3721       19.9900         6.5750         6.4550
HiPt          18.7414  40.4389       14.6085        1.3700         0.1550         0.2850
HiSmth        18.6840  41.4721       15.8139        1.4100         0.1650         0.2550
Mean          20.0616  41.1851       25.5147        7.9500         0.2750         0.4050
95Lo          10.5905  21.5540        1.5211        1.0900         0.0250         0.0650
95Hi          36.0764  56.3963       55.5353       19.3900         1.1850         1.5250
Tail?        Complete  falling         flat          flat         complete       complete

MARGINAL HISTOGRAMS IN DEMOGRAPHIC UNITS
       Calculations use mutation rates (in years) and generation time (in years) input at
runtime
       Parameter      Meaning Units
       q1      Pop1 Ne Individuals
       q2      Pop2 Ne Individuals
       qa      Ancest.Pop Ne  Individuals
       t       Years Div.     Years
       Generation time in years specified at runtime: 2.000000
       Geometric mean of mutation rates per year (based on rates specified in input file):
1.884000e-005
       Geometric mean of ML estimates of relevant mutation rate scalars: 1.000000e+000
---------------------------------
 Summaries
                      q1                 q2          qA              t
       Minbin        24945.2855       53508.5896    190.4220        19639.0658
       Maxbin        379511.1000     380653.6321    380653.6321   1061040.3397
       HiPt          124345.5837     268304.6362    96924.8118      72717.6221
       HiSmth        123964.7396     275159.8291    104922.5369     74840.7643
       Mean          133104.9969     273255.6089    169285.1821    421974.5223
       95Lo          70265.7280      143006.9423    10092.3674      57855.6263
       95Hi          239360.4880     374179.2832    368466.6224   1029193.2059
       Tail?         complete        falling             flat          flat
```

Final Run 2:

```
Command line string :  -i controlgeoim.u -o Cerd7.out -b100000 -l3000000 -k10 -n10 -m110 -
m210 -t20 -q110 -u 2 -a 20 -p5

MARGINAL HISTOGRAMS
---------------------------------
 Summaries
                      q1             q2             qA             t              m1
       m2
       Minbin     4.9652         7.0890         0.0287         0.5500         0.0050
0.0050
       Maxbin    57.2573        57.3721        57.3721        19.9900         4.5350
4.3450
       HiPt      19.6024        46.9826        13.9197         1.6700         0.1450
0.2250
       HiSmth    18.8562        46.1790        14.7807         1.7500         0.1850
0.2550
       Mean      19.8894        41.7591        25.2851         6.9700         0.3250
0.4050
       95Lo       9.3276        21.8410         1.7507         1.2700         0.0250
0.0450
       95Hi      36.1338        56.5111        55.4779        19.2900         1.6150
1.4050
       Tail?    complete      falling         rising          flat        complete
complete
```

MARGINAL HISTOGRAMS IN DEMOGRAPHIC UNITS
(settings are the same as above)
Summaries

	q1	q2	qA	t
Minbin	32943.0107	47034.2407	190.4220	29193.2059
Maxbin	379891.9440	380653.6321	380653.6321	1061040.3397
HiPt	130058.2445	311720.8583	92354.6831	88641.1890
HiSmth	125107.2718	306389.0416	98067.3439	92887.4735
Mean	131962.4648	277064.0494	167761.8059	369957.5372
95Lo	61887.1588	144911.1626	11615.7437	67409.7665
95Hi	239741.3321	374940.9713	368085.7783	1023885.3503
Tail?	complete	falling	rising	flat

Capítulo IV

2º ARTIGO

a ser submetido para publicação na revista Journal of Mammalogy

POPULATION GENETIC STRUCTURE OF THE CRAB-EATING FOX (*Cerdocyon thous*) INFERRED FROM MICROSATELLITE *LOCI*

POPULATION GENETIC STRUCTURE OF THE CRAB-EATING FOX (*Cerdocyon thous*) INFERRED FROM MICROSATELLITE *LOCI*

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Key words: *Cerdocyon*, genetic structure, microsatellites

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High levels of gene flow are expected in generalist species such as the crab-eating fox, *Cerdocyon thous*. This canid ranges through nearly all of South America, and is found in a variety of vegetation domains, including human-impacted areas. A previous study using mitochondrial DNA segments indicated strong phylogeographic partitioning between the northeastern and southern parts of the species' distribution, which was not supported by nuclear intron sequence data. In the present study, we investigated ten microsatellite *loci*, which showed high levels of polymorphism in this species, and no indication of genetic structuring throughout Brazil. This result corroborates the hypothesis that male-biased gene flow has been a major factor leading to the erosion of a historical pattern of geographic subdivision in this species.

Canids in general are excellent dispersers: individuals are not strongly limited by topographic or habitat barriers, and may range over several hundred kilometers during their lifetime (Wayne 1996). Species with this pattern of movement tend to show relatively little genetic differentiation among populations, and their genetic structure is observable only in fine-scale studies (Geffen et al. 2004; Lehman and Wayne 1991; Roy et al. 1994; Sacks et al. 2004). In contrast, some species of small canids with more limited vagility may be composed of different genetic units (e.g. Mercure et al. 1993).

High levels of gene flow would be predicted for habitat generalists with continuous distributions, such as the crab-eating fox. This fox is among the most versatile of canids, as evidenced by its ability to exploit a wide variety of habitat types and food sources (Courtenay and Maffei 2004). *Cerdocyon thous* ranges through almost all of South America (Fig. 1). It is found in tropical and subtropical forests, forest edges, open woodlands, savannas, and human-impacted areas (Berta 1982, 1987; Langguth 1975; Medel and Jaksic 1988). Individuals are opportunistic hunters and dietary generalists, eating fruits, eggs, crabs, small mammals and insects (Juarez and Marinho-Filho 2002; Macdonald and Courtenay 1996).

Social groups of the crab-eating fox are composed of a couple and sometimes their juvenile or adult offspring, occupying territories that partially overlap. Detailed field studies of dispersal patterns are still scarce. The little available data suggest that both male and female juveniles disperse, while maintaining long-term interactions with their neighboring parents (Macdonald and Courtenay 1996).

Insights into numerous aspects of species biology can be provided by assessing the pattern of distribution of genetic variation. Previous phylogeographic research conducted for *C. thous* using mitochondrial DNA control region sequences indicated a strong genetic differentiation between northern and southern populations in Brazil: these groups were defined by two historical lineages that diverged *ca.* 400,000 - 600,000 years ago. In contrast, sequences of

three nuclear introns suggested no structure among regions (Tchaicka et al. in press; [ver Capítulo III]). Coalescent-based analyses performed in that study suggested that male-biased gene flow was a relevant factor potentially explaining the lack of structuring in the nuclear data set. Alternative hypotheses were the longer coalescent times for these loci, and their slower mutation rates relative to the mtDNA control region segment. Both of these factors might also have prevented the nuclear intron sequence data from capturing the historical subdivision process inferred from the mitochondrial segment. To further test these hypotheses, it is important to investigate nuclear markers that exhibit different evolutionary properties from the previously analyzed introns, such as faster mutation rates.

In this study, we assessed the pattern of genetic variation in free-ranging *Cerdocyon thous* populations using 10 microsatellite *loci*. These markers were found to be highly variable in this species, and provided adequate information levels for testing the hypothesis of no population structuring based on nuclear DNA.

MATERIALS AND METHODS

Biological material was obtained from 82 individuals, sampled at 17 sites across most of the species' range. To explore regional-level patterns of population structure, we selected three sites in Southern Brazil (MS, PR and RS) to perform more extensive sampling, including 16-19 individuals from each locale (Fig. 1 and Table 1).

Genomic DNA was extracted using a standard phenol/chloroform protocol (Sambrook et al. 1989) from blood samples collected from captive individuals and wild animals captured for ecological studies (DNA preserved in a salt-saturated solution; 100 mM Tris, 100 mM EDTA, 2% SDS), and muscle or skin tissue collected from road-killed individuals. Ten tetranucleotide microsatellites *loci* originally described by Francisco et al. (1996) for the domestic dog (*Canis familiaris*), and subsequently selected for optimized applicability in Neotropical canids (Rodrigues et al. in preparation), were employed in this study: 2100, 2006, 2054, 2004, 2001, 2010, 2132, 2137, 2140, 2088. Each primer pairs were linked to

a standardized M13 fluorescent-labeled tail added to its 5' end (Boutin-Ganache et al. 2001) and used in a flexible three-primer PCR reaction.

Polymerase chain reactions were performed in mixtures containing 2 μl of 10X buffer, 1.5 mM $MgCl_2$, 0.2 μM dNTPs, 0.2 μM each of the reverse primer and the fluorescent M13 primer, 0.013 μM of the forward primer, 0.75 unit Taq polymerase and 1-3 μl of template DNA. Thermocycling conditions began with 10 "touchdown" cycles, which each had a 30s denaturing step at 94ºC, 30s annealing at 60-51ºC, and 45s extension at 72 ºC. This was followed by 30 cycles of 30s denaturing at 94 ºC, 30s annealing at 50ºC and 45s extension at 72ºC.

The genotyping of the PCR products was performed in a MegaBACE 1000 (GE Healthcare) automatic sequencer, using the GENETIC PROFILER 1.5 software (GE Healthcare) and an internal size standard (ET Rox-400, GE Healthcare). Initial verification of possible genotype errors during data recording was performed with MICROCHECKER 2.2.3 (Oosterhout et al. 2004).

FSTAT 2.9.3.2 (Goudet 2002) was used to test the occurrence of linkage disequilibrium (153,000 permutations), and also to calculate allele frequencies, gene diversity (the probability that two randomly chosen alleles are different in the sample; Nei 1987), number of sampled alleles in each site, and observed heterozygosity values. A Hardy-Weinberg Equilibrium (HWE) exact test implemented by the Markov chain method was performed with GENEPOP web version 3.1c (Raymond and Rousset 1995).

To investigate the population structure across the sampled region, we used STRUCTURE 2.1 (Pritchard et al. 2000), a Bayesian model-based approach. The analyses were implemented in the population admixture model without prior geographic information. Probabilities of 1 to 5 and 17 clusters (K, i.e. groups of individuals that probably belong to the same population) were tested in two different sets of analyses: (i) a data set with equivalent number of individuals for all sites (i.e. using only 6 individuals for RS, PR and MS, and all sampled individuals for the other sites); and (ii) using all 82 individuals obtained in this study. Each run was replicated 10 times, with 100,000 steps of sampling following 100,000 generations of burn-in. A third set of analyses was performed focusing only in the RS, PR and MS sample-sites (using all sampled individuals from these locales; n=16, n=19 and n=17, respectively). In this case, we tested the fit of the data to

models with 1 to 5 different clusters; for these runs the MCMC replicates after burn-in were modified to 150,000.

An analysis of molecular variance (AMOVA, Excoffier et al. 1992) and estimates of population subdivision through F_{ST} indices (conventional F_{ST} statistics) were obtained with ARLEQUIN 2.000 (Schneider et al. 2000) and the statistical significance of these values was tested using 10,000 permutations in the same software.

RESULTS

All *loci* were polymorphic, with allele counts ranging from 4 to 24, average observed heterozygosity of 0.645, and high gene diversity, with a mean of 0.757 for all *loci* (Tables 2 and 3). Three of ten microsatellite *loci*, 2132, 2137 and 2140, exhibited a dinucleotide repeat pattern; all others behaved as tetranucleotide *loci*, as originally described in *C. familiaris*. Allele frequencies and allele distribution by sample site are shown in Figs. 2a-j.

After applying a sequential Bonferroni correction (Rice 1989), no linkage disequilibrium was observed between *loci* (global and intra-sample analysis; corrected $\alpha = 0.000065$). Under this same correction ($\alpha = 0.005$), a heterozygote deficit was detected in PR and MS sample sites, based on the Hardy-Weinberg test conducted for each sample using all *loci* ($p < 0.00001$). Testing for HWE in each single *locus* in these two sample sites, we detected a heterozygote deficit only for locus 2137 ($p< 0.00001$), which also showed departure from HWE in the global analysis of sample sites ($p<0.00001$). Exclusion of locus 2137 from the PR and MS populations, however, maintained the heterozygote deficit ($p=0.0008$ and $p=0.004$ respectively). Comparative subsequent analyses of the population structure without *locus* 2137 were conducted, and it was eventually kept in the data set because its exclusion did not affect the results.

The Bayesian approach implemented in STRUCTURE using equivalent samples for all sites indicated as most probable the existence of a single population cluster (Ln Prob -1490 to -1505 on 10 runs), suggesting that there is no genetic structure among all 17 sample sites (in this analysis, the MS, PR and RS sites were each represented by 6 individuals, similar to the sample size from the

other locales; see Materials and Methods). This same analysis, when performed using all 82 individuals, resulted in the highest probability values for K=2 and K=3 (Ln Prob – 2575 to – 2591 and Ln Prob – 2542 to – 2590, respectively), indicating that RS is a differentiated cluster for both K=2 and K=3. In the latter case, the other two clusters did not correspond to identifiable geographic locations. The search using only MS, PR and RS (all sampled individuals from these sites; see Materials and Methods) resulted in very similar probabilities for K=2 and 3 (with the upper and lower limits of Ln estimation overlapping; from Ln -1582 to Ln -1629). The K=2 analysis indicated that PR and MS are a single cluster, with only one individual in RS being inferred to be a migrant from this region. The K=3 exercise correctly assigned all RS individuals to this population, and suggested some level of structuring between PR and MS, although several individuals were inferred to be admixed between these two areas.

The estimated fixation index produced concordant results and confirmed the STRUCTURE inference. When we analyzed all 17 sample sites as different populations (all 82 individuals), low values of F_{ST}=0.00041 (from AMOVA) among units were obtained. In the pairwise comparisons of sample sites, genetic differentiation was observed only between RS and the other 16 sites, with F_{ST} = 0.001 (for all RS pairs) significant for almost all the site pairs ($p<0.05$), including PR but not MS. This value of RS differentiation was lost (F_{ST}=0) when we used an equivalent number of individuals for RS, PR, MS and the other sites. Considering only these three major samples, RS, PR and MS, the calculated index among populations was F_{ST}= 0.0005 (p=0.08; AMOVA).

Alternative population schemes were also tested, grouping samples by ecoregions (major vegetation domains, the southern Atlantic Forest region including marginal portions of adjacent biomes of Chaco and southern grasslands), northeastern X southern group detected with mtDNA data (Tchaicka et al. in press [ver Capítulo III]), with and without the Eastern Amazonia sample (PA; which shows the highest genetic diversity, Tchaicka et al. in press). For all these analyses, the estimated F_{ST} values were zero.

In the AMOVA analysis, 99.96% of the genetic variation was found to be within each local samples; comparing only RS, PR and MS, 99.94% of variation was found within local samples. Values of the F_{IS} index were high only for the BA

sample site (0.455), and low to negative for the other sample sites, as listed in Table 4.

DISCUSSION

The microsatellite *loci* used here were found to be highly polymorphic (average gene diversity of 0.757) in *C. thous*, and are thus informative markers for populational studies in this species. Observed levels of variability, such as 4-24 alleles per locus and average heterozygosity of 0.64 across loci, are comparable to those obtained for coyotes and American wolves by Roy et al. (1994), who reported 4-20 alleles per locus and average heterozygosis of 0.65 for both species (using different *loci*). These diversity levels are likely influenced by high mutation rates at these loci, as well as characteristics of the species' life history and evolutionary past, including large effective population sizes that directly affect genetic variability. Species with large effective population sizes, such as wolves, coyotes and probably *Cerdocyon*, are less influenced by genetic drift and maintain high diversity (Amos e Balmford 2001).

Genetic indications of effective population sizes can be compared to data from field population censuses. For *Cerdocyon*, no precise estimates are available, but populations are generally considered to be quite large. Average densities include 0.55 animals per km² in the savanna-scrub mosaic of Marajó Island, Brazil; 4 individuals /km² in the Venezuelan llanos, and 1 individual /km² in dry forest in Santa Cruz, Bolivia (Courtenay and Maffei 2004).

Previous genetic studies have already indicated that *C. thous* possesses high levels of genetic variability, as suggested by gene diversity estimates of 0.83 ± 0.032 for mtDNA control region sequences, and 0.13 ± 0.061 to 0.20 ± 0.06 for three nuclear intronic segments (Tchaicka et al. in press [ver capitulo III]). In the same study, discrepant patterns of nucleotide diversity (π) for mtDNA were found between northern (π=0.021±0.001) and southern (π = 0.008 ±0.0008) population groups. This was also observed in the coalescent-based estimates θ for both mtDNA (South: 0.025 [0.015 – 0.044]; North: 0.126 [0.037 – 0,395]) and nuclear intron data (South - 0.008 [0.002 – 0.804] and North 0.032 [0.037 – 1.115] respectively); which were inferred to derive from contrasting population histories in

these regions. Data from the microsatellite *loci* indicated no considerable differences in diversity between these regions (mean gene diversity estimates of 0.64 and 0.70 for the northern and southern groups, respectively) or between sample-sites (Table 4).

A striking aspect observed in this previous study was that the mitochondrial DNA data revealed a strong geographic partition between northeastern Brazil and other parts of the species' distribution. F_{ST} values estimated between these areas were $\geq$ 0.68, and the phylogenetic analysis defined two monophyletic lineages occurring in a mostly non-overlapping phylogeographic fashion.

Despite the occurrence of exclusive alleles within the northern and southern regions (Figs. 2a-j) microsatellite data failed to support such a subdivision. The Bayesian approach implemented in STRUCTURE, as well as the F_{ST}–based inferences did not indicate the existence of geographical partitions, old population subdivision events, or barriers to gene flow across the species' range.

Comparing characteristics of mtDNA and intron sequences, discordant results can be attributed to slow accumulation of mutations and larger effective size of introns that delay the effect of genetic drift (Hare 2001). Microsatellite *loci*, however, show high mutation rates, which can promote more rapid differentiation between populations. Since mitochondrial markers have certain limitations because of their matrilineal inheritance (Avise 2000), the microsatellite - mtDNA discrepancy could be explained by the hypothesis that, in *Cerdocyon,* males disperse more often and/or farther than females.

No specific field study addressing this question has been performed so far, but available data (Macdonald and Courtenay 1996) reports dispersal of four juvenile males and one adult female. These juvenile males formed pairs with similar-aged females, who the authors suggest are also probably dispersers.

Our present data supports the view that males disperse more than females, which should now be tested in more detailed field studies addressing this issue.

To evaluate the effect of sampling on inferences of regional-level genetic structuring in *C. thous*, we selected three of our sites (PR, RS and MS - see Fig. 1 and Methods) to perform a more detailed assessment. These areas are thought to have been connected via continuous Atlantic Forest cover in the recent past, perhaps up to the end of the XXth Century. The PR sample site is in a central

position relative to the other two locales: it is *ca.* 650 km away from RS, and *ca.* 450 Km away from MS (see Fig. 1). The PR and RS are part of a formerly continuous Atlantic Forest biome spanning most of Southern Brazil, while the MS site lay on the western edge of this ecoregion, at the interface with more open habitats, but still fully connected to the other two locations in terms of suitable habitats for *C. thous*. Presently, the three sites are nearly completely separated by heavily impacted areas (e.g. urban environments, soy beans plantations and other agricultural areas), although the effects of such anthropogenic disturbances on *C. thous* demography have not yet been assessed. *C. thous* seems to adapt reasonably well to disturbed habitats, and may therefore remain continuously distributed across these regions. This has not been tested directly throughout the landscapes separating our sample sites. The MS site currently consists of patches of Atlantic Forest native vegetation interspersed with agricultural areas dominated by cattle ranching. The RS and PR sites are conservation units where the native Atlantic Forest biome still remains rather well preserved: RS is 5,000-ha Itapuã State Park; and PR is 185,000-ha Iguaçu National Park, which is adjacent to a large tract of additional conserved areas in Argentina.

Only a small degree of genetic structuring between RS and the other two sites was inferred from the F_{ST} pairwise analysis of these three sites, as also observed in the analyses including all other sites (Fst=0.001). STRUCTURE analysis failed to detect this partition in the global analysis, but clearly indicates this pattern on MS/PR/RS search (see results). The genetic similarities between MS and PR are probably due to the more recent fragmentation by agricultural development between these sites than between PR and RS.

Some species for which initial, large-scale, studies indicated no structure, when observed in detailed molecular investigations, with larger sample sizes, have shown some degree of genetic differentiation among sites, as observed in American wolves and coyotes (Geffen et al. 2004; Sacks et al. 2004; Vilà et al. 1999).

The departures from Hardy-Weinberg equilibrium inferred for PR and MS could be caused by the hypothesis of null alleles presence (inferred from heterozigosity deficits, see Results and Table 2) on 2137 *locus*. However, the deviations were maintained when this *locus* was excluded from the analysis.

Adding the observation of higher F_{IS} estimated from PR and RS than from RS (negative F_{IS}), we could explain this by either limited inbreeding within populations, or by a nonrandom sample of individuals within populations, which may be composed of family groups in PR and MS (Walhund Effect, Hartl e Clark 1997).

Intra-specific comparisons using molecular data for canid species have demonstrated how differences in dispersal ability, ecological constraints, historical demography and the nature extrinsic of barriers influence the degree of genetic structure within this group. The inferences from microsatellite data presented herein contribute to knowledge of the life history of *Cerdocyon thous*, and provide a basis for more detailed population-level studies in this species.

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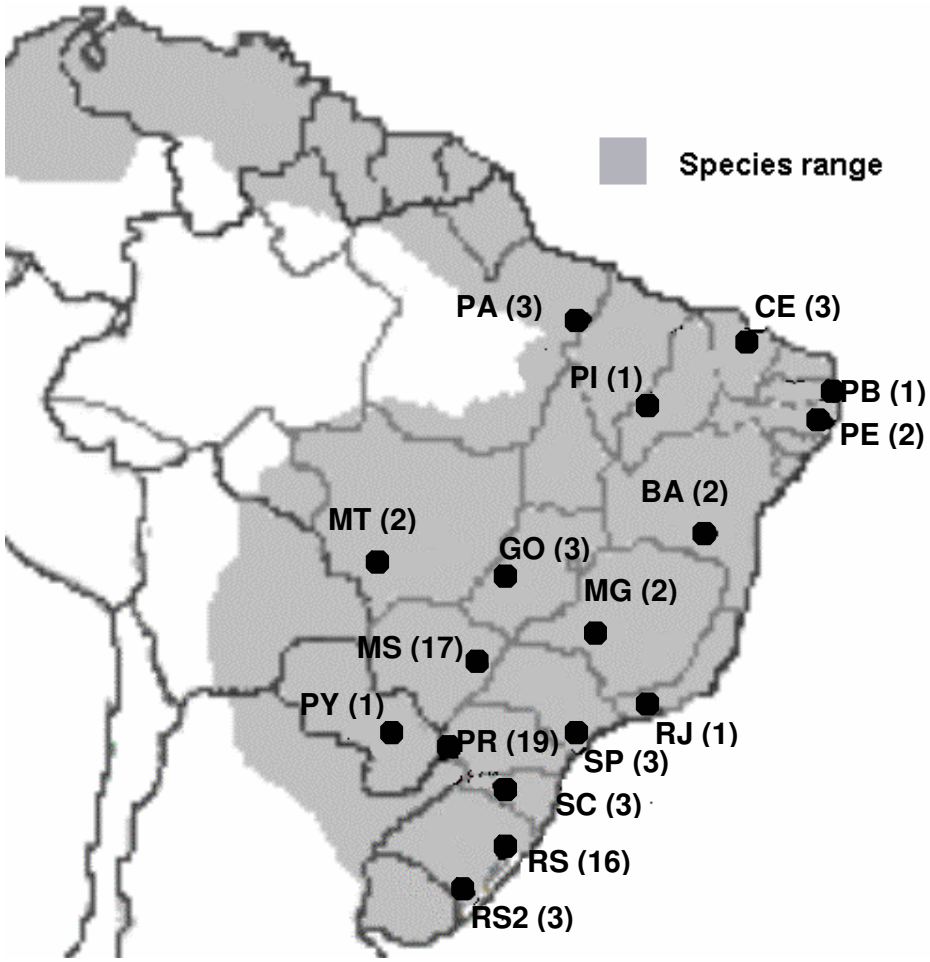
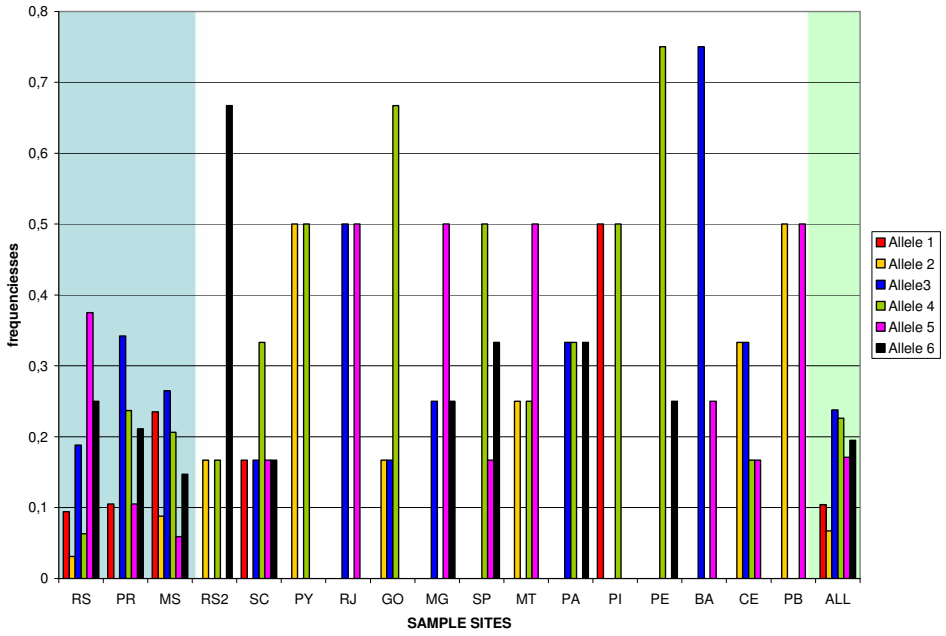
Fig. 1. — Map showing the current geographic distribution of *Cerdocyon thous* (modified from Sillero-Zubiri et al. 2004) and approximate sample collection sites, as coded in Table1, with sample sizes between parentheses.

Fig. 2. — Allele frequency distribution for each microsatellite locus analyzed in this study (a-j), for each of the 17 sampling sites (blue area comprises the three locales with larger sample sizes [RS, PR and MS]), and total frequency of alleles for each locus for the complete data set ("all" - green area on the right).
a) *Locus* 2100; b) *Locus* 2006; c) *Locus* 2054; d) *Locus* 2004; e) *Locus* 2001; f) *Locus* 2010; g) *Locus* 2132; h) *Locus* 2088; i) *Locus* 2137; j) *Locus* 2140.


Species range
PA (3)
CE (3)
PI (1)
PB (1)
PE (2)
BA (2)
MT (2)
GO (3)
MG (2)
MS (17)
PY (1)
RJ (1)
PR (19)
SP (3)
SC (3)
RS (16)
RS2 (3)

a)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frequenciesses
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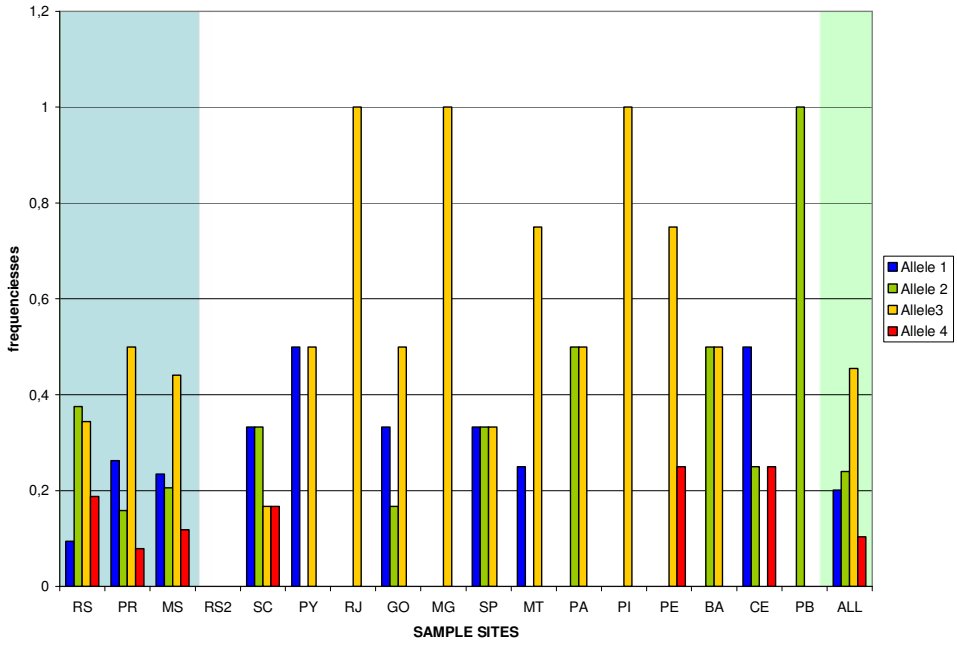
SAMPLE SITES
Allele 1
Allele 2
Allele3
Allele 4
Allele 5
Allele 6

b)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frequenciesses
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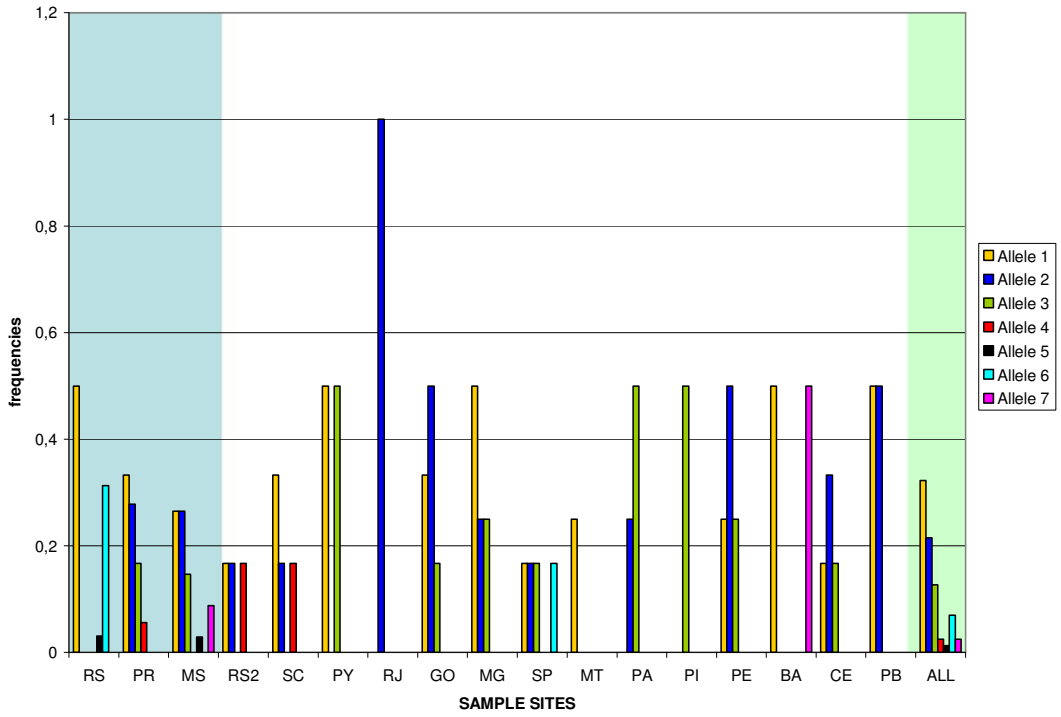
SAMPLE SITES
Allele 1
Allele 2
Allele3
Allele 4

c)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BA
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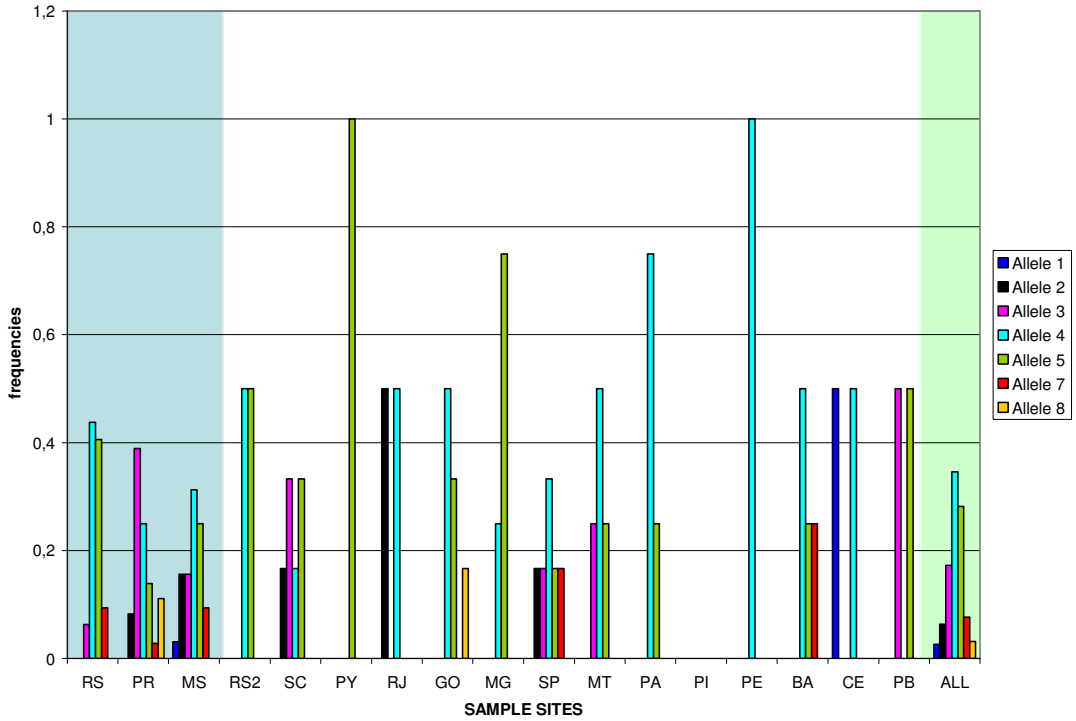
SAMPLE SITES
Allele 1
Allele 2
Allele 3
Allele 4
Allele 5
Allele 6
Allele 7

d)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frequencies
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BA
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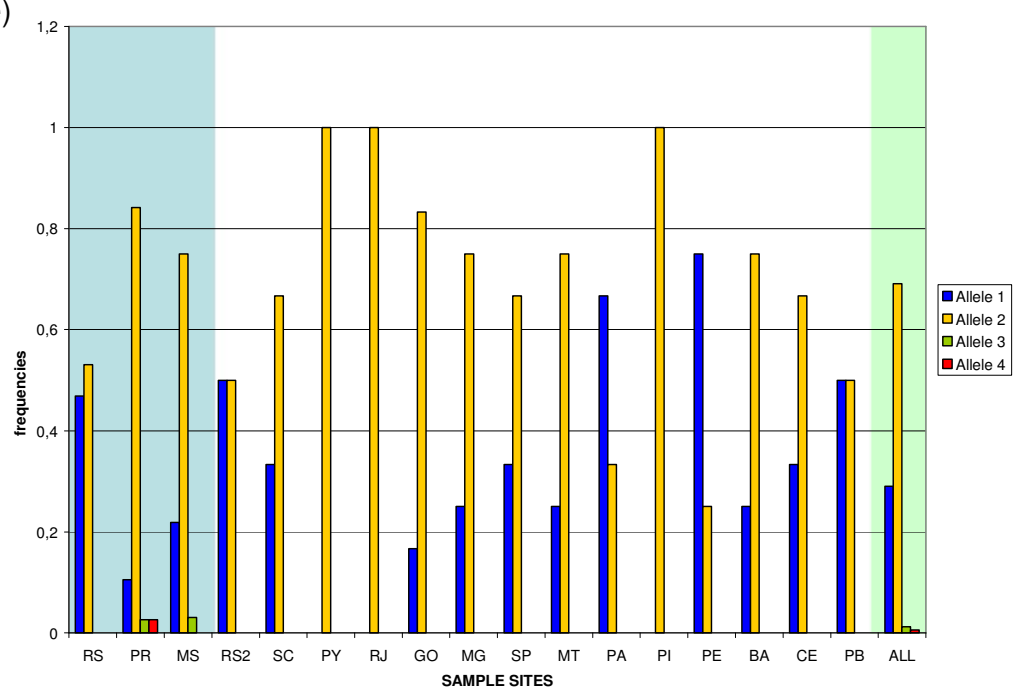
ALL
SAMPLE SITES
Allele 1
Allele 2
Allele 3
Allele 4
Allele 5
Allele 7
Allele 8

e)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SAMPLE SITES
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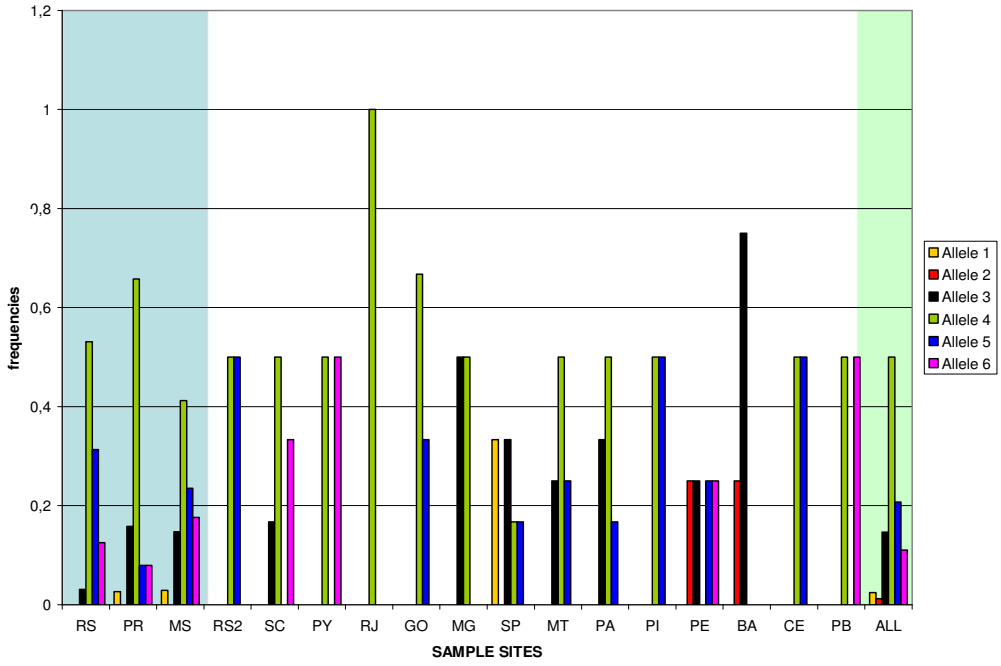
Allele 2
Allele 3
Allele 4

f)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frequencies
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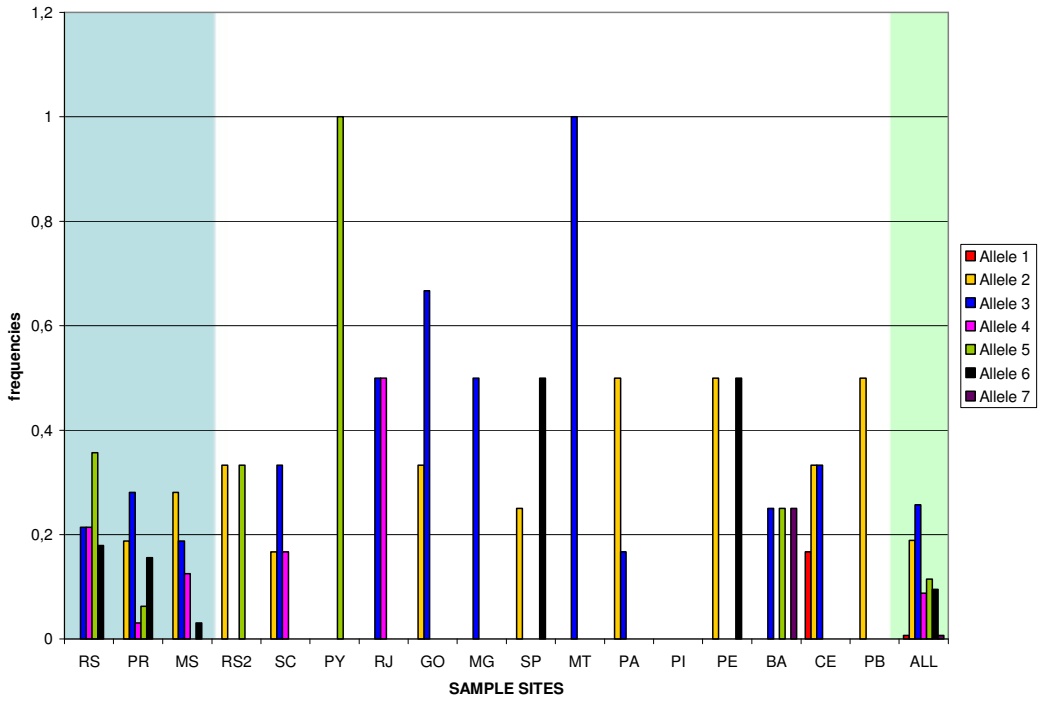
g)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SAMPLE SITES
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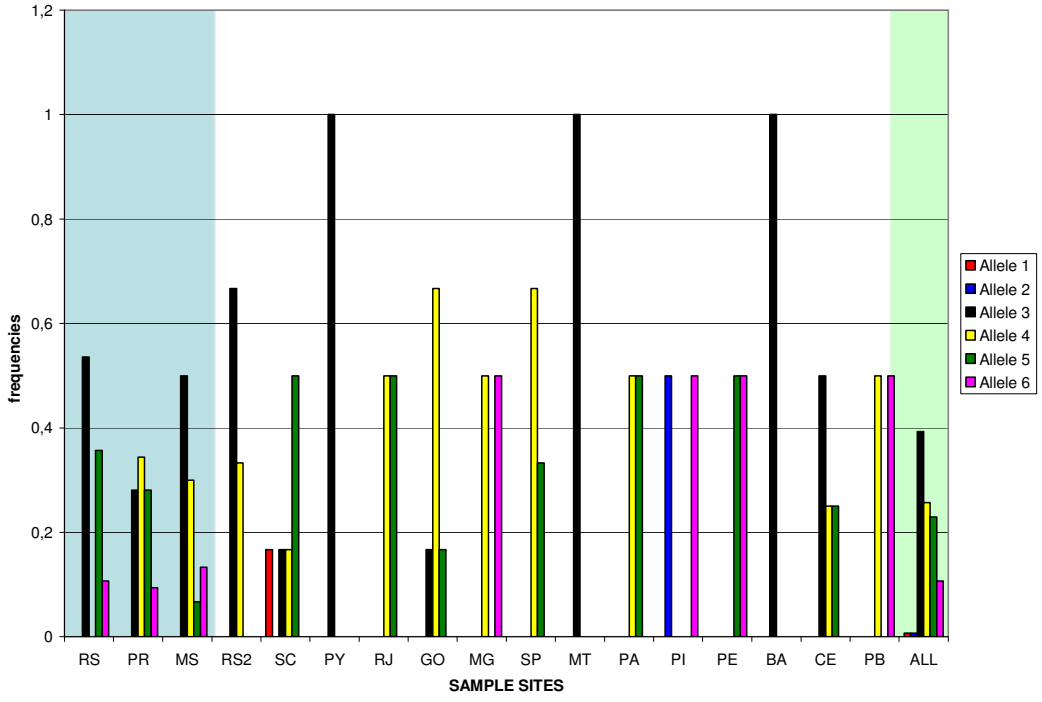
Allele 7

h)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SAMPLE SITES
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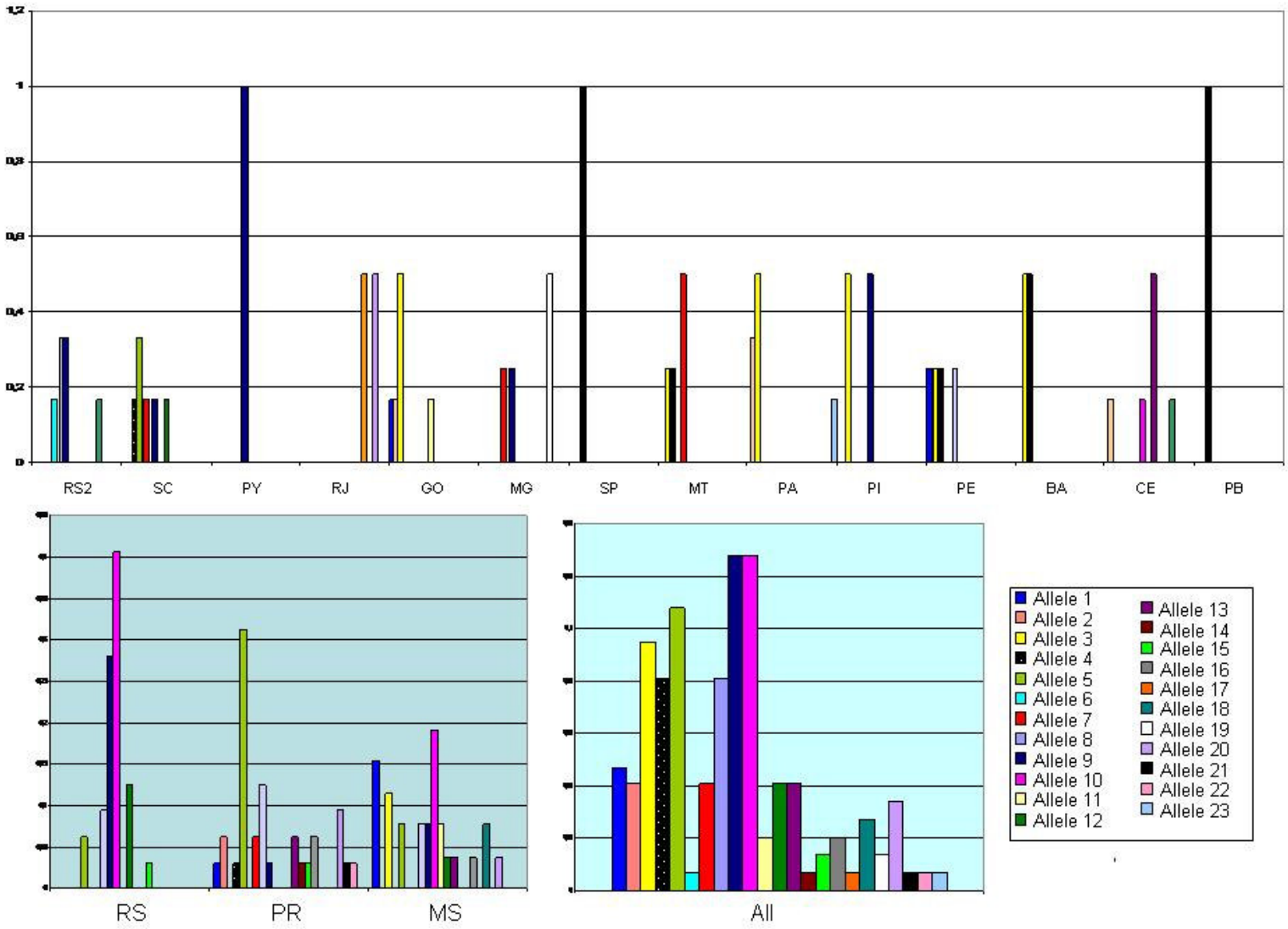
Allele 6

i)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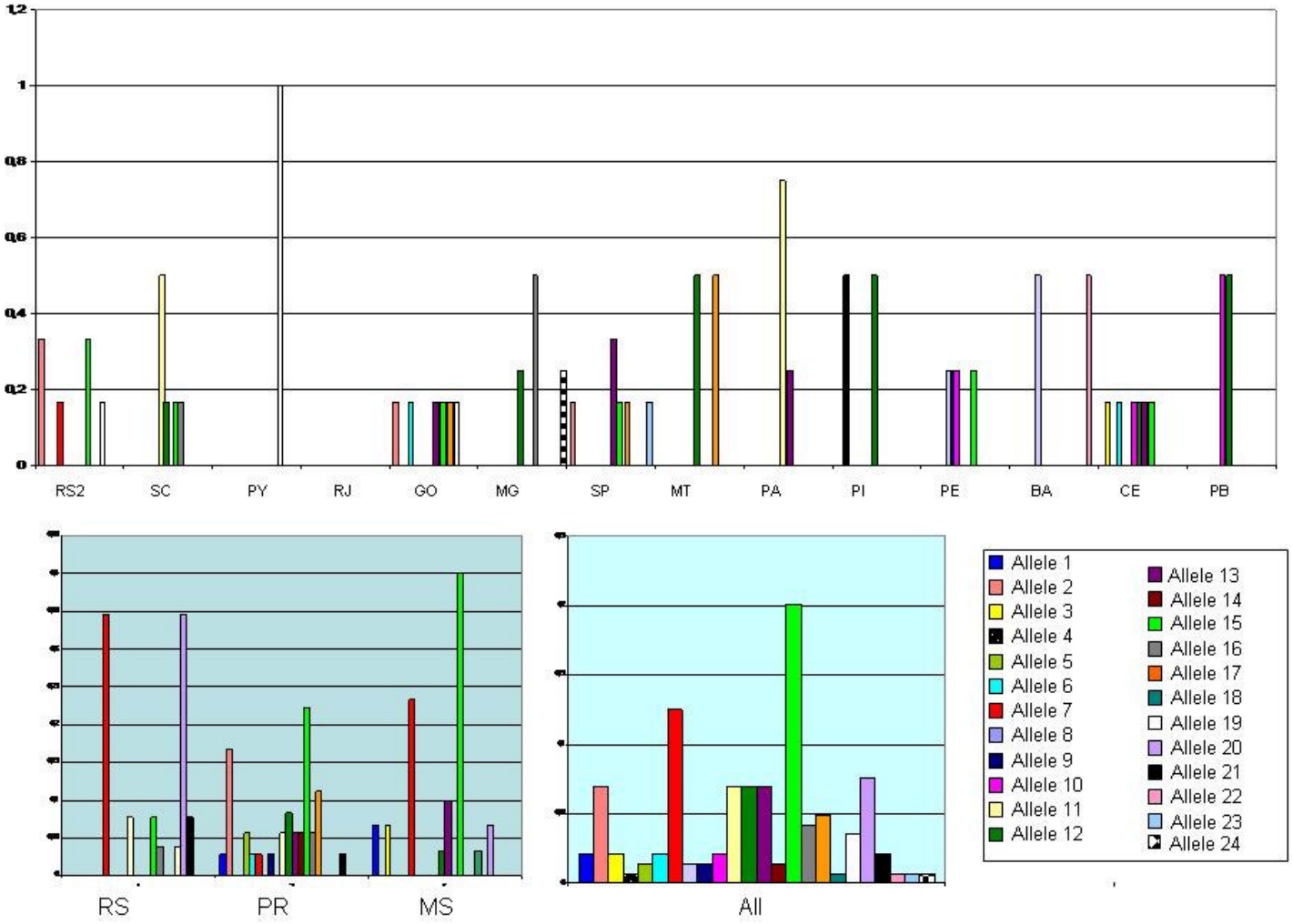
j)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Allele 24

Table 1. — Samples analyzed in the present study.

ECOREGION	*GEOGRAPHIC ORIGIN (SAMPLE SITE)*	*SAMPLES*	*INSTITUTION/CONTACT*
Southern Atlantic Forest	P.N. Iguaçu, Paraná State, S Brazil (PR)	bCth8, bCth9, bCth59, bCth60,bCth61, bCth62 bCth63, bCth64, bCth65, bCth67, bCth68, bCth69, bCth70, bCth71, bCth72, bCth74, bCTh75, bCth76, bCth77	UNIOESTE/José Flávio Cândido Jr. and Instituto Pro-Carnivoros
	Mato do Grosso do Sul State, SW Brazil (MS)	bCth164, bCth165, bCth166, bCth172, bCth173,bCth174,bCth175, bCth11,bCth232, bCth233, bCth234, bCth235, bCth236, bCth239, bCth240, bCth243, bCth244	Instituto Pró-Carnívoros/ Denis Sana
	Paraguay (PY1)	bCth91,	Guillermo D`Elia
	Santa Catarina State, S Brazil (SC1)	bCth154, bCth178, bCth210	Ligia Tchaicka
	Rio de Janeiro State, E Brazil (RJ)	bCth214	Zoológico de Pomerode
	São Paulo State, E Brazil (SP2)	bCth301, bCth305, bCth219	Cristiana Prada
	Rio Grande do Sul State, S Brazil (RS)	bCth80, bCth81, bCth82, bCth83, bCth84, bCth85, bCth99, bCth100, bCth134, bCth135, bCth136, bCth142, bCth143, bCth144, bCth145, bCth146	Mariana Faria-Corrêa and Instituto Pro-Carnivoros
	Rio Grande do Sul State, S Brazil (RS2)	bCth15, bCth39, bCth112	Alex Bager
Cerrado	Minas Gerais State, E Brazil (MG1)	bCth200, bCth336	Instituto Pro-Carnivoros
	Goiás State, Central Brazil (GO1)	bCth205, bCth208, bCth209	Zoológico de Goiânia/ Roberto Portela; Instituto Pro-Carnivoros
Caatinga	PN Serra da Capivara, Piaui State, NE Brazil (PI)	bCth201	FUNDHAM/ Vanderson C.Vaz and Paulo César D´Andrea
	Ceará State, NE Brazil (CE)	bCth192,bCth194, bCth197	Zoológico de Fortaleza/ Luiz C. Diniz

Table 1. — Continued.

Eastern Amazônia	Pará and Maranhão State, N Brazil (PA)	bCth221, bCth223, bCth228	Tadeu de Oliveira
Pantanal	Mato Grosso State, SW Brazil (MT2)	bCth51, bCth56	Rodrigo Jorge e Julio Dalponte
Northern Atlantic Forest	Paraíba State, NE Brazil (PB)	bCth309	Flavio Rodrigues
	Bahia State, NE Brazil (BA)	bCth186, bCth187	Zoológico de Salvador/Cláudio V. Lyra
	Pernambuco State, NE Brazil (PE)	bCth184, bCth185	Zoológico do Recife/ Poly- Ana Celina

Table. 2. — Characteristics of ten microsatellite *loci* amplified from *C. thous*.

Identification*	Number of Alleles	Allele Size Range	Ho**	Hs**
2100	6	176-196pb	0.774	0.784
2006	4	226-238pb	0.404	0.672
2054	9	158-194pb	0.824	0.817
2004	7	251-175pb	0.637	0.685
2001	4	145-161pb	0.443	0.443
2010	6	231-151pb	0.695	0.697
2132	11	156-196pb	0.683	0.726
2137	23	173-245pb	0.533	0.833
2140	24	121-171pb	0.850	0.840
2088	6	109-129pb	0.610	0.652

*Original *locus* identification by Francisco et al. (1996).
** Nei's estimation of Heterozygosity (Ho = observed values, Hs = expected values)
Indicate departure from HWE ($\alpha < 0.005$) for loci 2137.

Table. 3. — Gene diversity (Nei 1987) from microsatellite data in *Cerdocyon thous* (site names are coded as Fig. 1).

***Locus* ***	**Sample sites**													
	PR	**RS**	**MS**	**PA**	**MT**	**RS2**	**SC**	**PE**	**BA**	**CE**	**GO**	**MG**	**SP**	**ALL**
2100	0.787	0.769	0.827	0.750	0.750	0.667	0.917	0.500	0.500	0.833	0.667	0.750	0.833	0.816
2006	0.674	0.721	0.721	1.000	0.500	n.a	0.917	0.500	1.000	1.000	0.667	0.000	0.833	0.690
2054	0.789	0.642	0.838	1.000	0.750	0.750	0.917	0.750	n.a	0.917	0.750	0.750	1.000	0.810
2004	0.773	0.644	0.804	0.500	0.750	0.667	0.917	0.000	1.000	0.500	0.833	0.500	0.917	0.764
2001	0.287	0.517	0.398	0.500	0.500	0.667	0.500	0.500	0.500	0.500	0.333	0.500	0.500	0.440
2010	0.545	0.621	0.746	0.667	1.000	0.500	0.750	1.000	0.500	0.500	0.500	1.000	0.917	0.677
2132	0.827	0.788	0.752	0.833	0.000	0.917	0.833	n.a	1.000	0.833	0.500	0.500	1.000	0.830
2137	0.896	0.752	0.942	0.750	1.000	0.917	1.000	1.000	1.000	0.833	0.833	1.000	0.000	0.930
2140	0.913	0.763	0.788	0.500	n.a	0.917	0.750	1.000	n.a	1.000	1.000	0.750	0.917	0.917
2088	0.742	0.588	0.667	n.a	n.a	0.667	0.750	0.500	0.000	1.000	0.667	n.a	0.500	0.722

* *Locus* identification follows the original *locus* description (Francisco et al. 1996);
n.a. samples not analyzed

Table. 4.— Population comparison of diversity measures: total number of alleles, gene diversity (Nei, 1987) and F_{is} (heterozygote deficit) across all ten microsatellites *loci* studied for *C. thous* (the top line samples sites are coded as Fig. 1)

	RS	*PR*	*MS*	*RS2*	*PY*	*SC*	*MT*	*GO*	*MG*	*SP*	*RJ*	*PA*	*BA*	*CE*	*PE*	*PI*	*PB*
Allele Number	45	69	60	27	14	40	23	31	23	34	14	26	22	34	26	15	18
Gene Diversity	0,68	0,72	0,74	0,66	n.a	0,82	0,65	0,67	0,63	0,76	n.a.	0,72	0,55	0,79	0,52	n.a.	n.a.
F_{is}	-0.058	0.183	0.146	0.250	n.a	0.010	0.048	0.111	0.043	0.124	n.a.	0.154	0.455	0.011	0.130	n.a.	n.a.

n.a.samples not analyzed

Capítulo V

3º ARTIGO

a ser submetido para publicação na revista Journal of Heredity

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Molecular phylogeny of a recently diversified endemic group of South American canids (Mammalia: Carnivora: Canidae)

Molecular phylogeny of a recently diversified endemic group of South American canids (Mammalia: Carnivora: Canidae)

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ABSTRACT

In order to understand the phylogeny and phylogeography of an endemic group of South American canids, we examined 588pb of the mitochondrial DNA control region from a total of 142 individuals from six species of the genus *Lycalopex*. Phylogenetic analyses indicated monophyly of the genus, with *L. vetulus* as the most basal species (time of divergence from other *Lycalopex ca*. 1,000,000 years), a recent origin of the other species (*ca*. 500,000 years) and a very close relationship between *L. griseus* and *L. culpaeus* (having diverged *ca*. 350,000 years ago). Intra-specific analysis indicated that most of these species experienced population expansions, and that *L. gymnocercus* and *L. fulvipes* show indications of genetically differentiated geographic groups. The history of these events and their biogeographic and taxonomic implications are discussed.

INTRODUCTION

The first representatives of the family Canidae entered South America in the late Pliocene and early Pleistocene, coming from North America through the Panama Isthmus (formed approximately 3 million years ago [MYA]) and then radiated to achieve their present diversity (Berta 1987). Currently there are ten canid species endemic to South America, which is the most diverse continent with respect to this family. This diversity is at least in part due to a generalist and opportunistic feeding strategy that utilizes small prey as well as fruits and grains, and the potential to inhabit all types of habitats in the continent (Berta 1987, Ginsberg and Macdonald 1990, Wozencraft 2005).

Morphological and molecular evidence generally agree that living South American foxes belong to a monophyletic group (*Cerdocyon, Lycalopex, Atelocynus and Dusicyon*; *e.g* Wayne et al. [1997]; Zrzavy and Ricankova [2004]). These species have similar karyotypes, suggesting recent divergence (2n=74; NF=76 - *L. gymnocercus, P griseus, L. culpaeus, L. sechurae, A. microtis, P gymnocercus* and *L. vetulus*; 2n= 74; NF=110 - *Cerdocyon thous* [Brum-Zorrila and Langguth 1980; Wayne et al. 1987; Wayne 1993]). Although several previous studies have addressed the evolutionary relationships among these foxes (using morphological and/or molecular data; e.g. Bardeleben et al. 2005, Lindblad-Toh et al., 2006, Lyras and Van-Gelder 2003, Wayne et al. 1997, Zrzavy e Ricancova 2004), their relationships are still incompletely understood, especially with respect to the species usually included in the genera *Pseudalopex* and/or *Lycalopex*. These canids will be treated here as *Lycalopex vetulus* (Hoary fox), *L. gymnocercus* (Pampas fox), *L. culpaeus* (Culpeo fox), *L. fulvipes* (Darwin's fox), *L. griseus* (Chilla fox) and *L. sechurae* (Sechuran fox), following Wozencraft (2005).

These foxes exhibit variable body sizes, with *L. culpaeus* weighing up to 11Kg and *L. gymnocercus* 4 to 6.5 kg, while the other species are smaller, with mean weights of ca. 3.5Kg (Sillero-Zubiri et al. 2004). Patterns of coloration and morphology are so similar between some of these species, and in some cases between them and the related crab-eating fox (*Cerdocyon thous*), that it is often difficult to arrive at precise species identification based on visual criteria alone (L. Tchaicka and E. Eizirik, personal observation). This is especially problematic with respect to pelage colors, which can exhibit great geographic and/or seasonal variation, leading to potential confusion among species.

The precise geographic range of these species is still not known in full detail, but some major distributional patterns are clearly documented (Fig. 1). *L. culpaeus* is distributed along the Andes and hilly regions of western South America, from Southern Colombia to Tierra del Fuego. *L. fulvipes* is endemic to costal Chile. *L. griseus* is widespread in areas of plains and mountains on both sides of the Andes, from Northern Chile south to Tierra del Fuego. *L. gymnocercus* ranges from eastern Bolivia and western Paraguay to Northern provinces of Argentina and to the Atlantic coast in Southern Brazil. *L. sechurae* occurs in habitats near the Pacific coast of Peru. Finally, *L. vetulus* is endemic to the Cerrado biome in Central Brazil (Sillero-Zubiri and Macdonald, 2004).

Several taxonomic schemes for these species have been suggested in the past based on different methods. Langguth (1975), based on ecological and morphological data, suggested two taxa for this group: the *Lycalopex* genus and *Pseudalopex* as a subgenus of *Canis*. The former contained only *Lycalopex vetulus*, the type species for this genus, while the latter contained *Canis* (*Pseudalopex*) *culpaeus, C.* (*P.*) *gymnocercus, C.* (*P.*) *griseus* and *C.* (*P.*) *sechurae.* Subsequently, Clutton-Brock et al. (1976), using morphological and behavioral data, included all these species in the genus *Dusicyon*, originally proposed for the now extinct

Falkland Island "wolf", *D. australis* (Wozencraft 2005). Berta (1987), based on the fossil record and cladistic analyses of morphological data, proposed that the genus *Pseudalopex* should include *P. griseus, P. gymnocercus, P. culpaeus, P. vetulus, P. sechurae* and the extinct species *P. peruanus*. More recently, Zunino et al. (1995) grouped *P. gymnocercus* and *P. griseus* into a single species, *Lycalopex gymnocercus*, supporting the use of *Lycalopex* as the generic name for *L. culpaeus*, *L. vetulus* and *L. sechurae* (*L. fulvipes* was also considered to be a synonym of *L. gymnocercus* in this study).

L. fulvipes was first considered to be a subspecies of *L. griseus*, and thought to be endemic of Chiloe Island, in Chile (Nowak 1999, Redford and Eisenberg 1992, Wilson and Reeder 1993). Recently, a mainland population of *L. fulvipes* has been discovered, and molecular genetic analyses indicated that this fox is a distinct species that probably had a much broader distribution in the past (Yahnke et al. 1996).

Additional classifications of this group have been suggested by Thomas (1914); Kraglievich (1930); Cabrera (1931); Osgood (1934); Hough (1948); Thenius (1954); Van Gelder (1978), but classification remains unclear due to remaining uncertainties in the phylogenetic relationships among these taxa.

Recent intra-specific studies on Neotropical canids have concentrated mostly on ecological aspects such as diet and habitat use (*e.g.* Crespo 1971, Dotto 1997, Courtenay et al. 2006, Jaksic et al. 1998, Pia et al. 2003), and few papers have used molecular data to investigate these species (*e.g.* Vilà et al. 2004 [addressing species distribution]; Farrell et al. 2000 [addressing dietary separation among species]) A comparative phylogeographic approach can permit interesting studies of evolution, including patterns of speciation and the underlying processes, as well as the effects of habitat use and dispersal capabilities on the genetic structure of related taxa. These investigations may shed light on the links between

population processes and regional patterns of diversity and biogeography (Bermingham and Moritz 1998).

Mitochondrial segments are useful in evolutionary studies of recent divergence processes in animals due to their relatively high substitution rate, maternal inheritance, and absence of recombination (Schlotterer 2004). In spite of limitations derived from these same features, mtDNA segments remain an important source of information in the case of phylogenetic studies of closely related species or intra-specific phylogeography, since these rapidly evolving sequences (with lower effective population size) are often quite informative in attempts to capture recent episodes of population divergence.

In this study we have used mitochondrial DNA (mtDNA) control region sequences to investigate the evolutionary history of an endemic clade of South American foxes. These closely related species, whose classification has remained controversial for decades, pose an interesting challenge to phylogenetic reconstruction, due to their rapid and recent divergence, and unclear classification at specific and generic levels.

MATERIALS AND METHODS

Biological material was collected from a total of 142 Neotropical canids, including *Lycalopex culpaeus* (n=53), *L. gymnocercus* (n=24), *L. vetulus* (n=26), *L. fulvipes* (n=6) and *L. griseus* (n=32; nine individuals had been initially identified as *L. gymnocercus*; see Fig. 1 and Discussion). In addition, nine *Cerdocyon thous* individuals were included as an outgroup (two of them were previously sequenced by Tchaicka et al. [in press]; Capítulo III).

Blood samples (preserved in a saturated salt solution: 100mM Tris, 100mM EDTA, 2% SDS) were collected from captive individuals of known origin, as well as wild animals captured for field ecology studies. Tissue samples were obtained from road-killed individuals

and preserved in 95% ethanol. Genomic DNA was extracted from samples using a standard phenol/chloroform protocol (Sambrook et al. 1989).

The 5' portion of the mtDNA control region, containing the first hypervariable segment (HVS-I), was amplified by the Polymerase Chain Reaction (PCR; Saiki et al. 1985) using specific primers MTLPRO2 (5'-CACTATCAGCACCCAAAGCTG) and CCR-DR1, (5'-CTGTGACCATTGACTGAATAGC) (or H16498 [Ward et al. 1991] as an alternative reverse primer). PCR reactions contained 2µl 10X buffer, 1.5 mM $MgCl_2$, 0.2 µM dNTPs, 0.2 µM each primer, 0.75 unit Taq polymerase and 1-3 µl of empirically-diluted template DNA. Thermocycling conditions included 10 initial cycles of "touchdown", with 45'' denaturing at 94ºC, 45'' annealing at 60-51ºC, and 1'30'' extension at 72 ºC. This was followed by 30 cycles of 45'' denaturing at 94 ºC, 30'' annealing at 50ºC and 1'30'' extension at 72ºC. Products were examined on a 1% agarose gel stained with ethidium bromide, purified using shrimp alkaline phosphatase and exonuclease I and sequenced with ABI chemistry and analyzed with an ABI-PRISM 3100 automated sequencer. Sequences generated for this study have been deposited in GenBank (accession numbers XXXXX-XXXXX). In addition, one previously published partial sequence of the mtDNA control region of *Lycalopex sechurae* (Yahnke et al. 1996) was included in the analyses.

Sequences were verified and corrected by eye using Chromas (available from *www.tchnelysium.com.au*) or Sequencher (Gene Codes Inc.), aligned using the ClustalW algorithm implemented in MEGA 3.0 (Kumar et al. 2004) and visually checked. Sites or segments that could not be unambiguously aligned were excluded from all analyses. Initial sequence comparisons and measures of variability, such as the number of variable sites and nucleotide diversity (π per nucleotide site, the probability that two randomly chosen homologous nucleotides are different in the sample) were performed in Mega 3.0 using Kimura 2-

parameter distances and 1000 bootstrap replicates. Estimates of gene diversity (h, the probability that two randomly chosen mtDNA lineages were different in the sample) and tests of neutrality such as Tajima's D (Tajima 1989) and Fu's F_S (Fu 1997) were computed in Arlequin 2.0 (Schneider et al. 2000) with 10,000 permutations.

Phylogenetic relationships among haplotypes were inferred with PAUP 4.0 (Swofford 1998), using three different approaches: (i) maximum parsimony (MP), (ii) maximum likelihood (ML), and (iii) distance-based phylogeny with the Neighbor-Joining (NJ) algorithm (Saitou and Nei 1987). MP trees were estimated with heuristic searches implementing a random addition of taxa and TBR branch swapping. For the likelihood-based analyses, the appropriate model of sequence evolution, along with its parameters, were estimated from the data set with Modeltest 3.6 (Posada and Crandall 1998), using the Akaike Information Criterion, or directly with PAUP. ML heuristic searches started from an NJ tree and used NNI branch-swapping. Distance-based trees were inferred using the NJ algorithm, with distance measures chosen based on the selected model of sequence evolution for each data set. For each of the three methods, one hundred bootstrap replicates were used to evaluate nodal support. Three different data sets were analyzed: (i) all haplotypes, including incomplete sequences (this set was investigated with NJ alone, due to the existence of >>10,000 equally parsimonious trees, and the computational burden to run ML analysis in this case); (ii) complete sequences alone, leading to increased stability; and (iii) a mixture of the former two sets, in which three short haplotypes (representing *L. sechurae* and *L. fulvipes*) were added back to the more stable matrix, to test their phylogenetic placement.

Haplotype networks were generated with sequences from each species (except *L. sechurae* [n=1]) using statistical parsimony and the program TCS 1.18 (Clement et al. 2000), with a 95% threshold for parsimonious connections between haplotypes. To evaluate possible

events of population expansion and decline, mismatch distribution analyses were computed in DnaSP 4.0 (Rozas et al. 2003) and Harpending's Raggedness Test of goodness-of-fit was performed in Arlequin 2.0 (1000 replicates; species with smaller sample sizes [*L. fulvipes* and *L. sechurae*] were excluded from this analysis).

To estimate the time of divergence among mitochondrial lineages, we used the mutation rate μ estimated by Tchaicka et al. (in press [ver Capítulo III]) based on available data from grey wolf and coyote. We then used this substitution rate to calculate (with Mega) the time of divergence between species, based on the equation $d_{xy}=2\mu T$ (Nei 1987).

RESULTS

Seventy-eight different haplotypes were identified with the 588-base pair (bp) segment sequenced for *Lycalopex* species (nine additional haplotypes were included from *C. thous*), defining 220 variable sites and 193 parsimony-informative sites (Table 1). Base composition was biased, with a deficiency of guanine (T=31.1%; C=24.6%; A=26.6%; G=17.6%). No haplotypes were found to be shared between species.

Using all 87 haplotypes, the NJ analysis generated a tree topology that indicated *L. vetulus* as the most basal species in the genus *Lycalopex*, *L. fulvipes* and *L. gymnocercus* as distinct monophyletic groups, and a shallow internal cluster containing both *L. culpaeus* and *L. griseus* haplotypes, whose reciprocal monophyly was unclear (Fig. 2). *L. fulvipes* was placed as a sister-group to the (*L. griseus + L. culpaeus*) group, but with low support. Since some of the inferred clades showed low bootstrap values, we performed a second round of analyses excluding sequences with more than 10% missing data, generating a more robust data set of 49 haplotypes (including those of *C. thous*). This second set of analyses retrieved congruent topologies with all three of the approaches employed, which were also similar to

the findings with the full data set, albeit with higher support (Fig. 3). These analyses revealed reciprocally monophyletic clades for *L. culpaeus* and *L. griseus*, which are the most internal sister taxa in the genus. In these analyses, the single long *L. fulvipes* sequence was not placed as sister to the (*L. griseus + L. culpaeus*) group, but rather at a deeper position external to *L. gymnocercus*. However, support for this position was low, rendering the exact placement *L. fulvipes* unclear. The basal position of *L. vetulus* was strongly supported, but the relationships of *L. sechurae* could not be assessed, as it was represented by a single short sequence that was removed from this analysis. To address the outstanding issues, a third set of phylogenetic analyses was performed, keeping the 49 longer haplotypes from the second set, and returning the *L. sechurae* and two short *L. fulvipes* sequences, to test their placement given a more stable overall topological framework (Fig. 4). The position of *L. fulvipes* was the same as in the second set (compare to Fig. 3), indicating consistency of the information from the three sampled haplotypes, but support for this placement remained low. Likewise, no conclusive support could be obtained for the placement of *L. sechurae* (Fig. 4), although the results suggest that it is a rather basal species in this group, and not immediately related to *L. culpaeus* as might be expected (see Fig. 1).

Intra-specific analyses of sequences indicated that *L. culpaeus* and *L. fulvipes* showed less genetic variability than the remaining species (Table 2). Haplotype networks built for each species (Figs. 5, 6 and 7) indicated a geographic pattern in the distribution of variability in *L. fulvipes* and *L. gymnocercus* (Fig. 6 and 7). In the former species, continental haplotypes are separated from island haplotypes by four mutational steps, while between the latter there is only one step (the small sample sizes warrant cautious interpretation of this inference). All *L. fulvipes* samples with unknown origin had the same haplotype F03, which may suggest that these samples are from Chiloe Island, as no other copy of this haplotype

was found in mainland individuals. The Culpeo network was characterized by few mutational steps, as expected for recently diversified species. The *L. gymnocercus* network suggests a possible geographic partition between TA (Taim Ecological Station, in Southernmost Brazil) and other samples, as inferred from the phylogenetic analyses (Figs. 2, 3 and 7).

Despite no clear indication on the network, population expansion was inferred for *L. culpaeus* (r= 0.05; p=0.33), *L. vetulus* (r=0.033, p= 0.10), *L. griseus* (r=0.03, p=0.22) and *L. gymnocercus* (r=0.02, p=0.59), based on mismatch distribution analyses and the fit to a population growth model (Fig. 8). This was supported by Fu's F_s test only in the case of *L. griseus* (F=-8.17, significant), while all neutrality tests were not significant for the other species.

Divergence time among mtDNA lineages was calculated considering 95% confidence interval (CI:±2SE) from all values of d_{xy}. Using this interval we obtained low, medium and fast mutation rate estimations ($2.02x10^{-8}$, $3.68x10^{-8}$ and $5.34x10^{-8}$ respectively) that were combined with the same interval to the d_{xy} estimated for each node. By this procedure we estimate that the divergence between *Cerdocyon* and *Lycalopex* took place *ca.* 1,100,000 years ago. *L. vetulus* and *L. sechurae* seem to have diverged from other *Lycalopex* species *ca.* 1,000,000 years to 900,000 years ago, while the divergence among the latter is inferred to have occurred more recently, *ca.* 500,000 years ago (Table 3).

DISCUSSION

The trees produced using the full data set of 82 haplotypes (Fig. 2) recovered the main patterns of divergence in this group of canids, but contained some weakly supported nodes that proved to be unstable when compared to the results of the second set of analyses (with 49 longer haplotypes). This is likely due to missing data present in the first data set, which may have confused the search when very similar sequences were compared. The second round of analyses, using only the longer sequences, resolved some relationships more clearly, and in many cases generated higher support values (Fig. 3).

Our results indicate that *L. griseus* and *L. culpaeus* are sister taxa (Figs. 2 and 3) that probably diverged *ca.* 350,000 ybp, corroborating a previous molecular study conducted by Yahnke et al. (1996), that reported *ca.* 250,000-500,000 ybp for this event. In that study, a shorter segment of the mtDNA control region (344pb) was used, and led to the inference that *L. griseus* and *L. culpaeus* are not reciprocally monophyletic clades, possibly deriving from such a recent divergence that they had not yet achieved complete differentiation at this level. Our first phylogenetic analysis, which included some sequences with much missing data, produced the same result, with *L. griseus* appearing paraphyletic with respect to *L. culpaeus* (Fig. 2). In contrast, when only the longer sequences are considered (Fig. 3), the two species are supported as monophyletic groups, with bootstrap values above 79% for *L. culpaeus* and between <50 and 88% for *L. griseus*.

Initial results obtained from *L. griseus* prompted a more careful comparison with the *L. gymnocercus* data. Pampa fox haplotypes from Brazilian areas formed a well-supported monophyletic group (Figs. 2 and 3), but sequences from nine individuals initially recorded as *L. gymnocercus* in the field, collected in Bolivia and Argentina (Fig.1), were strongly placed as

members of the chilla fox clade (Figs. 2 and 3). There are no formal reports of *L. griseus* occurring in these regions (González del Solar and Rau 2004), but this species is sympatric with *L. gymnocercus* in several areas, and information on the precise distribution limits of both species is still scarce. Our results suggest that the areas where these samples were collected may be in fact inhabited by *L. griseus* instead of *L. gymnocercus*. Since color patterns of pelage are very similar between these foxes (Zunino et al. 1995), identifying them can be challenging, which suggests that recording errors may have confused historical reports on these species' natural history and the delimitation of their ranges. A non-exclusive alternative hypothesis is that hybridization between these species in their areas of contact could account for these observations, as well as for their morphological similarity. Zunino et al. (1995), analyzing pelage characters and cranial measures of Argentinean *L. griseus* and *L. gymnocercus*, observed a clinal variation in size and color, and concluded that these foxes are conspecific (thus calling them *L. gymnocercus*, the senior name). In this context, the samples initially labeled as *L. gymnocercus* that grouped with *L. griseus* might be hybrids between male pampa's foxes and female chillas.

Another supported clade is formed by the *L. fulvipes* samples. This fox lives in costal temperate rainforests of Southern Chile, where it inhabits Chiloe island and also occurs in sympatry with the chilla and culpeo in a small continental area (Medel et al. 1990, Vilà et al. 2004). Initially, it was described as an endemic insular canid and considered a subspecies of continental *L. griseus* (Nowak 1999; Wilson and Reeder 1993; Redford and Eisenberg, 1992).

A molecular genetic analysis conducted by Yahnke et al. (1996) revealed that Darwin's fox is a distinct species, a monophyletic clade that was a sister taxon to a (*L. griseus* + *L. culpaeus*) cluster, from which it diverged in the Pleistocene, *ca.* 275,000 to 667,000 years ago. These conclusions are broadly corroborated by the present study, as we estimate (using

the longer sequences) a divergence time of *ca.* 500,000 between Darwin's fox and the (culpeo + chilla) clade. However, the exact position of *L. fulvipes* in this recent clade was not completely consistent among our analyses: while the larger data set (including short sequences – Fig. 2) agreed with the position obtained by Yahnke et al. (1996), the other two data sets (relying on longer sequences and in general more stable – Figs. 3 and 4) suggested that this species is even less closely related to the Andean cluster, as it is placed outside the group that also includes *L. gymnocercus*. Since support values for either alternative are low, further accumulation of data will be required to settle this issue.

Our results strongly support a basal position of *L. vetulus* in the genus, and we estimate its origin to have been *ca.* 1,000.000 ybp. This is in agreement with a recent phylogeny based on multiple nuclear segments (Lindblad-Toh et al. 2005). The conclusion is that *L. vetulus* represents a unique lineage (>97% bootstrap support), distinct from the *griseus-gymnocercus-culpaeus-fulvipes* group.

Debate about the proper usage of *Lycalopex* or *Pseudalopex* for this group has been going on for many years (e.g., Cabrera 1931; Osgood 1934; Langguth 1975; Berta 1987, 1988; Tedford et al. 1995). The basal phylogenetic position of the hoary fox implies that it could be kept in its own genus (*Lycalopex*), while the other species move back to *Pseudalopex*, or that the whole cluster could be considered a single genus (*Lycalopex*), as in Wozencraft (2005). Both schemes are compatible with the phylogeny, and this decision will be arbitrary. We recommend that this decision be based on criteria such as clade age, morphology, and present usage, which should be established in a broader comparison across all lineages in the family Canidae.

Combined analysis of morphological and molecular data (Zrzavy and Ricankova 2004) and fossil data (Berta 1987) reported that *L. sechurae* and *L. vetulus* share a common

lineage. If this is true, the estimated time of divergence between these species (Table 3) indicates that they split at roughly the same time as the *L. vetulus* X *griseus-fulvipes-gymnocercus-culpaeus* separation, and that *L. sechurae* is also rather basal in this clade. However, the exact phylogenetic position of *L. sechurae* could not be completely defined by this mtDNA data set (Figs. 2 and 4), likely due at least in part to the lack of longer sequences available for this species.

Individual history from each species

The results obtained from the haplotype networks, along with the internal branch structure of the generated trees, indicate the occurrence of two partitioned genetic groups of *L. gymnocercus* in the sampled area (Figs. 3 and 7). Three pampa's fox subspecies have been proposed by Massoia (1982). Their geographic limits are not precise and there is no data regarding the taxonomic position of Bolivian foxes (our samples from Bolivia [BO] were ultimately considered to be *L. griseus*). All included *L. gymnocercus* samples (*i.e.* those composing its monophyletic clade; AR animals coming from this area were also grouped with griseus; see Fig.1) were collected from the *L. gymnocercus gymnocercus* range, that includes Southern Brazil and Northeastern Argentina (Massoia, 1982). Since TA (Taim Ecological Station, in Southernmost Brazil) appears to be a differentiated area, it appears that the classical subspecies' distribution does not reflect inferred patterns of historical population subdivision, as reported for other Neotropical carnivores (e.g. Eizirik et al. 1998; Tchaicka et al. in press).

The TA sample-site is located in a region of marshlands and lakes that has been intensively changed between the Middle Pleistocene and the Holocene, when sea level

variation created geographic barriers, probably including the event that created the Patos and Mirim Lagoons (Vilwock et al. 1986). These processes may have influenced the genetic structure and demographic history of some native species. Due to the matrilineal inheritance of mtDNA, this pattern may be influenced by the possible occurrence of female philopatry in this species, which should be investigated by in-depth field studies targeting this issue.

Some inference of genetic structure related to geographic distribution can also be observed in the *L. fulvipes* phylogenetic and network analyses, which indicate that mainland haplotypes are more similar to each other than to the island haplotype (Fig. 6). As suggested previously (Vilà et al. 2004; Yahnke et al. 1996) the island population appears to be isolated from remnant continental populations, and may have experienced genetic loss during its history (only one haplotype is present in Yahnke's data). This possibility must be investigated in more detail with expanded sampling in this region, and with the use of additional markers (e.g. microsatellites). The *L. griseus* results (Figs. 3 and 6) show no evidence of geographic structure among haplotypes (with only some support for BO branches), but the complex pattern of the network suggests that these inferences can be changed in the future by more detailed studies. The *L. culpaeus* haplotypes, are quite similar to each other, which is suggestive of a young age of the species, and compatible with a population expansion process inferred from the shape of its phylogeny (e.g. Fig. 2).

The clade formed by *L. vetulus* samples presents some well-supported intra-specific groups, which showed no obvious geographic orientation, and nor did the haplotype network for this species (Fig. 5). This pattern may derive from historical geographic structuring that has been obliterated by subsequent gene flow. This is not expected from small canids that generally disperse rather short distances, leading to some level of genetic isolation among areas (Mercure et al. 1993). However, field observations of hoary foxes report that all

offspring disperse by the time they are *ca.* 10 months old (Courtenay et al. 2006), and the current species' range seems to be rather continuous, probably offering sufficient opportunity for genetic admixture over time.

Inferences on the history of South American foxes

Three different canid invasions from North to South America in the Pliocene or Early Pleistocene have been proposed on the basis of previous inferences of the phylogenetic relationships among extant species. One of them would include the ancestor of fox group that includes *Lycalopex, Cerdocyon, Atelocynus and Dusicyon* (Langguth 1975; Wang et al. 2004). Genetic divergence values obtained from *cytocrome b*, *COI* and *COII* segments (Wayne et al. 1997) between Cerdocyon+Atelocynus and the other species suggest that this divergence occurred before the opening of the Panama land bridge, requiring one more invasion event.

Contrary to this view, our mtDNA control region data indicate that the divergence between *Cerdocyon* and *Lycalopex* took place *ca*. 1.2 mybp, suggesting that this episode of speciation may have occurred in South America, after immigration of a single ancestor through the Isthmus of Panama. In fact, the oldest fossils assigned to *Lycalopex* (*L. gymnocercus*) are reported from Argentinean deposits of the Uquian age (2.5 to 1.5 mybp), while those of *Cerdocyon thous* are recorded only from the Lujanian (800,000-10,000 ybp). Interestingly, there are North American canid fossils reported from the Miocene/Early Pliocene boundary (6-3mypb - Berta 1987) that have been assigned to the genus *Cerdocyon*, which would challenge this hypothesis. The present dating results, which are congruent with

our previous analyses (Tchaicka et al. [in press], ver Capítulo III), suggest that the identification and phylogenetic affinities of these fossils should be reassessed.

It is generally assumed from the fossil record (Berta, 1987; Langguth 1975) and supported by molecular data (Wayne et al. 1997) that, subsequently to divergence with *Cerdocyon*, the diversification of *Lycalopex* occurred in South America in the in the Pleistocene. Our results agree with a Pleistocene radiation of *Lycalopex*, indicating that: (i) the oldest extant lineages gave rise to *L. vetulus* and possibly also to *L. sechurae* (or perhaps both lineage are directly related; see Berta 1987; Zrzavy and Ricankova 2004), in the Early-Middle Pleistocene; (ii) this was followed by the diversification of the *griseus-culpaeus-gymnocercus-fulvipes* clade, in the Middle Pleistocene; and (iii) finally, by the *griseus-culpaeus* recent split in the Middle-Late Pleistocene.

In the Neotropical region, extensive environmental changes took place in the Pleistocene, which may have influenced this canid radiation. Climatic changes affected the vegetation domains as well as the sea level, producing potential geographic barriers to dispersal or confining species to habitat refuges (Marroig and Cerqueira 1997; Withmore and Prance 1987; Eisenberg and Redford 1999). Canids that had crossed Panama bridge and dispersed trough Andean savanna corridors, expanded their range to Patagonian and Brazilian lands by the Early Pleistocene (Langguth 1975). At this time, the La Plata- Paraguay depression suffered a marine invasion, and possibly became connected to the Amazon Basin, isolating a large region of Brazil (Marroig and Cerqueira 1997). This may account for the isolation of canid species in two groups: Brazilian and Argentinean, corresponding to *L. vetulus vs.* the remaining lineages.

During the Pleistocene glacial phases, arid climates dominated the equatorial areas and savanna corridors were broken. Some mammal species that became restricted to tropical

humid regions may have become savanna-adapted, and now occur in areas consisting of Cerrado habitat (Eisenberg and Redford 1999; Whitmore and Prance 1987). This may be the case of the Hoary fox: its small carnassials, wide crushing molars and the exceptionally large auditory bullae (Clutton-Brock et al. 1976) suggest adaptations to a predominantly insectivorous diet. Their preference for insects now allows them to partition food resources and to coexist with other sympatric canids such as the maned wolf (*Chrysocyon brachyurus*) and the crab-eating fox (*Cerdocyon thous*) (Juarez and Marinho-Filho 2002).

The center of the *Lycalopex* radiation has been proposed by Berta (1987) to have been in central Argentina, whereas Langguth (1975) suggested central Brazil are the most likely region. These two hypotheses are here reconciled, since the second episode of speciation in this group probably did take place in Argentina (*gymnocercus-fulvipes-[griseus+culpaeus*]). The origin of these species was followed by population expansion processes that in some cases are still detectable (Fig. 8). As suggested by Yahnke et al. (1996), *L. fulvipes* may represent a set of relict populations of a once more widely distributed species, whose phylogenetic affinities within this group are still not completely settled. Future work should use increased sampling of individuals and characters to attempt to clarify this issue, as well as the exact phylogenetic position of *L. sechurae*, so that a more complete biogeographic inference can be devised for this group. Overall, the prospect of understanding this recent radiation in South America promises to shed light on some of the processes shaping the mammalian biodiversity in this region during the Pleistocene.

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Figure legends

Figure 1. Map showing the current geographic distribution of *Lycalopex* species (modified from Courtenay and Maffey 2004) and approximate sample collection sites (polygons) identified as ■ *L. culpaeus*; ○ *L. fulvipes*; ▼ *L. griseus*; ● *L. gymnocercus*; ▲ *L. vetulus*; □ *L. sechurae*; and ★*L. griseus* initially labeled as *L. gymnocercus.* The number of samples per site are indicated (individuals with unknown geographic origin are not included; see Methods).

Figure 2. Neighbor Joining tree (built with Kimura81 [3-Parameter] distances) of 78 *Lycalopex* spp. haplotypes based on 588 bp of the mitochondrial DNA control region. Nine *Cerdocyon thous* haplotypes are used as the outgroup (CE, see Table 1). Labels are haplotype identification numbers (see Table 1) and sites of geographic origin of haplotypes (see Fig. 1; haplotypes without indication are samples of unknown origin that are also present on G04 and G08). Values above branches indicate bootstrap support for each node (only values above 50% are indicated).

Figure 3. Maximum likelihood tree (built using the GTR+G+I model) of 49 *Lycalopex* spp. mtDNA haplotypes, based on 588 bp of the control region (only complete sequences were used). *Cerdocyon thous* haplotypes (CE) are used as the outgroup. Labels are haplotype identification numbers (see Table 1) and sites of geographic origin (see Fig. 1; haplotypes without any indication are samples of unknown origin that are also present on G04 and G08). Values above branches indicate bootstrap support for selected nodes obtained from MP, NJ and ML, respectively (asterisks indicate values below 50).

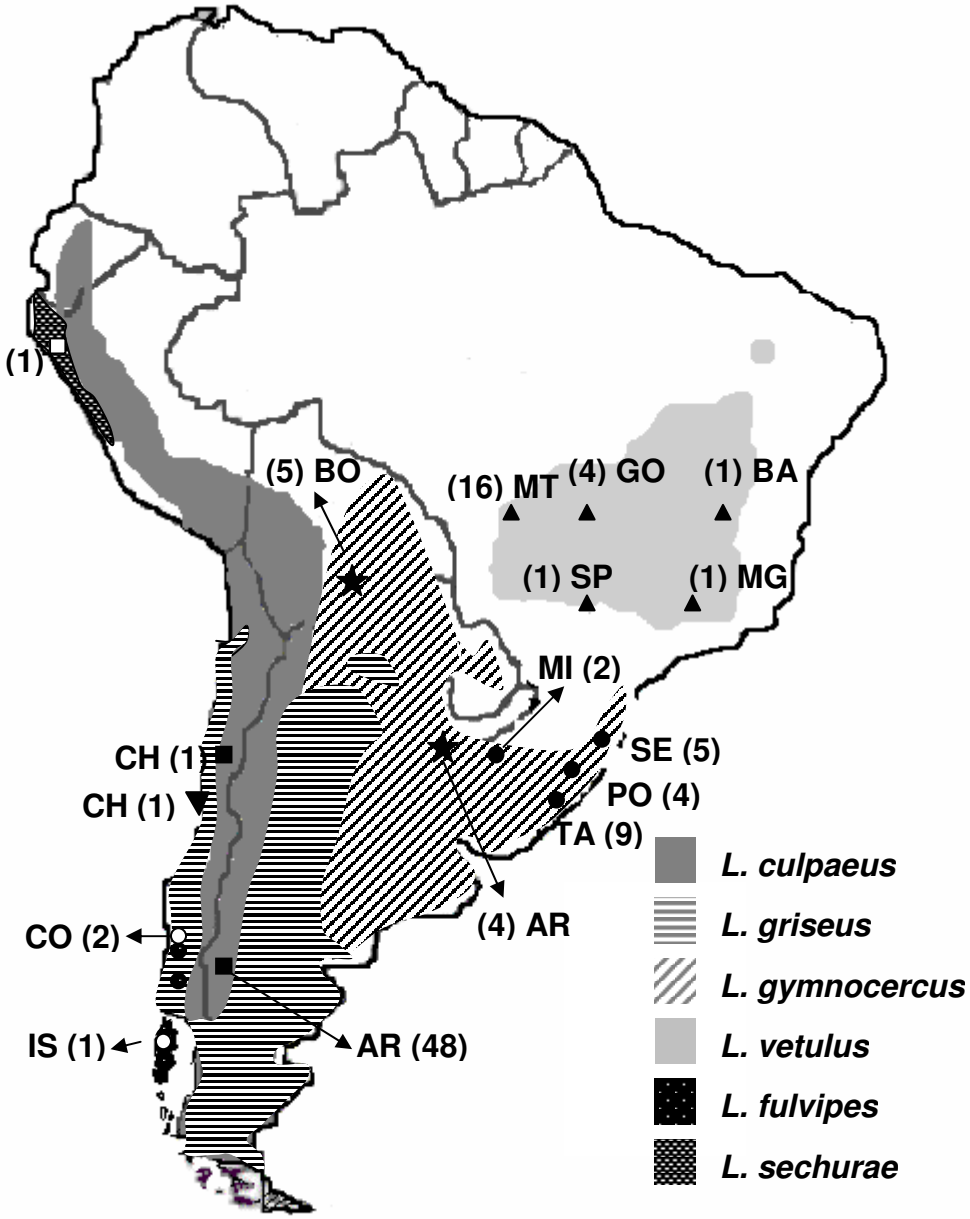
Figure 4. Maximum likelihood tree (reconstructed with the GTR+G+I model) of *Lycalopex* species mitochondrial DNA haplotypes (52 haplotypes [complete sequence data + short *L. sechurae* and *L. fulvipes* haplotypes]) identified in this study, based on 588 bp of the control region. *Cerdocyon thous* haplotypes are used as outgroup (CE, see Table 1). Labels are haplotype identification numbers (see Table 1). Values above branches indicate support for each node obtained from MP, NJ and ML respectively; values under 50% are indicated as *.

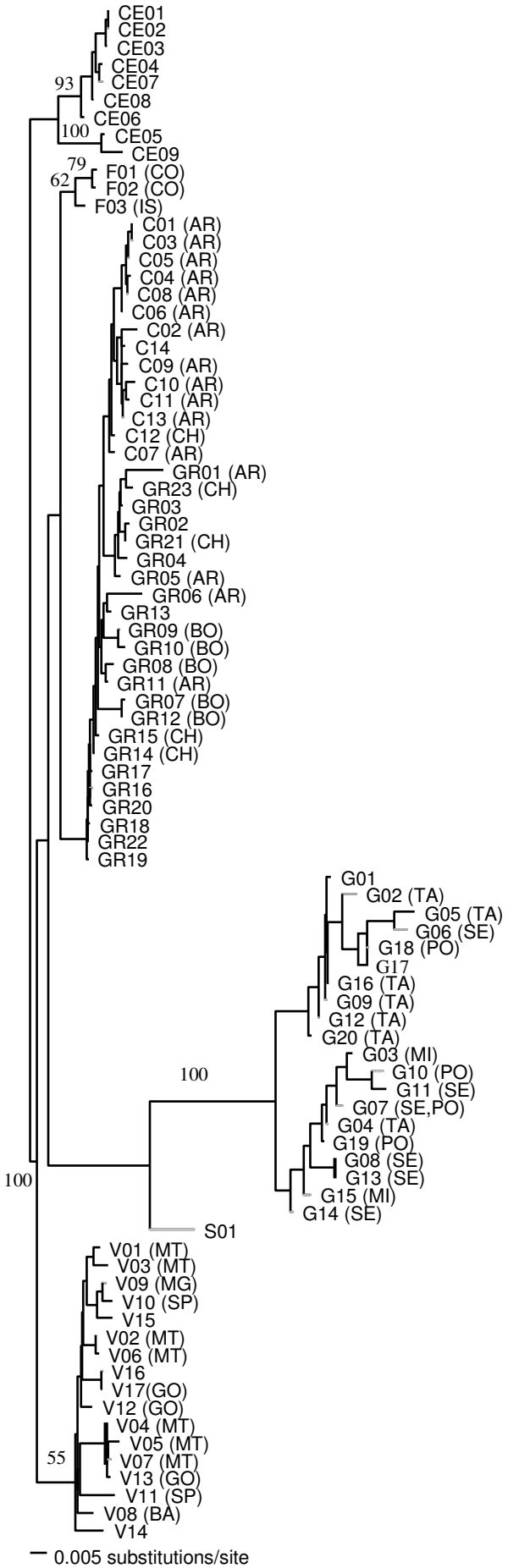
Figure 5. Networks of *Lycalopex vetulus* (a) *and Lycalopex culpaeus* (b) mtDNA control region (588bp and 626pb respectively) haplotypes generated with TCS using a 95% threshold for parsimony-based connections. Squares indicate haplotypes likely to be ancestral in each network (highest outgroup probability). The area of circles and squares is roughly proportional to the frequency of each haplotype. Haplotypes are identified as in Table 1 and geographic origin are indicated on right (see Fig. 1; haplotypes without indication have unknown geographic origin).

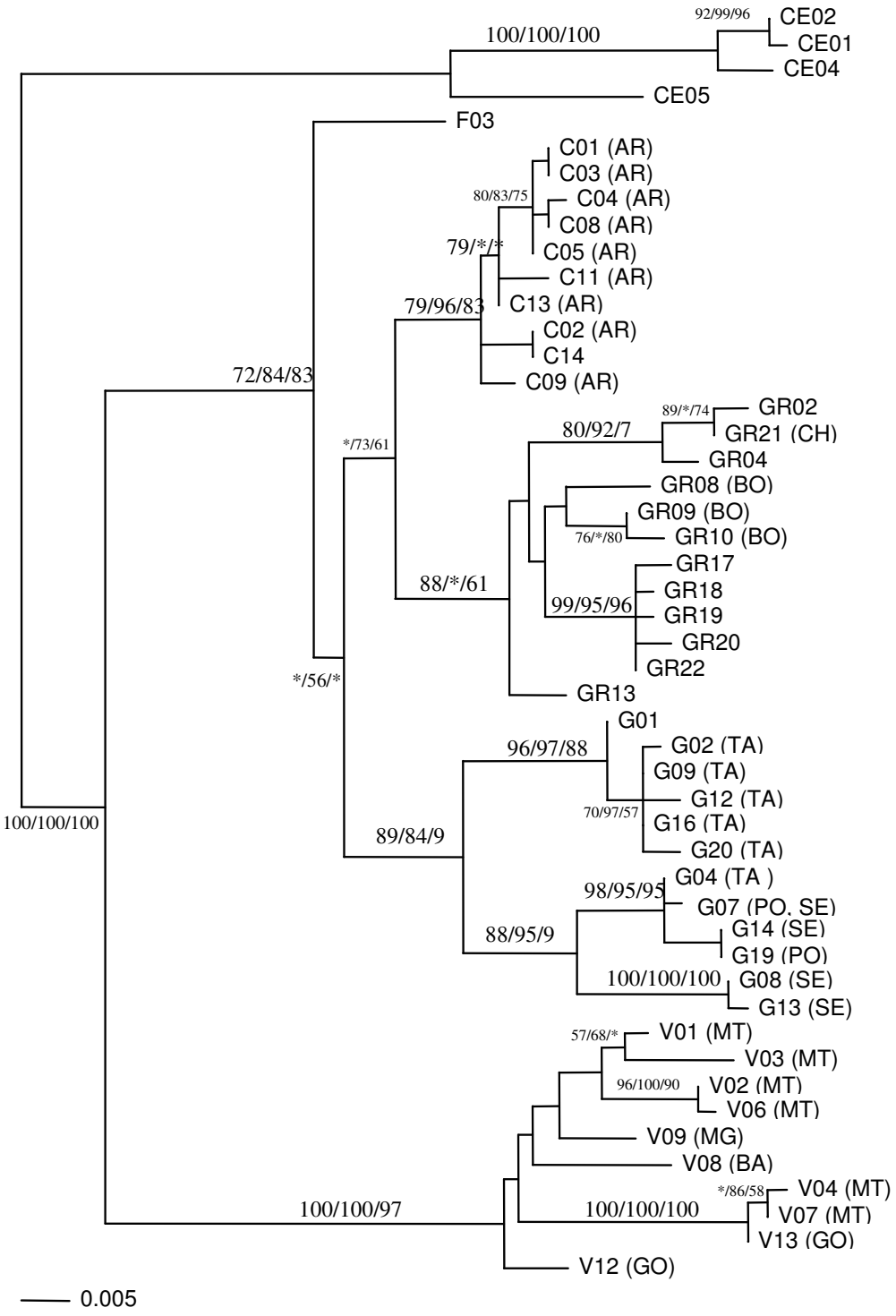
Figure 6. Networks of *Lycalopex fulvipes* (a) *and Lycalopex griseus* (b) mtDNA control region (627bp and 624pb respectively) haplotypes generated with TCS using a 95% threshold for parsimony-based connections. Squares indicate haplotypes likely to be ancestral in each network (highest outgroup probability). The area of circles and squares is roughly proportional to the frequency of each haplotype. Haplotypes are identified as in Table 1 and geographic origins are indicated on the right (see Fig. 1; haplotypes without indication or bearing an * have unknown geographic origin).

Figure 7. Networks of *Lycalopex gymnocercus* mtDNA control region (588pb) haplotypes generated with TCS using a 95% threshold for parsimony-based connections. Squares indicate haplotypes likely to be ancestral in each network (highest outgroup probability). The area of circles and squares is roughly proportional to the frequency of each haplotype. Haplotypes are identified as in Table 1 and geographic origin are indicated on right (See Fig. 1; haplotypes without indication and those with an * have unknown geographic origin).

Figure 8. Graphics depicting the result of the Mismatch Distribution Analysis performed with *Lycalopex* species based on their mtDNA control region sequences.


(1)
(5) BO
(16) MT
(4) GO
(1) BA
(1) SP
(1) MG
MI (2)
SE (5)
PO (4)
TA (9)
CH (1)
CH (1)
(4) AR
CO (2)
IS (1)
AR (48)
L. culpaeus
L. griseus
L. gymnocercus
L. vetulus
L. fulvipes
L. sechurae


CE01
CE02
CE03
CE04
93
CE07
CE08
CE06
100
CE05
CE09
79
62
F01 (CO)
F02 (CO)
F03 (IS)
C01 (AR)
C03 (AR)
C05 (AR)
C04 (AR)
C08 (AR)
C06 (AR)
C02 (AR)
C14
C09 (AR)
C10 (AR)
C11 (AR)
C13 (AR)
C12 (CH)
C07 (AR)
GR01 (AR)
GR23 (CH)
GR03
GR02
GR21 (CH)
GR04
GR05 (AR)
GR06 (AR)
GR13
GR09 (BO)
GR10 (BO)
GR08 (BO)
GR11 (AR)
GR07 (BO)
GR12 (BO)
GR15 (CH)
GR14 (CH)
GR17
GR16
GR20
GR18
GR22
GR19
G01
G02 (TA)
G05 (TA)
G06 (SE)
G18 (PO)
G17
G16 (TA)
G09 (TA)
G12 (TA)
G20 (TA)
G03 (MI)
G10 (PO)
G11 (SE)
G07 (SE,PO)
G04 (TA)
G19 (PO)
G08 (SE)
G13 (SE)
G15 (MI)
G14 (SE)
100
100
S01
V01 (MT)
V03 (MT)
V09 (MG)
V10 (SP)
V15
V02 (MT)
V06 (MT)
V16
V17(GO)
V12 (GO)
V04 (MT)
V05 (MT)
55
V07 (MT)
V13 (GO)
V11 (SP)
V08 (BA)
V14
0.005 substitutions/site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100/100/100
CE01
CE04
CE05
F03
C01 (AR)
C03 (AR)
80/83/75
C04 (AR)
C08 (AR)
79/*/*
C05 (AR)
C11 (AR)
C13 (AR)
79/96/83
C02 (AR)
C14
72/84/83
C09 (AR)
89/*/74
GR02
80/92/7
GR21 (CH)
*/73/61
GR04
GR08 (BO)
GR09 (BO)
76/*/80
GR10 (BO)
GR17
88/*/61
GR18
99/95/96
GR19
GR20
GR22
/56/
GR13
G01
96/97/88
G02 (TA)
G09 (TA)
G12 (TA)
70/97/57
G16 (TA)
100/100/100
89/84/9
G20 (TA)
G04 (TA)
98/95/95
G07 (PO, SE)
G14 (SE)
88/95/9
G19 (PO)
100/100/100
G08 (SE)
G13 (SE)
57/68/*
V01 (MT)
V03 (MT)
96/100/90
V02 (MT)
V06 (MT)
V09 (MG)
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V04 (MT)
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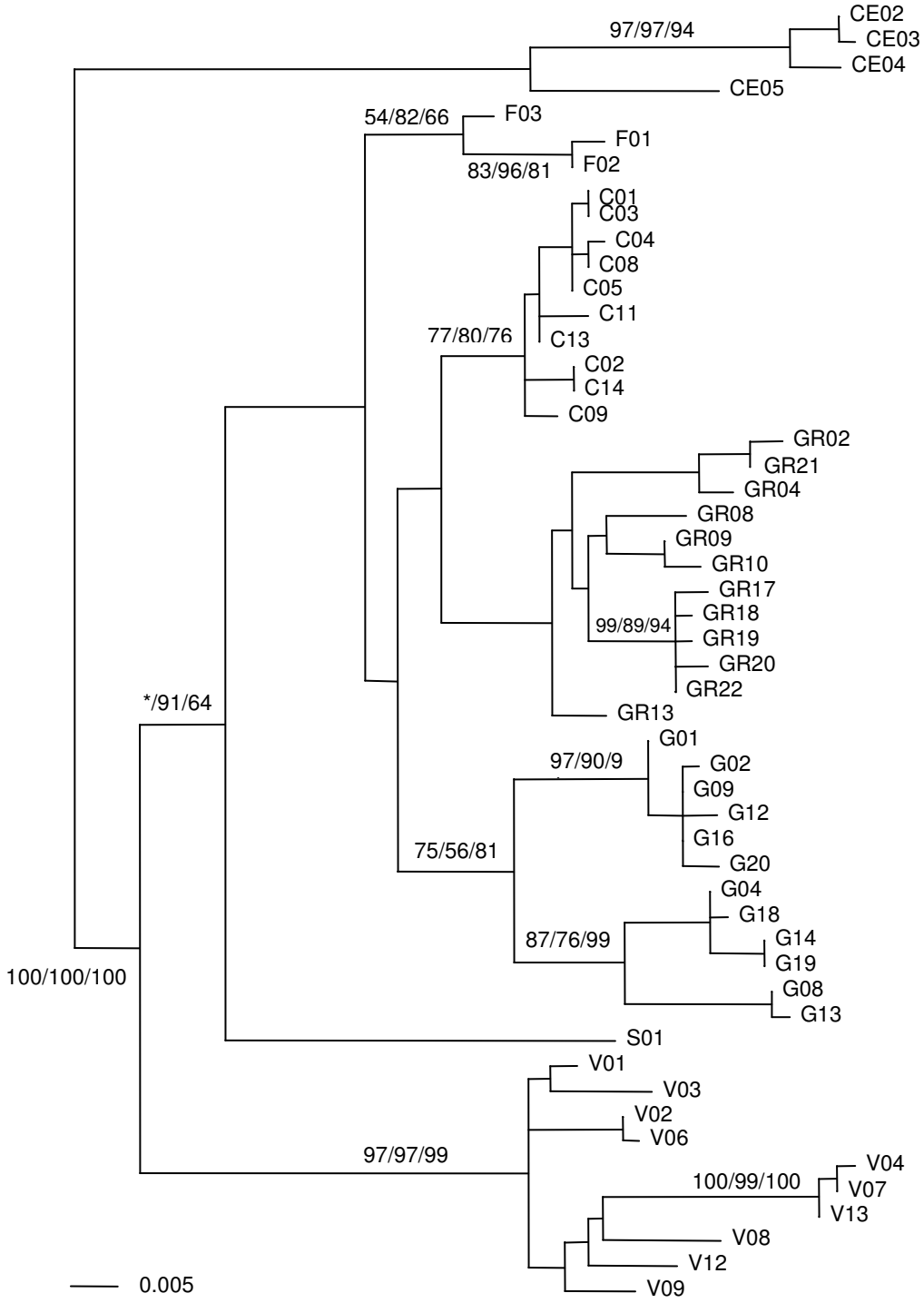
100/100/100
V07 (MT)
V13 (GO)
V12 (GO)
0.005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CE03
CE04
97/97/94
CE05
54/82/66
F03
F01
83/96/81
F02
C01
C03
C04
C08
C05
C11
77/80/76
C13
C02
C14
C09
GR02
GR21
GR04
GR08
GR09
GR10
GR17
GR18
99/89/94
GR19
GR20
GR22
GR13
*/91/64
G01
97/90/9
G02
G09
G12
G16
75/56/81
G20
G04
G18
87/76/99
G14
G19
G08
G13
100/100/100
S01
V01
V03
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V06
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V04
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V07
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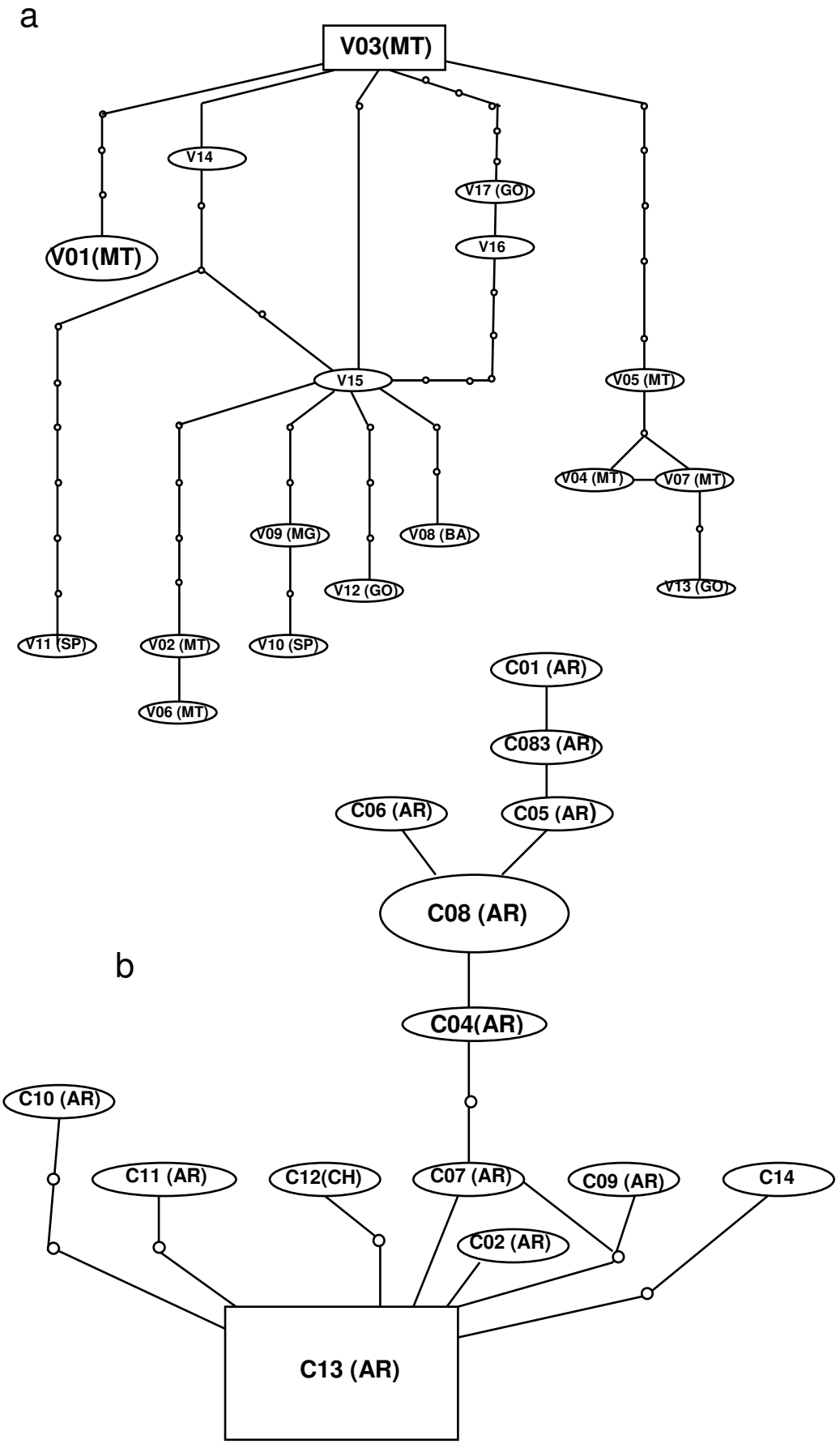
V12
V09
0.005


a
V03(MT)
V14
V17 (GO)
V01(MT)
V16
V15
V05 (MT)
V04 (MT)
V07 (MT)
V09 (MG)
V08 (BA)
V12 (GO)
V13 (GO)
V11 (SP)
V02 (MT)
V10 (SP)
V06 (MT)
C01 (AR)
C083 (AR)
C06 (AR)
C05 (AR)
C08 (AR)
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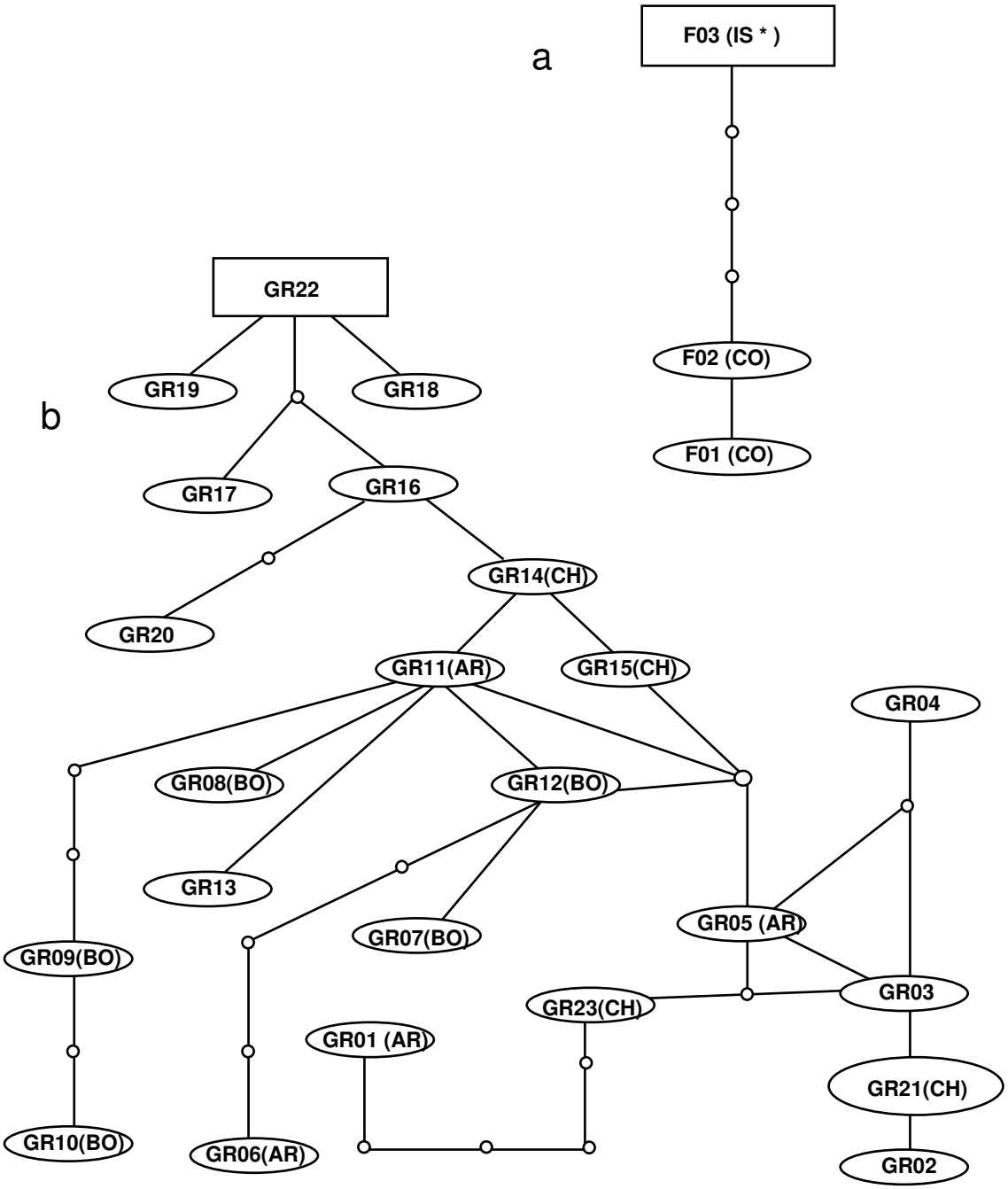
C14
C02 (AR)
C13 (AR)


a
F03 (IS *)
F02 (CO)
F01 (CO)
b
GR22
GR19
GR18
GR17
GR16
GR20
GR14(CH)
GR11(AR)
GR15(CH)
GR04
GR08(BO)
GR12(BO)
GR13
GR07(BO)
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GR01 (AR)
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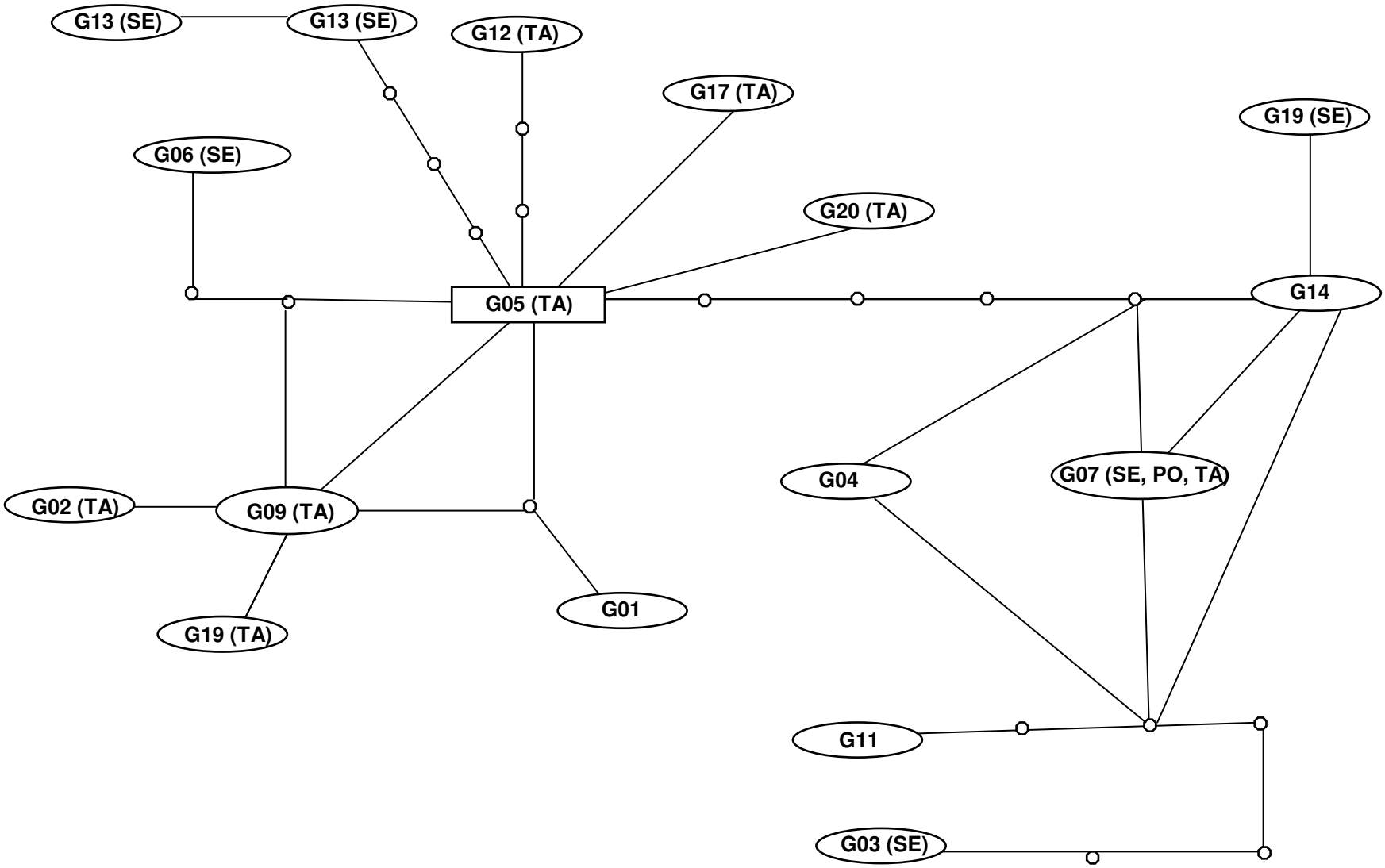
GR06(AR)
GR02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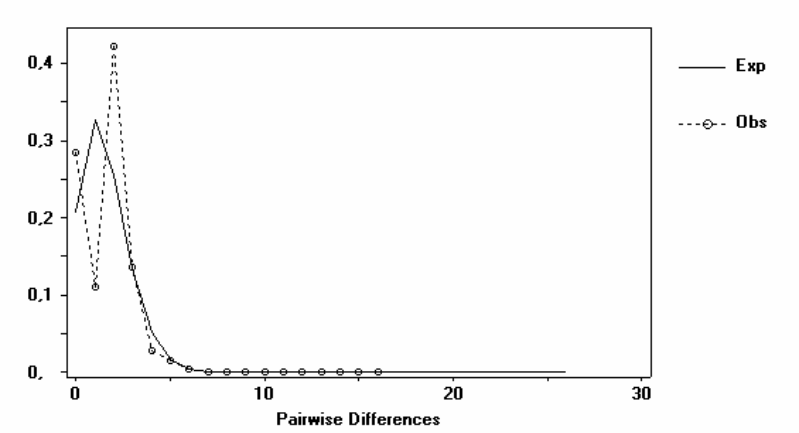
G05 (TA)
G14
G04
G07 (SE, PO, TA)
G02 (TA)
G09 (TA)
G01
G19 (TA)
G11
G03 (SE)

L. culpaeus



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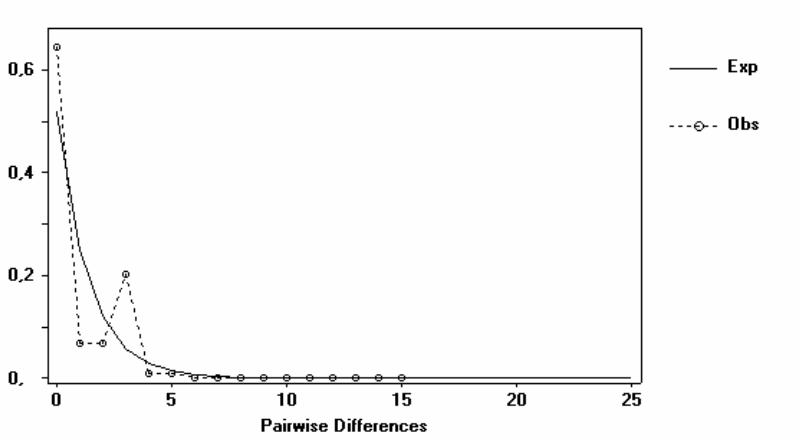

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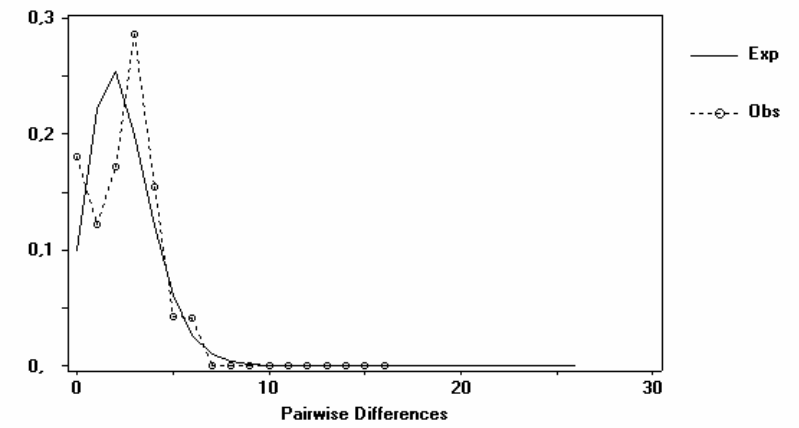

L. vetulus

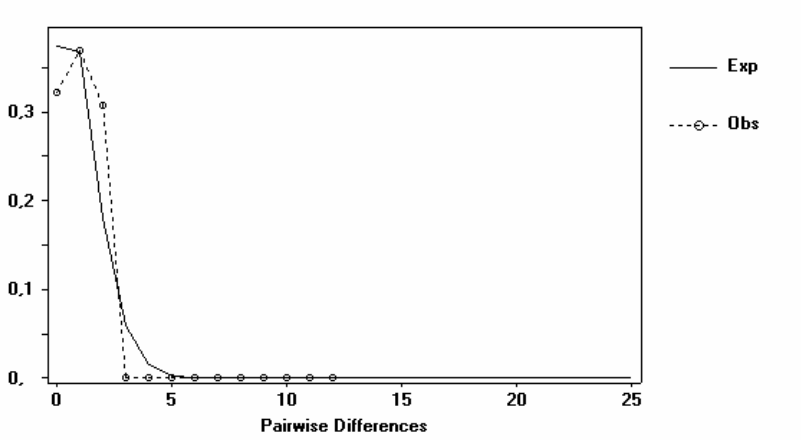

Table 1a. Mitochondrial DNA control region haplotypes identified from *Lycalopex* spp. and *Cerdocyon thous* samples. Only first 110 variable sites are shown. Site numbers (vertical notation) refer to the aligned position in our 588 bp data set

Species	Haplotipe (Fr)	Nucletide Position 4444444444444444444444444444444444444444444444455555555555555555555555555555555555555555555555555555555555555555555555555 4555555555666666666677777777788888888999999990000000011111111222222233333333444444444555555555666666677777788 9123456789012345678901245678912345789013467891234567890345678923456792345678901234568901234567901346783456787 8
C. thous	CE01 (1)	AAACACTATACGCTCTTCTTCGGAAGCTTGATTTAGGAAATATACCCTAGCTCAGGAATAAATAAACCATTCTCGTCACGCCGCTCGCAACTATTCCCGAACCTGTATCT
	CE02 (1)	.....
	CE03 (1)	.....
	CE04 (1)	....T.....A.....C.....T.....
	CE05 (1)	.....T.....T.....T.....A.....G...T...A.....C.....CTC.T.T.T.....T..TC.....
	CE06 (1)	??????????????????????.....TG...A.....A.....C.....T.....
	CE07 (1)	?????????.....A.....C.....T.....
	CE08 (1)	???????????.....G.....T..A.....C.....T.....
	CE09 (1)	???????????.....T.....T.....C.A.....G...T...A.....G....C.....CTC.T.T.....T...T..T?G...T.....
L.fulvipes	F01 (1)	.....GC.TA..G..T.CT..GG.T...T....A....A...GTT.TAT....A....T.....A...C.CT.??????????????????????????????????????
	F02 (1)	.....GC.TA..G..T.CT..GG.T...T....?....A...GTT.TAT....A....T.....C.CT??????????????????????????????????????
	F03 (4)	.....GC.T...G..T.CT...G.T...T....A....A...GTT.TAT.T..A....TG.....C.CT.CT.T.....T..G.TC...T.....AA.....
L.culpaeus	C01 (1)	.....GC.TA..G..T.CT...G..C..T....A....A...G.T.TATCT..A.G..C.....CT.CT.T.....T..G..C.....
	C02 (1)	.....GC.TA..G..T.CT...G.....C....A....A...A.T.TATCT..A.G..C.....C.CT.CT.T.....T..G..C...T.....AAGTATCTG
	C03 (2)	.....GC.TA..G..T.CT...G..C..T....A....A...G.T.TATCT..A.G..C.....CT.CT.T.....T..G..C.....
	C04 (5)	.....GC.TA..G..T.CT.....C..T....A....A...A.T.TATCT..A.G..C....G.....CT.CT.T.....T..G..C.....
	C05 (2)	.....GC.TA..G..T.CT...G..C..T....A....A...A.T.TATCT..A.G..C.....CT.CT.T.....T..G..C.....
	C06 (6)	.....GC.TA..G..T.CT.....C..T....A....A...A.T.TATCT..A.G..C.....CT.CT.T.....A??????????????????????????
	C07 (1)	.....GC.TA..G..T.CT.....T....A....A...A.T.TATCT..A.G..C....G....C.CT.??????????????????????????????????
	C08 (12)	.....GC.TA..G..T.CT.....C..T....A....A...A.T.TATCT..A.G..C.....CT.CT.T.....T..G..C.....
	C09 (2)	.....GC.TA..G..T.CT...G.....T....A....A...ATT.TATCT..A.G..C....G....C.CT.CT.T.....T..G..C...T.....
	C10 (1)	.....GC.TA..G..T.CT.A.G.....?....A....A...A.T.TATCT..ACG..C.....G..C.CT.CT.T.....T..G..C.....
	C11 (4)	.....GC.TA..G..T.CT...G.....C....A....A...A.T.TATCT..A.G..C.....C.CT.CT.T...A..T..G..C.....
	C12 (1)	.....GC.TA..G..T.CT.....T....A....A...G.T.TATCT..A.G..C.....C.CT.??????????????????????????????????
	C13 (20)	.....GC.TA..G..T.CT...G.....T....A....A...A.T.TATCT..A.G..C.....C.CT.CT.T.....T..G..C.....
	C14 (1)	.....GC.TA..G..T.CT...G.....T....A....A...A.T.TATCC..A.G..C.....C.CT.CT.T.....T..G..C.....
L.vetulus	V01 (6)	?.....-GC.T...AA.TC.T...G.T.....A..T.GGC.TAT.CAT....A....TG.....CC.C.ACTCT.....G..C..CT.T.....
	V02 (2)	?.....GC.T...AA.TC.T...G.T.....C.A..T.AGC.TAT.CAT....A....TG.....CC.CT.CTCT.....G..C..CT.T.....
	V03 (4)	?.....GC.T...AA.TC.T...G.T..A.....A..T.GGC.TAT.CATC.....TG.....CC.C..CTCT.....G.TC..CT.T.....
	V04 (1)	?....T.GC.T...AA.TC.T...G.T.....AAGT.AGC.TATCCAT.T..A....C.....CC..TACTCT.....C..CT.T.....
	V05 (1)	??????????????????????T..G.T.....AAGT.AGC.TATCCAT.T..A....C.....CC..TACTCT.A.....C..CT.T.....???????
	V06 (1)	?..A...GC.T...AA.TC.T...G.T.....C.A..T.AGC.TAT.CAT....A....TG.....CC.CT.CTCT.....G..C..CT.T.....
	V07 (1)	?....T.GC.T...AA.TC.T...G.T.....AAGT.AGC.TATCCAT.T..A....C.....CC..TACTCT.....C..CT.T.....
	V08 (1)	?....T.GC.T...AA.TC.T..G..T.....A..T.GGC.TAT.CAT.T..A....T.....CC..T.CTCT.....T....TC..CT.T.....
	V09 (1)	?....T.GC.T...AA.TC.T...G.T.....A..T.GGC.TAT.CAT....A....TG.....CC..T.CTCT.....G.TC...T.TA.....
	V10 (1)	???????????...AA.TC.T...G.T.....A..T.GGC.TAT.CAT...AA....TG.....CC..T.CTCT.....G.TC...T.TA.....
	V11 (1)	?????????.T...AA.TC.T.AG..T.....A..T.AGC.TAT.CAT...AA....CG.....CC..T.CT.T...A.....G..C...TTT.....
	V12 (1)	.....T?GC.T...AA.TCC?...G.T.....C.A..T.GGC.TAT.CAT....A....C.....CC.CT.CTCT.....G.TC..CT.T.....
	V13 (1)	?....T.GC.T...AA.TC.T...G.T.....AAGT.AGC.TATCCAT....A....C.....CC..TACTCT.....C..CT.T.....
	V14 (1)	.....T.GC.T...A..TC.T.....T...T..C.A....GGC.TAT.CAT....A....C.....CC..T??????????????????????????????????
	V15 (1)	.....T.GC.T...AA.TC.T.....T.....C.A..T.GGC.TAT.CAT....A....T.....CC..T.CTCT.....??????????????????????
	V16 (1)	????????????????.TC.T...G.T.....C.A..T.AGC.TAT.CAT....A.G..T.....CC.CTA.TCT.....T..G.TC..CT.T.....?
	V17 (1)	????????CGTA..AA.TC.T...G.T.....C.A..T.AGC.TAT.CAT....A.G..T.....CC.CTA.TCT.....T..G.TC..CT.T.....

Table 1a. continued

L.griseus	GR01	(1)	.......GC.TA..G..T.CT.........T....A....AG..A.T.CAT..G.A....CG....??
	GR02	(1)	.......GC.TA..G..T.CT...G.....T....A....A...A.T.TA.CTG.A....C......G.CC.CT..T.T......TA....C...T..............
	GR03	(1)	.......GC.TA..G..T.CT........?T....A....A...A.T.TA.CTG.A....C........CC.CT.??????????????????????????????????
	GR04	(1)	.......GC.TA..G..T.CT.........T....A....A...A.T.TA.CTG......C.........C.CT..T.T......TA....C...T..............
	GR05	(1)	.......GC.TA..G..T.CT.........T....A....A...A.T.TA.CT..A....C.....??
	GR06	(1)	.......GC.TA..G..T.CT...G.....T..C.A....AG..A.T.TATCT..A....CG...G??
	GR07	(1)	???????????A.GG..TCCT...G.T...C....A....A...A.T.?ATCT..A...GC.........C.CT..T.T......TA...TC...T..............
	GR08	(1)	??G....GC.TA..G..TCCT.........T....A....A...A.T.TATCT..A....C...?.....C..T.CT.T......TA...T....T..............
	GR09	(1)	?......GC.TA..G.CTCCT.........T....A....AG..A.T.TATCTG.A....C.........C..T.CT.T......TA...TC...T..............
	GR10	(1)	??....?GC.TA..G.CTCCT.........?....A....AG..A...TATCTG.A....CG..?.....C..T.CT.T......TA...TC...T..............
	GR11	(1)	.......GC.TA..G..TCCT.........T....A....A...A.T.TATCT..A....C.....??
	GR12	(1)	??????????????????????????????????.A....A...A.T.TATCT..A...GC.........C.CT..T.TT.....TA...TC...T..............
	GR13	(1)	.......GC.TA..G..TCCT.........T....A....AG..A.T.TATCT..A....C.........C.CT.CT.T......T....TC...T...G........T.
	GR14	(1)	.......GC.TA..G..TCCT.........T....A....A...A...TATCT..A....C.........C.CT.??????????????????????????????????
	GR15	(1)	.......GC.TA..G..T.CT.........T....A....A...A...TATCT..A....C.........C.CT.??????????????????????????????????
	GR16	(1)	.......GC.TA..G..TCCT...G.....T....A....A...A...TATCT..A....C.........C.CT.??????????????????????????????????
	GR17	(1)	.......GC.TA..G..TCCT...G.....T....A....A...A...TATCT..A....C.........C.CT.CT.T......TA...TCG..T.....AA.......
	GR18	(1)	.......GC.TA..G..TCCT...G.....T....A....A...A...TATCT..A....C.........C.CT.CT.T......TA...TCGG.T.....AA.......
	GR19	(2)	.......GC.TA..G..TCCT...G.....T....A....A...A...TATCT..A....C.........C.CT.CT.T......TA...TCG..T.....AA.......
	GR20	(1)	.......GC.TA..G..TCCT...G.....T....A....A...A...TATCT..A....C.........C.CT.CT.T......TA...TCG..T.....AA.......
	GR21	(4)	.......GC.TA..G..T.CT...G.....T....A....A...A.T.TA.CTG.A....C........CC.CT..T.T......TA....C...T..............
	GR22	(4)	.......GC.TA..G..TCCT...G.....T....A....A...A...TATCT..A....C.........C.CT.CT.T......TA...TCG..T.....AA.......
	GR23	(2)	.......GC.TA..G..T.CT...G.....C....A....A...A.T.TATCT..A.G..C.........C.CT.CT.T......T..G..C...T.....AA.......
L.gymnocercus	G01	(1)	.......GC.T...G..T.CT...G.T...T....A....A...ATT.CATCTG....C.C..G......C.CT...TT......T..G.TC.......GG..GTATCTG
	G02	(1)	?G.....GC.T...G..T.CT.....T...T....A....A...ATT.CATCTG....C.C..G......C.CT...TT......T..G.TC.......GG..GTATCTG
	G03	(1)	??????????????????????.....T...T..C.A....A...A.TCTAT.T..A....C..G.....CC.CT...TT..T...T..GGTC.....T.....GTATCTG
	G04	(1)	.......GC.T.T.G..T-C?.....T...C.CC.A....A...A.TCTAT.T..A....C..G......C.CT...TT......T..GGTC...........GTATCTG
	G05	(1)	??.C..G?...?.C.CT?????......T..G.TC.?.....GG?.GTATCTG
	G06	(1)	??????????????????????????TT..?T....A....A...ATT.CATCTG....C.C..G......C.CT...TTT.....TC.G.TC.......G?..GTATCTG
	G07	(2)	G......GC.T.T.G..T.CT.....T...C.CC.A....A...A.TCTAT.T..A....C..G......C.CT...TT......T..GGTC...........GTATCTG
	G08	(2)	?......GC.T...G..T.CT...G.T...T.CC.A....AG..A.TCTA..TG.A....T..G......CTC....TT......T..G.TC.....T.....GTATCTG
	G09	(1)	.......GC.T...G..T.CT.....T...T....A....A...ATT.CATCTG....C.C..G......C.CT...TT......T..G.TC.......GG..GTATCTG
	G10	(1)	??????????????????????.....T.G.C.CC.A....A...A.TCTAT.T..A....C..G......C.CT...TT......T..GGTC...........GTATCTG
	G11	(1)	??..A....C..G......C.CT...TT......TC.GGTC...........GTATCTG
	G12	(1)	.......-C.T...G..T.CT.....T...T....A....A...ATT.CATCTG....C.C..G......C.CT...TT......T..G.TC.......GG..GTATCTG
	G13	(1)	.......GC.T...G..T.CT...G.T...T.CC.A....AG..A.TCTA..TG.A....T..G......CTC....TT......T..G.TC.....T.....GTATCTG
	G14	(1)	......GGC.T.T.G..T.CT.....T...C.CC.A....A...A.TCTAT.T..A....C..G......C.CT...TT......T..GGTC...........GTATCTG
	G15	(2)	?????????.G.T.G..T.C?.....T...C.CC.A....A...A.TCTAT.T..A....C..G......C.CT...TT......T..GGTC...........GTATCTG
	G16	(2)	.......GC.T...G..T.C?.....T...T....A....A...ATT.CATCTG....C.C..G......C.CT...TT......T..G.TC.......GG..GTATCTG
	G17	(1)	??????-GCCT...G..T.C?.....T...T...TA....C..TATT.CATCTG....C.C..GC...C.C.CT...TT......T..G.TC.......GG..GTATCTG
	G18	(1)	??????????????????????.....T...TG..?A....A...ATT.CATCTG....C.C..G......C.CT...TT......T..G.TC.......GG..GTATCTG
	G19	(1)	......-GC.T.T.G..T.CT.....T...C.CC.A....A...A.TCTAT.T..A....C..G......C.CT...TT......T..GGTC...........GTATCTG
	G20	(1)	......GG?.T...G..T.C?.?...T...T....A....?...ATT.CATCTG....C.C..GC.....C.CT...TT......T..G.TC.......GG..GTATCTG
L.sechurae	S01	(1)	......GC.T..CG..T..T.....T...T..C.A...TG.CTT?TCTATC...A....C.........C..T.??????????????????????????????????

Fr frequency on the sample

Table 1b. Mitochondrial DNA control region haplotypes identified from *Lycalopex* spp. and *Cerdocyon thous* samples. Only latter 110 variable sites are shown. Site numbers (vertical notation) refer to the aligned position in our 588 bp data set.

Species	Haplotipe	Nucleotide Position
		4444444444444444444444444444444444444444444455555555555555555555555555555555555555555555555555555555555555555555555555
		4555555555666666666677777777788888888999999990000000001111111122222223333333344444444555555555666666677777788
		9123456789012345678901245678912345789013467891234567890345678923456792345678901234568901234567901346783456787 8
C. thous	CE01 (1)	GATCTGCTATCACTCACCTATGACACGACGCACTACTCTACTATCCTTGCTTTCAGAATATGCGTCGTGCTAA??
	CE02 (1)	..........CGCAGTCAGATATATGTAGCTGACTATCACATTTAGC
	CE03 (1)	..........G.....CGCAGTCAGATATATGTAGCTGACTATCACATTTAGC
	CE04 (1)	..........CGCAGTCAGATATATGTAGCTGACTATCACATTCGGC
	CE05 (1)	..........T.......GT..........C....G.........AC.....TGCAGTCAGATATATGTAGCTGACTATCACATTTAGC
	CE06 (1)	..........CGCAGTCAGATATATGTAGCTGACTAT?????????
	CE07 (1)	..........?????..........??????????????????????????????????
	CE08 (1)	..........?????????????????????????????????????
	CE09 (1)	..........?.......GT..........C.............AC.?????????????????????????????????????
L.fulvipes	F01 (1)	??
	F02 (1)	??
	F03 (4)	..........A.......G.............T.G..TC....C.............C.....TGCAGTCAGATATATGTAGCTGACTATCACATATAGC
L.culpaeus	C01 (1)	..........G.......G.A.............G..TC....C.............C.....CGCAGTCAGATATATGTAGCTGACTATCATATAT???
	C02 (1)	-.........G.......G.A.............G..TC....C.............C.....CGCAGTCAGATATATGTAGCTGACTATCATATATAGC
	C03 (2)	..........G.......G.A.............G..TC....C.............C.....CGCAGTCAGATATATGTAGCTGACTATCATATATAGC
	C04 (5)	..........G.......G.A.............G..TC....C.............C.....CGCAGTCAGATATATGTAGCTGACTATCATATATAGC
	C05 (2)	..........G.......G.A.............G..TC....C.............C.....CGCAGTCAGATATATGTAGCTGACTATCATATATAGC
	C06 (6)	??
	C07 (1)	??
	C08 (12)	..........G.......G.A.............G..TC....C.............C.....CGCAGTCAGATATATGTAGCTGACTATCATATATAGC
	C09 (2)	..........G.......G.A.............G..TC....C.............C.....CGCAGTCAGATATATGTAGCTGACTATCATATATAGC
	C10 (1)	..........G.......G.A.............G..TC....C.............C.....CGCAGTCAGATATATGTAGCTG??????????????
	C11 (4)	..........G.......G.A.............G..TC....C.............C.....CGCAGTCAGATATATGTAGCTGACTATCATGTATAGC
	C12 (1)	??
	C13 (20)	..........G.......G.A.............G..TC....C.............C.....CGCAGTCAGATATATGTAGCTGACTATCATATATAGC
	C14 (1)	..........G.......G.A.............G..TC....C.............C.....CGCAGTCAGATATATGTAGCTGACTATCATATATAGC
L.vetulus	V01 (6)	..........T.......G.............TC....C.............C.....CGCAGTCAAATACATGTAGCTGAC?????????????
	V02 (2)	..........GT..........TC..........C.....CGCAGTCAAATACATGTAGCTGAC?????????????
	V03 (4)	..........T...T.......G.............TC....C.............AC.....CGCAGTCAAATACATGTAGCTGAC?????????????
	V04 (1)	..........T.....TC....C.....G..C..??????????????????????????????????????
	V05 (1)	??
	V06 (1)	..........GT..........TC..........C.....CGCAGTCAAATACATGTAGCTGAC?????????????
	V07 (1)	..........T.....TC....C.............C...G.CGCAGTCAA??????????????????????????
	V08 (1)	..........G.....TC..........C.....CGCAGTCAAATACATGTAGCTGAC?????????????
	V09 (1)	..........T.......G.....TC....C.............C.....CGCAGTCAAATATATGTAGCTGAC?????????????
	V10 (1)	..........G.....TC....C.............C.??????????????????????????????????????
	V11 (1)	..........C.TC....C.............C.....CGCAGTCAGATACATGTAGCTGAC?????????????
	V12 (1)	..A.....G.....TC....C.............C.....CGCAGTCAAATACATGTAGCTGAC?????????????
	V13 (1)	..........T.....TC....C.............C.-.G.CGCAGTCAAGTACATGTAGCTGAC?????????????
	V14 (1)	??
	V15 (1)	??
	V16 (1)	..........T.......G.....TC....C.............C.-...CGCAGTCAAATACATGTAGCTGAC?????????????
	V17 (1)	..........T.......G.....TC....C.............C.....??????????????????????????????????

Table 1b. continued

L.griseus	GR01	(1)	???
	GR02	(1)	.................A.......G.A...................TC....C..............C.....TGCAG-CAAGTACATGTAGCTGACTATCACATATAGT
	GR03	(1)	???
	GR04	(1)	.................A.......G.A...................TC....C..............C.....CGCAG-TAAG-ATATGTAGCTGACTATCACATATAGC
	GR05	(1)	???
	GR06	(1)	???
	GR07	(1)	.................G.......G.A...................TC.....................C.....CGCAGTCAAATATATGTAGCTGAC?????????????????
	GR08	(1)	.................A.......G.A...................TC.........G..........C.....TGCAGTCAAATATATGTAGCT???????????????????
	GR09	(1)	.................A.......G.A...................TC.....................C.....TGCAGTCAAATACATGTAGCTGACTAT??????????
	GR10	(1)	.................A.......G.A...................TC.....................C.....TGCAGTCAAATACATGTAGCTGACTAT??????????
	GR11	(1)	???
	GR12	(1)	.................G.......G.A...................TC.....................C.....CGCAGTCAAATATATGTA?????????????????????
	GR13	(1)	.................A.......G.A...................TC....C..............C.....TGCAGTCAAATACATGTAGCTGACTATCACATATAGC
	GR14	(1)	???
	GR15	(1)	???
	GR16	(1)	???
	GR17	(1)	.................A.......G.A...................TC.....................C.....TGCAGTCAAATACATGTAGCTGACTATCACATATACG
	GR18	(1)	.................A.......G.A...................TC.....................C.....TGCAGTCAAATACATGTAGCTGACTATCACATATAGC
	GR19	(2)	.................A.......G.A...................TC.....................C.....TGCAGTCAAATACATGTAGCTGACTATCACACATAGC
	GR20	(1)	...............A.A.......G.A...................TC.....................C.....GGCAGTCAAATACATGTAGCTGACTATCACATATAGC
	GR21	(4)	.................A.......G.A...................TC....C..............C.....CGCAG-CAAGTACATGTAGCTGACTATCACATATAGT
	GR22	(4)	.................A.......G.A...................TC.....................C.....TGCAGTCAAATACATGTAGCTGACTATCACATATAGC
	GR23	(2)	.................G.......G.A................G..TC....C..............C.....CGCAGTCAGATATATGTAGCTGACTATCATATATAGC
L.gymnocercus	G01	(1)	ATCTGCTATCACTCA..TATGACGCAACGCACTACTCTACTGTCT..GCTC.CAGA.TATGCGTCGCGCTA.TGCAGTCAGATATATGTAGCTGACTATCAT????????
	G02	(1)	ATCTGCTATCACTCA..TATGACGCAACGCACTACTCTACTGTCT..GCT..CAGA.TATGCGTCGCGCTA.TGCAGTCAGATATATGTAGCTGACTAT??????????
	G03	(1)	ATCTGCTATCACTCAG.TATGACGCAACGCACTACTCTACTGTCT..GCT..CAGA.TATGCGTCGCGCTA.TGCAGTCAGATATATGTAGCTGACTAT??????????
	G04	(1)	ATCTGCTATCACTCAG.TATGACGCAACGCACTACTCTACTGTCT..GCT..CAGA.TATGCGTCGCGCTA.TGCAGTCAGATATATGTAGCTGACTAT??????????
	G05	(1)	ATCTGCTATCACTCA..TATGACGCAACGCACTACTCTACTGTCT..GCT..CAGA.TATGCGTCGCGCTA.TGCAGTCAGATATATGTAGCTGACTAT??????????
	G06	(1)	ATCTGCTATCACTCA..TATGACGCAACGCACTACTCTACTGTCT..GCT..CAGA.TATGCGTCGCGCTA.TGCAGTCAGATATATGTAGCTGACTAT??????????
	G07	(2)	ATCTGCTATCACTCAG.TATGACGCAACGCACTACTCTACTGTCT..GCT..CAGA.TATGCGTCGCGCTA.TGCAGTCAGATATATGTAGCTGACTAT??????????
	G08	(2)	ATCTGCTATCACTCAG.TATGACGCAACGCACTACTCTACTGTCT..GCT..CAG..TATGCGTCGCGCTA.TGCAGTCAGATATATGTAGCTGACTAT??????????
	G09	(1)	ATCTGCTATCACTCA..TATGACGCAACGCACTACTCTACTGTCT..GCT..CAGA.TATGCGTCGCGCTA.TGCAGTCAGATATATGTAGCTGACTAT??????????
	G10	(1)	ATCTGCTATCACTCAG.TATGACGCAACGCACTACTCTACTGTCT..GCT..CAGA.TATGCGTCGCGCTA.TGCAGTCAGATATATGTAGCTGACTAT??????????
	G11	(1)	ATCTGCTATCACTCAG.TATGACGCAACGCACTACTCTACTGTCT..GCT..CAGA.TATGCGTCGCGCTA.TGCAGTCAGATATATGTAGCTGACTAT??????????
	G12	(1)	ATCTGCTATCACTCA..TATGACGCAACGCACTACTCTACTGTCT..GCT..CAGA.TATGCGTCGCGCTA.T?CACTCCGAT?TATGTAGCTGACT?????????????
	G13	(1)	ATCTGCTATCACTCAG.TATGACGCAACGCACTACTCTACTGTCT..GCT..CAG..TATGCGTCGCGCTA.TGCAGTCCGATATATGTAGCTGACTATCAT????????
	G14	(1)	ATCTGCTATCACTCAG.TATGACGCAACGCACTACTCTACTGTCT..GCT..CAGA.TATGCGTCGCGCTA.TGC??????????????????????????????????
	G15	(2)	ATCTGCTATCACTCAG.TATGACGCAACGCACTACTCTACTGTCT..GCT..CAGA.TATGCGTCGCGC??????????????????????????????????????
	G16	(2)	ATCTGCTATCACTCA..TATGACGCAACGCACTACTCTACTGTCT..GCT..CAGA.TATGCGTCGCGCTA.TGCAGTCAGATATATGTAGCTGACTATCAT????????
	G17	(1)	ATCTGCTATCACTCA..TATGACGCAACGCACTACTCTACTGTCT..GCT..CAGA.TATGCGTCGCGCTA.TGCAGTCAGATATATGTAGCTGACTATCAT????????
	G18	(1)	ATCTGCTATCACTCA..TATGACGCAACGCACTACTCTACTGTCT..GCT..CAGA.TATGCGTCGCGCTA.TGCAGTCAGATATATGTAGCTGACTATCAT????????
	G19	(1)	ATCTGCTATCACTCAG.TATGACGCAACGCACTACTCTACTGTCT..GCT..CAGA.TATGCGTCGCGCTA.TGCACTCCGAT?TATGTAGCTGACTA????????????
	G20	(1)	ATCTGCTATCACTCA..TATGACGCAACGCACTACTCTACTGTCT..GCT..CAGA.TATGCGTCGCGCTA.TGCAGTCAGATATA??????????????????????
L.sechurae	S01	(1)	???

Fr frequency on the sample

Table 2. Diversity indices (nucleotide [π] and gene [h] diversity) observed in *Lycalopex* species control region

	N	h	π	Number of haplotypes	Number of Variable sites	Number of Parsimony informative sites
L. culpaeus	53	0.8004 +/- 0.0407	0.005 +-0.002	13	16	10
L. vetulus	26	0.9323 +/- 0.0352	0.023 +-0.004	17	51	33
L.griseus	31	0.9398 +/- 0.0311	0.023 +-0.005	23	50	27
L. gymnocercus	26	0.9723 +/- 0.0209	0.022+-0.004	20	44	29
L. fulvipes	6	0.6000 +/- 0.2152	0.009+-0.004	3	5	4

Table 3. Nucleotide divergence between pairs of species (Dxy, with standard error [se] included) used to infer the intervals of divergence time (reported in years [y]) in this clade of Neotropical canids.

Lineages compared	Dxy (se)	Lower bound (y)	**Mean time (y)**	Upper bound (y)	Inferences
Cerdocyon X *Lycalopex species*	0.088 (0.013)	580,524	**1,125,652**	2,821,782	Genera divergence on Early-Middle Pleistocene
L. gymnocercus X *L. vetulus*	0.080 (0.011)	543,071	**1,086,956**	2,524,752	*L. vetulus* divergence from other *Lycalopex* on Early-Middle Pleistocene
L. griseus X *L. vetulus*	0.079 (0.013)	496,254	**1,076,369**	2,599,009	
L. fulvipes X *L. vetulus*	0.078 (0.016)	430,711	**1,059,782**	2,722,772	
L. sechurae X *L. vetulus*	0.078 (0.016)	430,711	**1,059,782**	2,722,772	
L. culpaeus X *L. vetulus*	0.071 (0.011)	552,434	**964,673**	2,301,980	
L. griseus X *L. sechurae*	0.066 (0.015)	337,078	**896,739**	2,376,237	*L. sechurae* divergence from other *Lycalopex* (except *L. vetulus*) on Early-Middle Pleistocene
L. sechurae X *L. culpaeus*	0.064 (0.014)	337,078	**869,565**	2,277,227	
L. gymnocercus X *L. sechurae*	0.064 (0.014)	337,078	**869,565**	2,277,227	
L. fulvipes X *L. sechurae*	0.061 (0.011)	365,168	**828,804**	2,054,455	
L. fulvipes X *L. gymnocercus*	0.052 (0.013)	243,445	**706,521**	1,930,693	Early-Middle Pleistocene
L. griseus X *L. gymnocercus*	0.050 (0.008)	318,352	**679,347**	1,633,663	*L.* gymnocercus divergence from *L. griseus-culpaeus* on Middle Pleistocene
L. gymnocercus X *L. culpaeus*	0.041 (0.007)	243,445	**557,065**	1,361,386	
L. griseus X *L. fulvipes*	0.042 (0.010)	205,992	**570,652**	1,534,643	*L. fulvipes* divergence from *L. griseus-culpaeus* on Middle Pleistocene
L. fulvipes X *L. culpaeus*	0.036 (0.009)	168,539	**489,130**	1,336,633	
L. griseus X *L. culpaeus*	0.027 (0.006)	140,449	**366,847**	965,346	Middle- Late Pleistocene

Capítulo VI
DISCUSSÃO

O conjunto de dados obtidos no presente estudo descreve um panorama inicial para a filogeografia das raposas sul americanas, contribuindo para o preenchimento das lacunas de conhecimento existente neste grupo de canídeos. É abordado o gênero *Lycalopex*, sua especiação e algumas características genéticas intraespecificas, bem como, mais detalhadamente, a história evolutiva e dinâmica populacional de *Cerdocyon thous.*

As análises filogenéticas de seqüências da região controladora do DNA mitocondrial (mtDNA) indicaram que as espécies do gênero *Lycalopex* compõem um grupo monofilético. Neste, *L. vetulus* é a espécie mais basal e *fulvipes-griseus-culpaeus-gymnocercus* formam um ramo mais interno no qual se observa menor distância entre *L. culpaeus* e *L. griseus*.

Alguns estudos prévios haviam analisado as relações dentro do grupo utilizando dados genéticos, morfológicos ou ambos. A posição basal *L. vetulus* foi apoiada pela análise de 15 Kb de seqüências nucleares (Lindblad-Toh *et al*., 2006), em contradição as inferências de Wayne *et al*. (1997) com uso de dados de regiões codificadoras do DNA mitocondrial, Zrzavy e Ricancova (2004) em abordagem conjunta de dados moleculares, morfológicos e comportamentais, e Lyras e Van-Gelder (2003) com morfologia do cérebro.

A monofilia de *L. fulvipes* e sua distinção em relação a *L. griseus* foi anteriormente indicada por Yahnke *et al*. (1996), e também é confirmada pelo trabalho de Lindblad-Toh *et al*. (2006).

A relação entre as demais espécies é bastante discordante entre os diferentes estudos, no entanto, *griseus-gymnocercus-culpaeus* estão muito proximamente relacionados nas análises filogenéticas de Wayne *et al.* (1997), incluindo entre eles também *L. vetulus*; Lindbal-Toh (2006) incluindo *L. sechurae*; e Zrzavy e Ricancova (2004).

As estimativas de tempos de divergência entre os ramos das filogenias obtidas para *Lycalopex* spp. e *Cerdocyon thous*, indicam que para os canídeos, como para a maior parte da biota, o Pleistoceno foi um período decisivo para determinação dos padrões de distribuição de espécies que observamos. As datas aproximadas de divergência entre as espécies do gênero *Lycalopex* indicaram o Pleistoceno médio-inferior como período de origem de *L. vetulus e L. sechurae*; um pouco mais recente mais ainda neste período a origem de *L.*

gymnocercus e L. fulvipes; e o Pleistoceno médio-inferior para a divergência entre *L. griseus e L. culpaeus* (Tabela 3 Capítulo V; os intervalos para todas as análises estão compreendidos no período do Pleistoceno). Estas inferências são compatíveis com o registro fóssil que descreve neste período a radiação adaptativa do gênero: o registro mais antigo é de *L. gymnocercus* – 2,5 a 1,5 milhões de anos A.P. (antes do presente); para as outras espécies os registros estão relacionados ao Pleistoceno superior ou ao tempo recente (Berta, 1987).

Ainda neste período (*ca*. 400.000 anos A.P.), *Cerdocyon thous*, segundo nossos resultados, teria passado por processos demográficos que deram origem a uma estruturação genética muito forte entre o grupo das populações do nordeste X sul de sua distribuição (Figs. 1, 2, 3 e Tabela 3, 4 e 5 Capítulo III; as diferentes inferências obtidas através de marcadores nucleares são abaixo discutidas).

O Pleistoceno foi considerado por muito tempo um período de estabilidade para a América do Sul, Ásia e Africa (Marroig e Cerqueira, 1987). Dados posteriores, inclusive para a distribuição das espécies na América do Sul, indicaram que ao contrário, este foi um período de intensas variações climáticas que produziram fortes modificações ambientais. As oscilações de temperatura durante o período foram acompanhadas de mudanças no tipo e distribuição da vegetação e por alterações do nível do mar, que por sua vez modificaram a distribuição de águas continentais (Withmore e Prance, 1987; Marroig e Cerqueira, 1997; Eisenberg and Redford 1992; Costa 2003).

Durante a fase de maior transgressão marinha (aproximadamente 2,5 milhões de anos A.P., no inicio do Pleistoceno) a bacia Amazônica transformou-se em um grande lago e pode ter se ligado a Bacia do Prata-Paraguai e Paraná que tiveram seus níveis elevados. Esse evento deve ter gerado uma forte barreira entre as regiões de terras Argentinas, que se mantiveram conectadas aos Andes, e as terras Brasileiras (conforme revisão de Marroig e Cerqueira, 1997).

As linhagens de canídeos então existentes na América da Sul possivelmente sofreram um processo de vicariância, que pode ser sugerido como um dos fatores que influenciaram sua especiação. Especialmente a distância entre *L. vetulus* e os demais *Lycalopex* pode ser explicada por este evento, aliada as modificações de vegetação ocorridas no período.

As florestas tropicais e o Cerrado constituem formações bastante antigas que se originaram durante o Cretáceo e o Terciário, e tiveram sua extensão diminuída durante os períodos mais frios e secos do Pleistoceno, formando refúgios aos quais espécies tornaram-se adaptadas (Withmore e Prance, 1987; Eisenberg e Redford, 1992; Oliveira *et al*., 2005). O alto endemismo dos biomas Cerrado e Mata Atlântica são resultados desse processo que pode ter incluído *L. vetulus*, endêmico do cerrado (Eisenberg, 1981). Outro canídeo influenciado por estas modificações parece ser *C. thous*, que apresenta história discrepante para os dois clados intraespecíficos (Fig. 1, 2 e 3 Capítulo III). As populações a nordeste de sua distribuição apresentam-se como um grupo antigo (altos índices de diversidade – Tabelas 3 e 5, haplótipos bastante diferenciados – Figs. 2 e 3, Capítulo III), que manteve-se estável em tamanho efetivo (ausência de expansão populacional – Fig. 4 Capítulo III) e foi influenciado por processos de restrição ao fluxo gênico em algumas épocas (Fig. 3 Capítulo III). Já as populações do clado sul são resultantes de um evento recente de invasão da área e expansão no tamanho populacional (Figs. 2, 3 e 4; Capítulo III). Provavelmente o primeiro grupo esteja relacionado ao isolamento de um fragmento da floresta tropical no nordeste do Brasil durante períodos de glaciação, e o segundo aos padrões mais recentes de vegetação gerados ao final do Pleistoceno (Withmore e Prance, 1987).

Corroborando nossas inferências, Langguth (1975) sugere que as terras Brasileiras foram um importante centro para a evolução dos canídeos neotropicais, e Vanzolini (1988), Bates *et al.* (1998); Costa *et al.* (2000); Lara & Patton (2000), Ditchfield (2000), Costa (2003) descrevem padrões concordantes de distribuição de espécies e estruturação genética para diversos taxa (répteis, aves e pequenos mamíferos).

Outra região importante para a especiação dos Canidae, conforme sugerido por Berta (1987), parece ter sido a Argentina, onde provavelmente se diversificaram as espécies mais recentes de *Lycalopex* (o que é apoiado pelo maior número de espécies presentes atualmente), processo que deve estar relacionado com as modificações climáticas que perduraram até o último glacial (13,000-18.000 anos A.P. segundo Withmore e Prance, 1987). Nossos dados

sugerem que, após este período, várias espécies do gênero sofreram uma expansão populacional (Fig. 8, Capítulo V).

A divergência entre os gêneros *Lycalopex* e *Cerdocyon* foi estimada em cerca de 1milhões de anos antes do presente, tempo posterior ao fechamento do istmo do Panamá, que ocorreu há aproximadamente 3 milhões de anos A.P. Os resultados obtidos, assim, levantam a possibilidade de que a divergência entre os dois grupos tenha ocorrido já no continente sul-americano. Nossos dados não corroboram as inferências obtidas pela análise de 2001pb de seqüências codificadoras de proteínas do mtDNA, que indicaram idade mais antiga para o evento (cerca de 3 milhões de anos A.P.), e sugeriram que as espécies atuais dos dois gêneros provêm de diferentes eventos de invasão do continente (Wayne *et al*.,1997). As diferenças observadas para as duas estimativas podem estar relacionadas a diferentes taxas de mutação entre as duas regiões mitocondriais utilizadas e, mais provavelmente, a diferenças nos métodos de análise e nas calibrações fósseis utilizadas. Análises mais aprofundadas utilizando múltiplos segmentos e diferentes calibrações fósseis são necessárias para testar de forma mais conclusiva estes cenários.

Os resultados discutidos nos parágrafos acima podem ser aplicados às questões referentes à taxonomia do grupo, sugerindo a classificação de *L. fulvipes* como espécie distinta de *L. griseus* (ver Yahnke *et al*., 1996). A classificação de *L. vetulus* em um gênero distinto, como proposto por Langguth (1969,1975), mostra-se compatível com a filogenia obtida, suscitando um debate sobre a alternativa mais adequada para o uso neste grupo.

Indica-se ainda que *L. gymnocercus* e *L. griseus* (que representam ramos distintos bem definidos) devem ser mantidos na categoria de espécies distintas, contrariando Zunino *et al*. (1995), que propuseram sua união.

O agrupamento de animais inicialmente identificados como *L. gymnocercus* no ramo formado por *L. griseus* tem implicações importantes relacionadas à atual distribuição geográfica destas espécies, e também aos caracteres usados para sua identificação. Para as áreas onde estes animais foram coletados, não é considerada a ocorrência de *L. griseus* (Gonzáles del Solar e Rau, 2004) e, dada a similaridade de coloração das duas espécies (Zunino *et al*., 1995), estes animais podem estar sendo erroneamente identificados.

Outra possibilidade é a de que os animais sejam híbridos do cruzamento de fêmeas de *L. griseus* (tendo em vista a herança matrilinear do mtDNA) e machos de *L. gymnocercus*. Tal sugestão vem da comparação destes dados genéticos com a variação clinal descrita por Zunino *et al*.,1995 para a coloração e a morfologia das duas espécies na área Argentina de sua distribuição.

Para C. *thous* e *L. gymnocercus*, a estruturação genética observada questiona as propostas de distribuição de subespécies propostas por Cabrera (1931) e por Massoia (1982), para uma e outra espécie, respectivamente. A subespécie C. *thous entrerianus* deve ter sua distribuição aumentada para o norte (até a região do bioma Cerrado) em detrimento das áreas de C. *thous azarae*; e a região para onde era reconhecido C. *thous thous* é mais propriamente uma área onde se misturam os dois grupos anteriormente comentados (Fig.1 Capítulo III).

Para a raposa dos pampas pode ser questionada a distribuição de *L. gymnocercus gymnocercus*, dada a inferência a partir dos dados moleculares de uma estruturação genética dentro da área de ocorrência desta subespécie (na região do banhado do Taim, ao sul do Brasil; Fig. 2,3,4 e 7, Capítulo V). Este resultado deve ser explorado em mais detalhe em estudos futuros, utilizando um maior número de indivíduos e outros marcadores moleculares.

As inferências intraespecificas obtidas nos permitem comparar a história evolutiva de algumas das diferentes espécies abordadas. Enquanto um padrão de haplótipos intimamente relacionados, ligados a um haplótipo central mais freqüente, indica a idade recente dos grupos de populações ao sul da distribuição de *Cerdocyon*, a existência de vários passos mutacionais entre os haplótipos das populações ao norte desta espécie e de *L. vetulus* e *L. griseus* inferem uma história evolutiva mais antiga (Fig. 2 – Capítulo III; Fig. 5 e 6 – Capítulo V). A diferenciação dos haplótipos mostra relação com sua distribuição geográfica para *L. gymnocercus*, *Cerdocyon* e *L. fulvipes* indicando que barreiras históricas devem ter sido determinantes desses padrões (modificações ambientais do Pleistoceno para as duas primeiras; isolamento na Ilha Chiloé para *L. fulvipes*)

A inferência de partição genética norte-sul para C. *thous*, obtida através dos dados de mtDNA, foi amplamente investigada neste trabalho aplicando diferentes marcadores moleculares. Todos os marcadores utilizados mostraram-se informativos em relação às questões levantadas, e indicaram que *Cerdocyon*

thous apresenta altos índices de diversidade genética (4-24 alelos/*locus* e heterozigosidade média de 0.64, no caso dos microssatélites; $\pi = 0.83 \pm 0.032$ para região controladora do mtDNA; e $\pi=0.13 \pm 0.061$ a 0.20 ± 0.06 para os três seguimentos de introns).

As regiões nucleares, porém, não indicam nenhuma estruturação genética dentro da área de distribuição estudada para *C. thous*. Esta diferença em relação aos resultados provenientes do mtDNA, poderia resultar (ver Capítulo III), do maior tamanho efetivo e das taxas menores de mutação nas regiões de íntrons, o que faria com que os resultados destas seqüências mostrassem um panorama anterior, mais antigo, ao indicado pelo mtDNA, sem capturar este episódio histórico de subdivisão (Zhang e Hewitt, 2003). Tal hipótese foi testada pela utilização dos microssatélites (Capítulo IV), marcadores aplicáveis ao estudo de eventos recentes devido a suas altas taxas de mutação (Schlotterer, 2004). A ausência de estruturação mostrada também para os microssatélites indica então que o fluxo gênico diferencial entre os dois sexos é um importante fator determinante das diferenças observadas (Hare, 2001; Antunes *et al.*, 2002; Zhang e Hewitt, 2003).

O mtDNA, apresentando herança matrilinear e tamanho efetivo menor que o dos marcadores nucleares, é mais suscetível à deriva genética, capturando assim de forma mais sensível a diferenciação genética entre grupos em períodos de isolamento (Hare, 2001; Antunes *et al.*, 2002; Zhang e Hewitt, 2003), como provavelmente tenha acontecido neste caso. A manutenção desses padrões no mtDNA no tempo recente, após o desaparecimento da barreira de isolamento entre os dois grupos, deve-se provavelmente à maior filopatria das fêmeas, que impediram a dissolução da estruturação. A ausência desses padrões nos marcadores nucleares parece ser produto da influência da transmissão desses caracteres também por machos, que teriam maiores taxas de dispersão nesta espécie, conforme indicado pelas análises comparativas baseadas em coalescência (Tabela 5, capítulo III).

Não existem dados precisos, obtidos por observação direta, para as taxas de dispersão de machos e fêmeas nesta espécie. O trabalho que reporta em mais detalhes eventos dessa natureza é o de Macdonald e Courtenay (1996), que monitoraram grupos familiares de C. *thous* na região da Ilha de Marajó, no norte

do Brasil. Os autores reportam que entre os doze nascimentos durante o estudo (mais de uma estação de reprodução) apenas cinco dispersaram, sendo quatro machos juvenis e uma fêmea já adulta, porém, os machos formaram casais com fêmeas de idade semelhante à deles, o que sugere que estas também dispersem. Ambos estabeleceram territórios próximos ou adjacentes aos de seus pais, para onde continuaram voltando ocasionalmente.

Estas características de distribuição dos territórios parecem estar refletidas nos padrões de isolamento pela distância observados para as análises de mtDNA (Fig. 2 e 3, Capítulo III), porém são muito levemente indicadas pelos marcadores nucleares na abordagem de estruturação populacional, apoiando mais uma vez as inferências de fluxo gênico preferencial de machos. Apenas a população de RS (especialmente na análise bayesiana implementada no programa STRUCTURE) aparece com alguma diferenciação em relação as demais (Capítulo IV). A investigação mais completa desta e de mais duas populações da espécie (MS e PR), indicaram que, mesmo distando aproximadamente 500Km entre si, estas últimas comportam-se como uma única unidade evolutiva.

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