Diversidade Genética e Padrões Filogeográficos da Lontra Neotropical (*Lontra longicaudis* [Olfers, 1818]); (Mammalia: Mustelidae)

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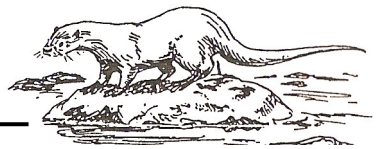
Sumário

Resumo

O conhecimento sobre a estruturação geográfica da diversidade genética em populações naturais permite inferir os processos históricos atuantes sobre as espécies e é fundamental para o planejamento de estratégias eficazes de conservação biológica. Neste contexto, o presente estudo é o primeiro a identificar e caracterizar a variabilidade genética, padrões de estruturação populacional e história demográfica de *Lontra longicaudis*. Para tanto, foram utilizados três segmentos do DNA mitocondrial (mtDNA; porção hipervariável I da região controladora, gene *ATP8* e gene *ND5*), bem como 12 locos de microssatélite, em indivíduos amostrados em diferentes regiões de sua distribuição geográfica. Ambos os marcadores revelaram moderados a altos níveis de variabilidade genética e padrões filogeográficos claros, os quais sugerem que as populações brasileiras desta espécie encontram-se geneticamente diferenciadas das outras regiões amostradas a Noroeste da América do Sul. As análises de mtDNA indicam a provável existência de quatro entidades filogeográficas: Colômbia, Bolívia, Guiana Francesa/Peru e Brasil. Colômbia e Bolívia foram representadas por apenas um indivíduo cada, os quais revelaram grande divergência genética dos outros indivíduos amostrados, sugerindo profunda subdivisão filogeográfica envolvendo estas regiões. A alopatria entre Guiana Francesa e Brasil é quase completa, sugerindo que a incongruência entre filogenia e geografia possa ser decorrente de um processo de colonização ancestral no sentido Norte-Sul. A inferência de diferenciação genética entre Brasil e as outras áreas amostradas na América do Sul são apoiadas pelas análises de microssatélite. Os resultados obtidos a partir das análises de mtDNA indicam ausência de estruturação genética no Brasil e são indicativos de um cenário de expansão populacional recente nesta região. Os padrões observados neste estudo têm implicações para a conservação de populações naturais de *Lontra longicaudis*. As quatro entidades filogeográficas reconhecidas demonstram-se suficientemente diferenciadas e deveriam, portanto, ser conservadas e manejadas independentemente. Estudos adicionais são necessários para melhorar o conhecimento sobre estas populações, bem como para investigar a existência de outras unidades demográficas ao longo da distribuição da lontra Neotropical.

Capítulo I – Introdução Geral



A Família Mustelidae

A Família Mustelidae compreende atualmente 25 gêneros e 67 espécies (Nowak 1999) colocadas em cinco subfamílias: Mustelinae, Melinae, Taxiidinae, Mellivorinae e Lutrinae (Dragoo & Honeycutt 1997; Koepfli & Wayne 2003; Flynn *et al*. 2005; Fulton & Strobeck 2006). Esta família compreende carnívoros de médio porte, dentre os quais se encontram os furões, texugos, lontras, martas e doninhas. Os membros desta família apresentam geralmente o corpo alongado, as patas relativamente curtas, e normalmente possuem uma cauda longa (Eisenberg & Redford 1999).

Mustelídeos habitam todos os continentes, exceto Austrália e Antártica, e não ocorrem em Madagascar nem em ilhas oceânicas. Membros desta família podem ser encontrados em ambiente terrestre, de água doce ou marinho. São principalmente carnívoros, e várias espécies incluem uma grande diversidade de vertebrados e invertebrados em sua dieta (Nowak 1999; Sato *et al*. 2003).

A Subfamília Lutrinae

A subfamília Lutrinae compreende 13 espécies de lontras, distribuídas em seis gêneros: *Aonyx, Enhydra, Lutrogale*, *Pteronura, Lutra* e *Lontra* (Kruuk 2006). Recentemente, Zyll de Jong (1987) forneceu evidências filogenéticas de que as lontras do Novo Mundo (exceto *Pteronura*), até então incluídas no gênero *Lutra*, deveriam passar a compor um novo gênero, *Lontra*, o qual inclui quatro espécies: *Lontra provocax*, *L. felina*, *L. canadensis* e *L. longicaudis* (Kruuk 2006).

As lontras encontram-se distribuídas por todos os continentes, exceto Austrália e Antártica (Foster-Turley *et al*. 1990). Na região Neotropical ocorrem quatro espécies de lontras: *Lontra provocax*, *L. felina*, *L. longicaudis* e *Pteronura brasiliensis* (Chehébar 1990). Duas destas espécies de lontras ocorrem no Brasil, a lontra neotropical (*Lontra longicaudis*) e a ariranha ou lontra gigante (*Pteronura brasiliensis*) (Chehébar 1990), sendo que em algumas regiões do país estas ocorrem em simpatria. Ambas foram intensamente caçadas para utilização da pele até a década de 1980 (Duarte & Rebelo 1985), apesar de a caça ter se tornado uma atividade proibida por lei desde 1967, em todo o território nacional.

A Lontra Neotropical

A lontra neotropical é um carnívoro de médio porte, de coloração marrom-pardacenta, quase preta, tendo apenas o lado ventral e o focinho amarelados. Possui grandes vibrissas que auxiliam na localização de presas embaixo d'água (Cimardi 1996). Suas patas apresentam

membranas interdigitais e sua cauda é longa e levemente achatada na extremidade, sendo ambas as estruturas adaptadas para a locomoção na água (Silva 1994).

É uma espécie de hábito semi-aquático, ocorrendo em uma grande variedade de habitats, em rios, lagos, pequenos canais, banhados e também ambientes marinhos associados a cursos d'água (p.ex. nos Estados de Santa Catarina e Rio de Janeiro) (Blacher 1987; Mason 1990; Fonseca *et al*. 1994); pode ser encontrada em uma ampla faixa de gradiente altitudinal (desde regiões litorâneas até cerca de 3.000 m de altitude) (Emmons 1990; IBAMA 1997). A dieta desta espécie, como a da maioria das espécies de lontras, consiste basicamente de peixes, crustáceos e moluscos, podendo ser complementada por aves, pequenos mamíferos, anfíbios e insetos (Chebez 1999).

Trata-se de uma das espécies de lontras menos conhecidas, sendo que as pesquisas realizadas até o momento são geograficamente restritas e direcionadas a estudos do hábito alimentar (Gallo 1986, 1989, 1997; Passamani & Camargo 1995; Spinola & Vaughan 1995; Parera 1996; Soldateli & Blacher 1996; Helder & De Andrade 1997; Pardini 1998; Colares & Waldemarin 2000; Quadros & Monteiro-Filho 2000, 2001; Utreras *et al.* 2002), distribuição local (Waldemarin 1997; Lacomba *et al.*, 2001), uso de abrigos como tocas e locais de descanso (Gallo 1989; Spinola & Vaughan 1995; Pardini 1999; Waldemarin & Colares 2000) e freqüência de marcação (Soldateli & Blacher 1996; Spinola & Vaughan 1995). Além disso, estudos sobre reprodução e fisiologia da espécie foram realizados apenas em cativeiro (Colares & Silva 1987; Colares & Best 1991; Parera 1996).

A distribuição geográfica atual da espécie é pouco conhecida, embora seja descrita como ocorrendo amplamente, ao longo de uma faixa contínua que inicia no México, cobrindo praticamente todo continente sul-americano e terminando no nordeste da Província de Buenos Aires (Argentina) e sul do Uruguai, alcançando também o norte do Peru (Chehébar 1990; Chebez 1999; Eisenberg & Redford 1999), e estando distribuída em todo o território brasileiro (Emmons 1990).

Em termos de *status* de conservação em nível internacional, *L. longicaudis* é considerada como tendo "Dados Insuficientes" pela IUCN (IUCN 2006), e está incluída no Apêndice I ("Espécies ameaçadas") da CITES ("Convention on International Trade in Endangered Species of Wild Fauna and Flora") (IUCN 2006). No Brasil, esta espécie é categorizada como "Quase Ameaçada" (IBAMA 2004), embora em Estados como Minas Gerais, São Paulo, Paraná e Rio Grande do Sul esteja classificada como "Vulnerável" (Paraná 1995; Machado *et al*. 1998; São Paulo 1998; Indrusiak & Eizirik 2003).

Dentre as principais ameaças a esta espécie encontram-se os conflitos com pescadores e proprietários de criadouros de peixes, o desmatamento e outras alterações antrópicas das

margens dos rios (Macdonald & Mason 1985). A poluição das águas também é uma grave ameaça à espécie, uma vez que pode ter influência indireta ou direta na estabilidade das populações de lontras. Danos indiretos referem-se à diminuição do estoque de alimento e contaminação do ambiente. Efeitos diretos causam impacto no animal, resultando em sua morte ou reduzindo seu sucesso reprodutivo (Macdonald & Mason 1990). Além disso, freqüentemente são mortas por atropelamentos em estradas (Macdonald & Mason 1990) e a alta densidade populacional humana também é citada como um fator que pode levar ao seu desaparecimento (Fonseca *et al*. 1994; IBAMA 1997).

Estruturação Geográfica de Populações Naturais – Inferências Evolutivas e Implicações para a Conservação

Uma das principais metas da Biologia da Conservação é a preservação da biodiversidade e a manutenção dos padrões e processos evolutivos que a geraram; para tal, é necessário elaborar programas adequados de conservação, nos quais deve ser decidido o que e como proteger (Johnson *et al*. 2001). Assim, uma área recente da Biologia, conhecida como Genética da Conservação, tem auxiliado na tomada destas decisões geralmente através da utilização de marcadores moleculares, os quais são em parte responsáveis pela possibilidade de estudar aspectos complexos de espécies e populações ameaçadas em condições naturais. A utilização de técnicas moleculares para este fim tem se expandido muito nas últimas duas décadas, sendo acompanhada também de uma crescente aceleração no desenvolvimento de métodos laboratoriais e analíticos empregados nestas abordagens (Frankham *et al.* 2002; Hey & Machado 2003; Luikart *et al.* 2003; DeSalle & Amato 2004; DeYoung & Honeycutt 2005; Kohn *et al*. 2006).

O conceito de filogeografia foi introduzido por Avise *et al.* (1987) para designar o estudo da distribuição da variabilidade genética de uma espécie em uma escala espacial e temporal. Os estudos filogeográficos têm por objetivo revelar a história evolutiva de uma linhagem, relacionando-a com sua distribuição geográfica, através, principalmente, das diferenças entre seqüências de DNA mitocondrial (mtDNA) (Avise 2000), utilizando as explicações geográficas históricas para interpretar as relações evolutivas entre os *taxa* (Stevens & Hogg 2003). Análises de padrões filogeográficos permitem a verificação de estruturação genética e a interpretação das possíveis barreiras ao fluxo gênico dentro e entre as espécies (p.ex. Eizirik *et al*. 2001), gerando um aumento do conhecimento sobre os processos históricos biogeográficos.

Além da análise dos níveis de diversidade genética, o acesso à distribuição geográfica desta variabilidade dentro de uma espécie é também de extrema importância para identificar e priorizar áreas nas quais programas de manejo e conservação devem ser elaborados (Moritz & Faith 1998). É fundamental avaliar se a espécie apresenta uma distribuição homogênea da

variabilidade genética ou algum grau de subdivisão, pois, no primeiro caso, qualquer área de sua distribuição é representativa da espécie enquanto que, havendo estruturação, a representatividade de cada sub-população deve ser preservada.

Assim, a partir de estudos de filogeografia podem-se identificar dois tipos de linhagens evolutivas para fins de manejo: (i) Unidades Evolutivamente Significativas ("Evolutionarily Significant Units" - ESUs) e (ii) Unidades de Manejo ("Management Units" - MUs). As primeiras são constituídas por unidades demográficas infra-específicas, distintas geograficamente e que se apresentam diferenciadas geneticamente (implicando isolamento histórico) de outras unidades semelhantes contidas na mesma espécie. As Unidades de Manejo estão contidas nas ESUs, e são formadas por populações regionais com restrita conexão demográfica entre si, mas não necessariamente com diferenciação genética profunda. Entretanto, em uma escala de tempo curta (uma ou poucas gerações), o contato entre estas populações através de migração ou re-colonização torna-se escasso, transformando-as em entidades ecológicas relativamente separadas, e que deveriam ser manejadas de forma independente ou coordenada (Ryder 1986; Moritz 1994; Eizirik 1996; Crandall *et al*. 2000; Fraser & Bernatchez 2001; Frankham *et al*. 2002)

Utilização de Marcadores Moleculares em Estudos Evolutivos e Populacionais

Entre os marcadores moleculares mais freqüentemente empregados em estudos populacionais, evolutivos e/ou voltados para conservação destaca-se o DNA mitocondrial (mtDNA), como marcador de diversidade genética, sendo também muito útil em investigações sobre relações filogenéticas entre diferentes *taxa* e identificação de subdivisão geográfica entre unidades populacionais (Avise *et al.* 1987; Bermingham & Moritz 1998; Avise 2000).

Duas razões justificam a ampla utilização do polimorfismo de mtDNA na reconstrução de filogenias moleculares: primeiro, o mtDNA representa a parte mais bem conhecida do genoma animal e, segundo, a taxa de evolução do mtDNA é 5 a 10 vezes mais rápida do que a dos locos nucleares de cópia única na maioria das espécies de mamíferos (Avise *et al.* 1992; Baker *et al.* 1993; Martin & Palumbi 1993; Ballard & Whitlock 2004).

De um modo geral, mtDNA animal apresenta duas características que podem ser vistas em alguns contextos como vantagens sobre os marcadores nucleares: (i) a relação filogenética do mtDNA reflete a história de linhagens maternas dentro de uma população ou espécie; (ii) o tamanho efetivo da população do genoma mitocondrial é de ¼ comparada aos genes autossômicos, levando a uma maior taxa de diferenciação local por deriva aleatória (Neigel & Avise 1986).

Além do mtDNA, o DNA nuclear tem se tornado cada vez mais utilizado em estudos evolutivos e populacionais. Dentre os marcadores nucleares mais amplamente empregados

encontram-se os microssatélites. Estes marcadores consistem de segmentos curtos de DNA (1-6 pares de bases), que se repetem em número variável em *tandem*, sendo em sua maioria, repetições de mono, tetra ou, principalmente, dinucleotídeos, apresentando-se como locos altamente polimórficos dispersos amplamente em genomas eucarióticos (Schlötterer 1998; Schlötterer 2004).

As características apresentadas pelos microssatélites (alto nível de polimorfismo, alta taxa de mutação, e tendência à neutralidade seletiva) permitem a sua utilização em estudos como comparação da variação genética entre espécies e populações (Johnson *et al.* 1999; Wisely *et al*. 2002), grau de estrutura das populações e migração (Ciofi & Bruford 1999; Waits *et al.* 2000; Cegelski *et al.* 2003) e determinação de parentesco e estrutura social (Nesje *et al.* 2000), os quais são extremamente necessários para o desenho de estratégias para a conservação de espécies ameaçadas. Além disso, permitem a utilização de DNA altamente fragmentado, em pequenas quantidades ou até mesmo de amostras antigas (Bruford & Wayne 1993).

Estudos Genéticos e Moleculares com Mustelídeos

O papel dos carnívoros na regulação de comunidades e manutenção da biodiversidade vem sendo amplamente discutido nas últimas décadas (Kitching 1986; Fonseca & Robinson 1988; Terborgh 1990, 1992; Wright *et al.* 1994; Asquith *et al.* 1997). Estudos realizados com a lontra marinha (*Enhydra lutris*), por exemplo, identificaram a importância desta espécie na manutenção da biodiversidade das comunidades marinhas onde vive (Kitching 1986). A lontra neotropical, por sua vez, além de ser um carnívoro, é uma das únicas espécies de maior porte que ocupa esta posição nas cadeias alimentares de ambientes aquáticos onde ocorre e, assim, pode desempenhar uma importante função na regulação das comunidades destes sistemas. Apesar de ter sofrido com a caça excessiva no passado, atualmente a lontra neotropical parece estar distribuída de forma ampla no Brasil, ocorrendo inclusive em áreas moderadamente urbanizadas e onde problemas ambientais como poluição orgânica e desmatamento da mata ciliar estão presentes. No entanto, os registros de extinções locais sofridas pela lontra euro-asiática (*Lutra lutra*) em vários países europeus, e pela lontra norte-americana (*Lontra canadensis*) em diversas localidades dos Estados Unidos, demonstram que mesmo espécies altamente adaptáveis e flexíveis podem sofrer declínios populacionais extremos perante modificações ambientais intensas (Foster-Turley *et al.* 1990; Swimley *et al.* 1998; Reuther *et al.* 2000). Embora a lontra neotropical seja uma espécie considerada como apresentando algum grau de tolerância à presença humana, pouco se sabe sobre os efeitos desta aproximação sobre as populações de *L. longicaudis.*

Estudos genéticos e moleculares sobre os mustelídeos realizados até o momento são principalmente direcionados à lontra euro-asiática (*Lutra lutra*) (Dallas & Piertney 1998; Dallas *et*

al. 1999; Mucci *et al.* 1999; Dallas *et al*. 2000; Dallas *et al.* 2002; Randi *et al*. 2003; Arrendal *et al*. 2004; Huang *et al*. 2005), lontra norte-americana (*L. canadensis*) (Serfass *et al*. 1998; Blundell *et al*. 2002; Beheler *et al*. 2004; Beheler *et al*. 2005), texugo euro-asiático (*Meles meles*) (Frantz *et al.* 2003; Pope *et al*. 2005), glutão ou carcaju (*Gulo gulo*) (Walker *et al*. 2001; Kyle & Strobeck 2001) e marta-americana (*Martes americana*) (Broquet *et al.* 2006).

Os únicos estudos genéticos incluindo *L. longicaudis* publicados até o momento são análises filogenéticas da família Mustelidae e da Subfamília Lutrinae como um todo, os quais não abordam quaisquer aspectos específicos desta lontra em particular (Zyll de Jong 1987; Koepfli & Wayne 1998; Marmi *et al*. 2004; Koepfli & Wayne 2003). Sendo assim, não existe até o momento qualquer estudo publicado voltado para a investigação de parâmetros genéticos das populações de *L. longicaudis*.

O presente estudo tem por objetivo, através da utilização da variação em seqüências do mtDNA e microssatélites, (1) identificar e caracterizar subdivisões geográficas em populações de *Lontra longicaudis*, e (2) a partir dos padrões observados inferir os processos históricos que atuaram sobre esta espécie, bem como comparar os padrões encontrados com aqueles descritos para outras espécies de vertebrados, além de avaliar o desempenho destes marcadores para estudos evolutivos e populacionais de mustelídeos.

Capítulo II - Artigo

“Phylogeographic Patterns and Evolutionary History of the Neotropical otter (*Lontra longicaudis*)”

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Phylogeographic Patterns and Evolutionary History of the Neotropical otter (*Lontra longicaudis*)

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Abstract

The Neotropical otter (*Lontra longicaudis*) is a medium-sized carnivore with a broad distribution in the Neotropical region. This species, as well as other otter species, suffered with hunting in parts of its range until 1960s due to the high value of its pelts. Despite being apparently common in many areas, it is one of the least known otters, and genetic studies on this species are scarce. Here we have investigated its genetic diversity, population structure and demographic history across part of its geographic range by analyzing 1472 base pairs (bp) of mitochondrial DNA (mtDNA) and 12 microsatellite loci. *L. longicaudis* exhibits high levels of mtDNA and microsatellite diversity. Both mitochondrial and nuclear data revealed a consistent phylogeographic pattern, indicating that Brazilian populations are genetically distinct from sampled populations distributed in Northwestern South America. The mtDNA data indicates the probable existence of four phylogroups occurring in Brazil, French Guiana/Peru, Bolivia and Colombia, respectively. The single Colombian haplotype was the most basal relative to all other lineages, suggesting a substantial isolation of this phylogroup. No substantial geographic substructure is observed within the Brazilian cluster, but a demographic expansion is inferred. Inferences on the history of these phylogroups and possible causes of such patterns are drawn, based on phylogenetic and population-genetic approaches. Implications of the observed patterns for the conservation of *L. longicaudis* are addressed, highlighting the finding that broad geographic regions contain differentiated population segments that likely warrant independent management status.

Introduction

Phylogeographic studies of widely distributed semi-aquatic mammals can provide a natural link between the geological and biotic evolution of an area, since dispersal of these species is largely related to the direct connection among basins, and the history of basin interconnections is a result of geological processes (Bermingham & Martin 1998). In this context, mitochondrial DNA (mtDNA) genealogies have been used extensively to infer processes at population levels and the phylogenetic diversification of taxa in relation to their geographical distribution (Avise *et al*. 1987; Avise 2000). However, mtDNA reflects only a portion of the total historical record of a matriarchal component of the organismal pedigree (Avise 1994), and thus a more complete view can be obtained by additional investigation of nuclear loci. In this context, microsatellites have an interesting informative potential for demographic analysis, due to their rapid mutation rate and a biparental inheritance (Frankham *et al*. 2002).

Processes in the demographic history of populations, such as expansions or contractions, leave recognizable signatures in the pattern of molecular diversity (Harpending *et al*. 1998; Schneider & Excoffier 1999). Historical events can affect population size, and may result in low levels of genetic diversity in current populations even if these populations do not present small size. Moreover, climatic changes as occurred during the Pleistocene have been responsible for the current patterns of genetic variation in many species (Lessa *et al*. 2003).

So far, few studies have addressed the evolutionary history of Neotropical taxa from an intra-specific phylogeography perspective, so that most of the evolutionary aspects still need to be investigated. Some studies have investigated phylogeographic patterns in Neotropical vertebrates (Eizirik *et al*. 1998; Lovejoy & de Araújo 2000; Eizirik *et al*. 2001; Cantanhede *et al*. 2005; Vianna *et al*. 2006; Grazziotin *et al*. 2006; Marquéz *et al*. 2006; Tchaicka *et al*. 2007), but the region has still been poorly characterized with respect to widely distributed semi-aquatic mammals.

Lontra Longicaudis is a medium-size semi-aquatic carnivore, a relatively common species widely distributed in the Neotropical region that exhibits a fish-based diet, complemented by crustaceans and mollusks (Chebez 1999). In the past, and especially during the last two centuries, humans made heavy use of otters for skins. Otter pelts were very much in demand in the international market during the first half of the 20th century (Chehébar 1990). Although some illegal hunting continues, this species has been relatively free of exploitation since the 1960s, when the hunting pressure declined significantly, due to the growing concern for wildlife conservation and because of the Latin American countries

enforcing CITES regulations (Chehébar 1990). However, throughout this range, this mustelid has been subjected to persecution because its supposed predation on fish stocks (Macdonald & Mason 1990) and are also heavily killed on roads. Despite that, the species is considered as "Data Deficient" by IUCN (2006), but in several localities of its distribution it is categorized as "Near Threatened" or "Vulnerable" (Chehébar 1990).

Most otter species are poorly known and are important targets for conservation due to be indicator of healthy aquatic environments. As otters are typical animals at the top of the food chain, they are among the first species to refuse and disappear when the environment is degraded (Foster-Turley *et al*. 1990). However, even basic information is difficult to obtain because otters are rarely observed, and are very difficult to trap, mark and recapture (Dallas & Piertney 1998). Some studies have addressed aspects of intra-specific genetic diversity and population structure of basically three otter species (Bodkin *et al*. 1999; Blundell *et al*. 2002; Dallas *et al*. 2002; Larson *et al.* 2002; Pérez-Haro *et al*. 2005); however, no large-scale study has yet been published on the Neotropical otter (*Lontra longicaudis*).

The present study aims to characterize the genetic structure and evolutionary history of Neotropical otter populations on a broad geographic scale, based on the analysis of independent loci that represent both the nuclear and mitochondrial genomes. Phylogeographic partitions and estimated population genetic parameters are used to infer the evolutionary history of this species. Since this is the first phylogeographic study of a Neotropical semi-aquatic mammal, we aimed to test whether the observed patterns were congruent with those observed in terrestrial taxa (e.g. other carnivores, small mammals - Eizirik *et al*. 1998; Eizirik *et al*. 2001; Costa 2003; Tchaicka *et al*. 2007) or aquatic species (e.g. manatees, fish, caimans – Lovejoy & de Araújo 2000; Sivasundar *et al*. 2001; Cantanhede *et al*. 2005; Vianna *et al*. 2006; de Thoisy *et al*. 2006), or yet presented unique features not yet detected in other organisms.

Materials and Methods

Sample collection

Biological samples were collected from 45 Neotropical otters across a large area of the species' range (Fig. 1; Table 1). Blood samples were obtained from captive individuals (with known geographic origin). Tissue samples were collected opportunistically by collaborators from road-killed animals. Scat samples were collected by field researchers and from captive individuals. A hair sample was obtained from one dead animal. Blood samples were preserved in a salt saturated solution (100mM Tris, 100mM EDTA, 2% SDS) and muscle and scat samples were preserved in ethanol 96%. All samples were stored at -20°C

prior to DNA extraction. Samples of *Lontra canadensis*, *Aonyx cinereus* and *Pteronura brasiliensis* were also included to be used as outgroups in some of the analyses.

DNA extraction and molecular analyses

Genomic DNA was extracted from blood and tissue samples using a standard Proteinase-K digestion and phenol-chloroform-isoamyl alcohol protocol (Sambrook *et al.* 1989). DNA from hairs was extracted using the ChargeSwitch® Forensic DNA Purification Kit (Invitrogen). DNA from scats was extracted using the QIAamp DNA Stool Mini Kit (Qiagen) following the manufacturer's instructions. The scat DNA extractions were carried out in a separate laboratory area, in a UV-sterilized laminar flow hood, dedicated to the DNA analysis of non-invasive samples.

Three segments of the mitochondrial DNA were amplified by the Polymerase Chain Reaction (PCR, Saiki *et al.* 1985): (I) the 5' portion of the mtDNA control region (CR), containing the first hypervariable segment, using primers MTLPRO2 (5'-CACTATCAGCACCCAAAGCTG-3') and CCR-DR1 (5'-CTGTGACCATTGACTGAATAGC-3'); (II) a segment of the *ATP8* gene using primers ATP8-DF1 (5'-AGAAGCTAAATAAGCATTACCTTTTA-3') and ATP8-DR1 (5'-CCAGTATTTGTTTTGATGTTAGTTG-3'); and (III) a segment of the *ND5* gene using primers ND5-DF1 (5'-TTGGTGCAACTCCAAATAAAAGT-3') and ND5-DR1 (5'-AGGAGTTGGGCCTTCTATGG-3').

Since fecal samples tend to present degraded DNA, hindering the sequencing of long fragments, we designed internal primers for the control region dividing it into three shorter, overlapping fragments (each one with *ca.* 250 bp). Through this strategy it was possible to obtain a complete sequence of the CR fragment from fecal samples. These primers are: LonCR-R1 (used in combination with MTLPRO2) (5'-ATGGTTTCTCGAGGCATGGT-3'), LonCR-F2 (used in combination with CCR-DR1) (5'-AACTATACCTGGCATCTGGTTCTT-3'), and the internal pair LonCR-F1 (5'- GGTTTGCCCCATGCATATAA-3') + LonCR-R2 (5'-TGTGTGATCATGGGCTGATT-3'). The scat samples were amplified only for the mtDNA control region.

Each 20ul PCR reaction contained 1-2 ul of DNA, 1x PCR Buffer (Invitrogen), 1.5 - 2.0 mM $MgCl_2$, 200 uM dNTPs, 0.2uM of each primer and 0.5 unit of Taq DNA Polymerase (Invitrogen). The PCR conditions were the same for the three mitochondrial segments, and began with 10 cycles (*Touchdown*) of 94°C for 45s, 60-51°C for 45s, 72°C for 1.5 min; this was followed by 30 cycles of 94°C for 45s, 50°C for 45s, 72°C for 1.5 min and final extension of 72°C for 3 min. Products were visualized on a 1% agarose gel stained with ethidium

bromide, purified with PEG8000, sequenced using the *DYEnamic ET Dye Terminator Sequencing Kit* (Amersham Biosciences), and analyzed in a MegaBACE 1000 automated sequencer (Amersham Biosciences). Sequences were deposited in GenBank under accession numbers XXXX-XXXX.

DNA extracts were also typed by PCR for 12 microsatellite loci (Lut453, Lut733, Lut782, Lut701, Lut818, RIO06, RIO07, RIO11, RIO17, RIO18, RIO19 and RIO20), developed for two other species of otters (*Lutra lutra* and *Lontra canadensis*) (Dallas *et al*. 1998; Beheler *et al*. 2004, 2005). Every forward primer was 5'-tailed with an M13 sequence (5'- CACGACGTTGTAAAACGAC-3') (Boutin-Ganache *et al*. 2001), and used in combination with an M13 primer that had the same sequence but was dye-labeled on its 5' end. The PCR reactions were performed in 10ul reactions containing 0.5 – 1.5 ul of empirically diluted DNA, 1x PCR Buffer (Invitrogen), 1.5 - 2 mM $MgCl_2$, 200 uM dNTPs, 0.2 uM of the reverse and M13-fluorescent primers, 0.0133 uM of the M13-tailed forward primer, and 0.5 unit of Taq DNA Polymerase. The PCR conditions were the same as for the mitochondrial segments, except for a 30min final extension at 72°C. PCR reactions were carried out for each locus separately, and products from 1 to 3 loci were diluted and pooled together based on yield, size range and fluorescent dye, and then analyzed in a MegaBACE 1000 automated sequencer.

Sequence analysis

Sequences were visually checked and manually corrected using CHROMAS 2.0 (http://www.thecnelysium.com.au/chromas.html) and aligned with the CLUSTALW algorithm implemented in MEGA 3.1 (Kumar *et al*. 2004), with the resulting alignments edited by hand. MEGA was also used to perform initial sequence comparisons and computations of variability.

Phylogenetic analyses were performed separately for the mtDNA control region (CR), coding fragments (*ATP8*+*ND5*), and full concatenation (CR+*ATP8*+*ND5*). The data sets were assessed for the most appropriate model of nucleotide substitution using the Akaike Information Criterion (AIC) as implemented in Modeltest 3.07 (Posada & Crandall 1998). We inferred phylogenetic relationships among haplotypes using PAUP* 4.0b10 (Swofford 2002) for three different optimality criteria: (i) maximum likelihood (ML) employing the selected model and estimated parameters, with a heuristic search started from a neighbor-joining (NJ) tree and using the nearest-neighbor interchange (NNI) branch-swapping method; (ii) distance-based, using the (NJ) algorithm and ML genetic distances; and (iii) maximum parsimony (MP) using heuristic searches with 50 replicates of random taxon addition and

tree-bisection-reconnection (TBR) branch-swapping. Group support for all of the above methods was evaluated with 100 nonparametric bootstrap replicates. Additionally, we performed Bayesian phylogenetic analyses (BI) with MrBayes 3.1 (Huelsenbeck & Ronquist 2001), with four Metropolis-coupled Markov Chain Monte Carlo (MCMC) chains run for 1 million generations. Trees were sampled every 100 generations, discarding the first 2,500 trees as burn-in. Two independent runs were performed for each data set to evaluate convergence. We used *Lontra canadensis, Aonyx cinereus* and *Pteronura brasiliensis* as outgroups in the phylogenetic analyses.

A median-joining network (Bandelt *et al* 1999) was constructed using Network 4.1.1.2 (www.fluxus-engineering.com) to depict phylogenetic, geographic, and potential ancestor-descendent relationships among the sequences. Divergence times between inferred clades were estimated using a distance-based linearized tree method implemented in MEGA, employing a molecular calibration of 5.9 million years ago (MYA) (credibility interval (CI): 4 - 8.3 MYA) for the evolutionary separation between the *Aonyx* and *Lontra* lineages (E. Eizirik *et al*. unpublished).

Population structure analyses were performed assuming broad geographic units based on the observed phylogeographic pattern (see Results). The testing of additional, alternative scenarios of geographic subdivision could not be adequately performed with this approach due to limitations of sample size for some of the included areas. As measures of differentiation among populations, we estimated fixation indices (F_{ST}) (Wright 1965), using an Analysis of Molecular Variance (AMOVA) approach (Excoffier *et al.* 1992) implemented in ARLEQUIN 2.0 (Schneider *et al*. 2000).

A Mantel test (Mantel 1967) as implemented in AIS 1.0 (Miller 2005) was performed to test the null hypothesis of no correlation between geographic and genetic distances. Statistical significance was tested using 1000 random permutations. Statistics such as nucleotide (π) and haplotype (h) diversity, neutrality tests such as Tajima's D (Tajima 1989), Fu and Li's F* & D* (Fu & Li 1993), and Fu's F_s (Fu 1997), and Mismatch Distribution Analyses (Rogers & Harpending 1992) were computed using DnaSP (Rozas *et al*. 2003) and ARLEQUIN.

Microsatellite analyses

Microsatellite genotyping was performed using the software Genetic Profiler 2.2 (Amersham Biosciences). We calculated observed (H_O) and expected (H_E) heterozygosity for each locus and tested for evidence of deviation from Hardy-Weinberg equilibrium (HWE) and linkage equilibrium (LE) using CERVUS 2.0 (Marshall *et al*. 1998) and ARLEQUIN. To

correct for multiple comparisons, Bonferroni adjustments (Rice 1989) with an original α level of 0.05 were carried out for all tabulated results. Using the unlinked loci that were in HWE, we performed a *G*-test to evaluate the null hypothesis that allelic frequencies were identical across populations (Sokal & Rohlf 1981).

The degree of population genetic structure was estimated with the F_{ST} index using an Analysis of Molecular Variance (AMOVA) approach implemented in ARLEQUIN. For comparison, we also calculated R_{ST}, an analogous measure designed for microsatellite data that incorporates a stepwise mutation model (Slatkin 1995). As an independent measure of the partitioning of genetic variation among groups, the program STRUCTURE 2.0 (Pritchard *et al.* 2000) was used to cluster individuals into subpopulations and to reveal patterns of gene flow across the sampled area. STRUCTURE uses an iterative approach to cluster microsatellite genotypes into *K* populations regardless of the geographic locations of individuals. The approach is based on the assumptions of Hardy-Weinberg and linkage equilibrium within the resulting clusters, so that the likelihood of K is estimated from the genotype data alone. The highest likelihood value indicates the most likely number of populations in the sample. Individuals can be assigned to one or more populations, including the possibility of admixture. The first step of this analysis involved estimating the numbers of populations (*K*). Five independent runs each of K = 1-5 were performed with 10^4 – 10^5 MCMC iterations after a burn-in of 10^4 – 10^5, using no prior information and assuming uncorrelated allele frequencies and allowing admixture. In the second step of the analysis, individuals were assigned to each original geographic sample group (using K = 2; see Results). To evaluate the STRUCTURE results in determining how indicative an individual's genotype was of the population from which it was sampled, we performed an assignment test (Paetkau *et al.* 1995) as implemented in ARLEQUIN.

A Mantel test was performed with the program AIS to assess the significance of the association between genetic and geographic distances. Statistical significance was tested using 1000 random permutations.

Results

mtDNA sequences

A 516 base-pair (bp) fragment of the control region (CR) was sequenced for 44 *L. longicaudis* individuals. Due to a portion of ambiguous alignment, 25 sites from the control region were excluded from all further analyses, totaling 491 bp. Sequences of the *ATP8* (329 bp) and *ND5* (651 bp) genes were obtained for 37 Neotropical otters each (Table 2). Outgroups were sequenced for these three fragments yielding the same sequence length,

except for *Aonyx cinereus* whose control region segment was 1 bp longer than the remaining individuals (so that the total alignment was 517 bp-long). The three segments were concatenated totaling 1472 bp.

Moderate to high levels of genetic diversity were observed in all three segments (Table 2).The CR was the most variable segment when *L. longicaudis* samples were analyzed separately, but showed clear indications of saturation when other species were also included in the comparisons (see Table 2). Separate analyses were conducted for three different mtDNA data sets: (i) control region alone; (ii) coding segments (*ATP8* + *ND5*); and (iii) full concatenation (CR + *ATP8* + *ND5*).

Control region data set

Sequences generated for this segment defined 23 different haplotypes (Table 3). A relatively high level of nucleotide diversity was observed among individuals (Table 2). The Hasegawa-Kishino-Yano (Hasegawa *et al.* 1985) substitution model with a proportion of invariant sites and a gamma distribution of rate heterogeneity across sites (HKY+I+G) provided the best fit to this data set (ln = -1363.4178). The MP, ML, NJ and Bayesian phylogenetic analyses did not conflict with each other in the inferred tree topology, but were unable to resolve the relationships among the haplotypes (Fig. 2). The only supported grouping obtained with this segment was a clade containing most haplotypes from French Guiana and one sequence from Peru (subsequently named Clade 2 – see below). The haplotype network also failed to show a clear pattern, with a considerable amount of reticulation indicating the occurrence of homoplasy and likely saturation at variable sites (Fig. 3).

In spite of the apparent lack of discernible phylogenetic structure, the F_{ST} between two broadly defined geographic groups (Brazil *vs.* Northwestern South America; see below) was quite high (F_{ST} = 0.45; p = 0.000). Of the neutrality tests performed, only Fu's F* was significantly negative (Fu's F*= -6.546, p=0.001) when the Brazilian samples were analyzed separately, while all other tests were non-significant for separate or combined population samples. The mismatch distribution showed a multi-modal pattern for most data sets, except for the analysis of Brazilian individuals by themselves, which resulted in a roughly unimodal peak (Fig. 4A).

Combined data sets

Sequences from the concatenations of the *ATP8* and *ND5* segments (*n* = 36; data set II) and *ATP8* + *ND5* + CR (*n* = 35; data set III) led to the observation of 19 and 26 unique

haplotypes, respectively (Tables 4 and 5). Moderate to high levels of nucleotide and gene diversity were observed for these data sets in Neotropical otter individuals (Table 6).

The TIM + I and the TIM + I + G models were selected for AIC as the best model of nucleotide substitution to data sets II and III, respectively (ln = -3260.5420; ln = -4453.25662), and were applied in all subsequent model-based analyses. All trees produced with different phylogenetic methods were congruent, with mostly subtle differences in nodal support (Figs. 5 and 6). With these data sets a clear phylogeographic pattern could be observed, with all samples from Brazil clustering in a single, well-supported and very shallow clade. The shape of its internal phylogeny, with very short branches, little structure and no evidence of geographic differentiation (in spite of a broad sampling across Brazil), is suggestive of a recent population expansion in this area (Lavery *et al*. 1996; Avise 2000).

The sister-group to this Brazilian clade was a single haplotype (Ll-AN15 and Ll-ANC23 in the data sets II and III, respectively) found in two different individuals from French Guiana (bLlo41 and bLlo66). Together, this haplotype and the Brazilian sequences formed a well-supported phylogenetic group (indicated as Clade 1 in Figs. 5 and 6). A second well-supported monophyletic cluster (Clade 2) included only haplotypes found in French Guiana and Peru, with some indication of possible sub-structuring in this area.

The haplotype found in Bolivia (Ll-AN16 and Ll-ANC24 in data sets II and III, respectively) was not contained in either Clade 1 or 2, but rather was similarly divergent from both of them (Figs. 5 and 6). Its exact placement was not identical with the two data sets: with *ATP8* + *ND5*, it was positioned in a trichotomy with Clades 1 and 2, whereas in the full concatenation (data set III), it was a sister-group to Clade 2, albeit with weak bootstrap support. This suggests that Bolivia may contain a third phylogenetic lineage separate from Clades 1 and 2, whose exact relationships should be further investigated with additional sampling. Furthermore, the individual from Colombia (bLlo23) contained a very distinct haplotype (Ll-AN14 and ANC21 in data sets II and III, respectively), which was consistently placed as the most basal lineage of all *L. longicaudis*.

The haplotype network produced with *ATP8* + *ND5* (Fig. 7) depicted a clear phylogeographic pattern, with at least 6 mutational steps separating the samples belonging to clades 1 and 2 recovered in the phylogenetic analyses. A star-shaped pattern with several localized lineages connected by short branches to a more common, widespread haplotype, is suggestive of a relatively recent population expansion in the Brazilian group. In contrast, the median-joining network produced with data set III (Fig. 8) was not efficient at resolving the relationships among the individuals, probably due to saturated mutation sites in the control region; nevertheless, a similar star-shaped pattern could be observed in some Brazilian

haplotypes. Again, individuals bLlo41 and bLlo66 were positioned near the Brazilian group instead of being placed in association with other samples from their geographic region (French Guiana).

The AMOVA results indicated that most of the genetic variability in *L. longicaudis* mtDNA could be explained by a single Northwest *vs.* Southeast partition (*i.e.* Brazil [SE] *vs.* all other sampled locations [NW]. The F_{ST} between these two geographic groups was high (0.65, $p < 0.001$; and 0.54, $p < 0.001$, for data sets II and III, respectively), as expected due to their almost perfect allopatry. Given this result and the available sample, we focused several geography-based analyses on these two broad units, even though the NW group seems to contain a diverse assembly of non-monophyletic historical lineages (Figs. 5 and 6).

For the complete *L. longicaudis* sample, as well as and for the NW geographic group, all neutrality tests were non-significant. In contrast, significantly negative results were obtained for the Brazilian group (Fu's $F^* = -8.6625$, $p < 0.001$, data set II; Fu's $F^* = -6.793$, $p < 0.001$; Tajima's $D = -5.872$, $p = 0.02$, data set III), congruent with the inference of a recent population expansion in this region. The mismatch distribution analyses taking into account the whole species and the NW geographic group showed a multimodal distribution (not shown), which is expected given the observed phylogenetic structure in this area. On the other hand, the Brazilian sample revealed a unimodal pattern (Fig. 4B,C), which is again consistent with a historical demographic expansion.

The Mantel test was performed only with data set III (CR + *ATP8* + *ND5*) and revealed a weak but significant relationship between genetic and geographic distances when the entire sample was compared ($r = 0.29$; $p = 0.02$). However, when the two geographic groups of samples were analyzed individually, the correlation was very low and non-significant (SE: $r = 0.054$, $p = 0.1$, $n = 29$; NW: $r = 0.034$, $p = 0.41$, $n = 7$).

The divergence between *Lontra longicaudis* and *L. canadensis* was dated to *ca.* 4.16 MYA (confidence interval [CI]: 2-82 – 5.85 MYA). Within *L. longicaudis*, the divergence between the Colombian haplotype and the other lineages was estimated to have occurred around 0.644 MYA (CI: 0.437 – 0.906 MYA), while the three-way split among the Bolivian haplotype and Clades 1 and 2 was estimated to have occurred at *ca.* 0.450 MYA (CI: 0.303 – 0.629 MYA). The diversification within clades occurred soon afterwards. Of particular interest is the age of the base of Clade 1 (separation between the haplotype from French Guiana and the Brazilian group), estimated at 0.422 MYA (CI: 0.286 – 0.594), and the coalescence of the Brazilian clade itself (dated at 0.196 MYA; CI: 0.133 – 0.276).

Microsatellite data set

All twelve microsatellite loci analyzed were polymorphic for *L. longicaudis* (n = 36); however, two loci (RIO17 and Lut818) presented low amplification efficiency and were thus excluded from analyses. All individuals genotyped for the remaining ten loci presented unique multilocus composite genotypes. Moderate levels of genetic variability were found, with each locus yielding between 6 and 11 alleles and an average expected heterozygosity of 0.766 (Table 7). All loci were in linkage equilibrium in both populations after Bonferroni adjustments (α = 0.05). No significant departure from Hardy-Weinberg expectations was observed in NW geographic group after the Bonferroni correction (α = 0.05), but three departures from HWE were found in the Brazilian [SE] group (RIO07, RIO18 and RIO20). All departures from HWE were found to be heterozygote deficits, which can imply the presence of null alleles in this group. Comparative analyses excluding the loci that were out of equilibrium in any geographic group (uncorrected assessment) indicated no significant change in the results relative to our complete data set (data not shown). We have, therefore, used the complete data set of 10 loci throughout the analyses presented here.

As with the mtDNA data, the statistical analyses of population differentiation were performed assuming broad geographic units (Brazil [SE] and NW). Both F_{ST} and R_{ST} values were significant, indicating low to moderate structuring in this data set (F_{ST} = 0.058, p = 0.004; R_{ST} = 0.085, p = 0.026). These results are in agreement with mitochondrial analyses, showing a partition between Brazil and Northwestern South America. However, the magnitude of the differentiation is almost 10-fold lower in the microsatellite markers relative to the mtDNA data sets.

The *G*-test did not support a significant difference between these two major groups in their allele frequency distributions (p > 0.05). However, in the assignment test 88.9% (n = 32) of individuals were correctly allocated in their geographic group of origin. This result indicates that there is sufficient genetic distinction between these groups to allow mostly accurate assignments, in agreement with the significant results observed with the AMOVA.

Using the Bayesian clustering procedure, we initially evaluated the most likely subdivision scenario without using the information of the known geographic origin of each individual, and the probability of the observed data was maximum with two populations (k = 2; ln = -1182.93). We observed that 65% of individuals known to be from the same broad geographic area were assigned to the same genetic cluster. To better investigate the genetic composition of our data set, we performed a second set of analyses with STRUCTURE using population information. In this case, we considered the two geographic groups as separate populations, and the results were more compelling than in the first set of analysis, with

individuals that were *a priori* grouped in the SE and NW groups being correctly assigned with 97% and 89% accuracy, respectively (Fig. 9). An interesting case was that of individual bLlo21, from French Guiana, which was estimated to have a 57% probability of belonging to the Brazilian genetic cluster. Since this individual had 30% of its microsatellite data missing, we repeated this analysis excluding the loci for which no genotypes had been obtained from this animal. The proportion of its genomic ancestry in the Brazilian group decreased to 49%, but remained too high to be easily dismissed, implying some level of recent gene flow between the two groups.

The Mantel test revealed a weak but significant correlation between genetic and geographic distances when the whole sample was analyzed (r = 0.33; p = 0.001). When each group was analyzed separately, the Mantel test indicated a significant correlation for the Brazilian group (r = 0.47, p = 0.003, n = 29), but not for the NW geographic group (r = 0.49, p = 0.13, n = 7).

Discussion

Genetic Diversity

Observed levels of genetic variability in Neotropical otters (Tables 2, 6 and 7) were considerably high when compared to other vertebrates. For example, analyses of mtDNA control region data sets (overlapping with the segment employed here) from sympatric mammals yielded nucleotide diversity (π) values of 0.0017 in marsh deer (*Blastocerus dichotomus*) populations of the Río de la Plata basin (Márquez *et al*. 2006), and 0.00771 in jaguars (*Panthera onca)* throughout their range (Eizirik *et al.* 2001), while in this study we obtained values of 0.011 (Tables 2 and 6).

Tchaicka *et al.* (2007) used the same control region segment in phylogeographic analyses of the crab-eating fox (*Cerdocyon thous*)*,* a genetically diverse Neotropical canid sympatric with *L. longicaudis* throughout most of its range. The comparison between these species indicates that haplotype diversity is higher in *L. longicaudis* (*h* = 0.8911 ± 0.0060) than in *Cerdocyon* (*h* = 0.83 ± 0.032), while nucleotide diversity is lower (π = 0.0113 ± 0.0027 *vs*. 0.019 ± 0.002).

Comparisons to other studies performed with mustelids suggest moderate to high levels of mtDNA diversity in *Lontra longicaudis*. Both nucleotide and haplotype diversity found in the Neotropical otter mtDNA CR (π = 0.0113 ± 0.0027; *h* = 0.8911 ± 0.0060) are similar to or higher than estimates reported for *Mustela lutreola* (π = 0.012 ± 0.058 to 0.0012 ± 0.088; *h* = 0.469 to 0.939, Northeast and Southeast Europe populations, respectively; Michaux *et al*. 2005), *Enhydra lutris* (π = 0.098 ± 0.029; *h* = 0.412; Larson *et al*. 2002) and *Lutra lutra* (π =

0.0006; *h* = 0.16 ± 0.06; Ferrando *et al*. 2004). Studies on European populations of *Lutra lutra* based on 300bp of the 5' hypervariable sement of the mtDNA control region have so far described only six haplotypes, all of which differ from each other by only one nucleotide, indicating a low genetic variability for that species (Effenberger & Suchentrunk 1999; Mucci *et al*. 1999; Cassens *et al*. 2000; Pérez-Haro *et al*. 2005). The absence of other genetic studies on otter or any mustelid using the genes *ATP8* and *ND5* precludes direct comparisons of these data to other species.

In spite of its high diversity relative to other mtDNA segments, our analyses indicate that the control region may not be the best mitochondrial marker for phylogeographic studies in *L. longicaudis* and perhaps other related species. Variable sites in this segment seem to be saturated even at the intra-specific level, leading to a lower signal-to-noise ratio than verified for the other two fragments. Conversely, the *ND5* segment used here seems to be a very informative segment, less prone to saturation at recent levels, which has also been observed for other carnivores studied by our group (unpublished data).

With respect to microsatellite diversity, Beheler *et al*. (2004) described 10 loci for *Lontra canadensis*, two of which were used in this study. These markers exhibited more alleles in *L. longicaudis* than reported for their original target species, whereas the observed heterozygosity was higher in the latter. Beheler *et al*. (2005) described an additional 10 microsatellites for *L. canadensis*, four of which were analyzed here for *L. longicaudis*. Again, more alleles were found for every locus in *L. longicaudis*, while the observed heterozygosity was relatively lower in this species. The remaining microsatellite markers used here were developed for *Lutra lutra* (Dallas & Piertney 1998); in these markers a similar number of alleles and higher heterozygosity were observed for *L. longicaudis* relative to the original target species. Other studies employing these markers include the following: Hung *et al*. (2004) used three of these loci (Lut701, Lut733 e Lut782) on *Lutra lutra* scat samples in Kinmen, and found fewer alleles than identified here; Dallas *et al.* (2002) also employed the same three markers in *L. lutra* and found fewer alleles than reported here for two of the loci (Lut701 e Lut733); finally, Randi *et al.* (2003) used four of these loci in European populations of *L. lutra* and found a higher allelic diversity but observed heterozygosity generally lower than we estimated for *L. longicaudis*. A practical implication of these results is that these markers are quite variable in *L. longicaudis*, are thus informative for further investigations of population genetics and molecular ecology in this species, and should provide useful comparisons to other related species.

Phylogeography and Demographic History

The phylogenetic and network analyses of *Lontra longicaudis* mtDNA sequences showed this species to be composed of at least four geographically structured phylogroups: (i) Colombia; (ii) Bolivia; (iii) French Guiana; and (iii) Brazil. Colombia and Bolivia were represented by a single sample each, whose genetic divergence from other individuals indicates deep phylogeographic partitions involving these areas, which need to be further ascertained by means of enhanced sampling.

The allopatry of the two better-sampled phylogroups (Brazil and French Guiana) is almost complete, with only two individuals (bLlo41 and bLlo 66, which share the same haplotype) found in a geographic region inconsistent with their phylogenetic placement. The position of these French Guiana samples in Clade 1 is intriguing, as they are solidly placed at the base of this clade. This suggests that this incongruence between geography and phylogeny may be due to an ancestral colonization process, instead of secondary mtDNA gene flow. In that case, it would imply that the Brazilian clade derives from a recent episode of colonization from the north, so that northern populations are paraphyletic with respect to more southerly ones. Another observation that is consistent with this inference is the position of the single haplotype (sampled in two different individuals) from the Brazilian northeast, which is the most basal of all lineages from Brazil (e.g. Ll-AN4B in Fig. 5 and Ll-ANC18 in Fig. 6). This hypothesis needs to be further investigated on the basis of additional sampling in northern Brazil and adjacent areas.

The mtDNA inference of a significant genetic partition between Brazil and the other sampled areas in South America is supported by the microsatellite data set. However, the magnitude of this difference is much lower with these fast-evolving nuclear markers than with the matrilineal mitochondrial sequences. This may be due to slower effects of historical genetic drift acting on two isolated groups, given the larger effective population size of nuclear markers, or (non-exclusively) to the occurrence of ongoing male-biased gene flow between these areas. Teasing apart these processes should prove to be an interesting avenue for research in this species, for which data on individual dispersal and social behavior is currently unavailable.

The pattern observed with the mtDNA phylogenies and haplotype networks is consistent with a recent population expansion in Brazil, with most haplotypes from this region differing from each other by only one or two mutational steps, often connected in a star-shaped fashion (e.g. Figs. 5 - 8). This inference is also supported by the mtDNA mismatch distribution analysis (Fig. 4) and the neutrality test results. The mismatch distribution results of the *ATP8* + *ND5* data set (seemingly “cleaner” in terms of phylogenetic signal, with less

homoplasy than inferred for the control region) fits particularly well the expected pattern under a sudden demographic expansion (Rogers & Harpending 1992), with a single, smooth prominent peak. The age of this expansion was estimated at *ca.* 200,000 years ago, possibly following colonization of this area from northern South America.

Implications for conservation

The results presented here have implications for the conservation and management of this species in the wild and in captivity. In spite of being represented by a single sample each, the divergence observed in Colombia and Bolivia suggests that those areas may be sufficiently differentiated to warrant recognition as separate Evolutionarily Significant Units (ESU – Ryder 1986). As such, they should be conserved and managed as distinct entities. Further studies are required to improve our knowledge about these populations and their evolutionary relationships to others.

French Guiana otters are not monophyletic in their mtDNA lineages trees, but should be viewed as separate conservation unit. This area seems to contain a very high level of genetic diversity, and may hold important clues to better understand the evolutionary history of this species. It should therefore be viewed as an important focus for additional research and conservation efforts. Other demographic units probably exist in the Neotropical otter, which should be investigated in more detailed population genetic studies involving an expanded geographic sampling of the species range.

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Figure Legends

Fig 1. Map depicting currently assumed geographic distribution (*shaded area*) of the Neotropical otter (modified from http://www.otterspecialistgroup.org/Species/Lontra_longicaudis.html), with approximate sample collection sites. Black circles represent individuals from Brazil and black squares indicate individuals from Northwestern South America (French Guiana, Colombia, Bolivia, Peru). Numbers next to the collection sites are sample identification labels (number after "bLlo" in Table 1) of *L. longicaudis* individuals in each area. Boxes indicate individuals from the same region.

Fig. 2. Maximum likelihood tree of *L. longicaudis* mtDNA CR haplotypes identified in this study, based on 492 bp. Labels are haplotype identification numbers (see Table 3). Values above branches indicate support for the adjacent node based on ML / MP / NJ / BI. Given the evidence for saturation in the control region data set (see text), only *L. canadensis* was used as outgroup in these analyses (see Figs. 5 and 6).

Fig. 3. Median-joining network of haplotypes of *L. longicaudis* mtDNA control region (using 438 bp, excluding all sites containing indels or missing information). Circle size indicates the frequency of each haplotype, which is identified as listed in Table 3. Bars on branches indicate nucleotide substitutions inferred to have occurred between the connected haplotypes. Vertically hatched circles are haplotypes sampled in Brazil; horizontally hatched symbols are haplotypes sampled in French Guiana; the light grey haplotype (Ll-C18) was sampled in Colombia; the dark grey haplotype (Ll-C21) was sampled in Bolivia and the white one (Ll-C22) was sampled in Peru.

Fig. 4. Mismatch distribution analysis for the Brazilian samples. (A) mtDNA control region; (B) *ATP8* + *ND5* data set; (C) CR + *ATP8* + *ND5* data set. The dotted line indicates the

observed frequency of pairwise differences among haplotypes, while the continuous lines depicts the expected frequency under the sudden population expansion model.

Fig. 5. Maximum likelihood tree of *L. longicaudis* mtDNA *ATP8* + *ND5* haplotypes identified in this study. Labels are haplotype identification numbers (see Table 4). Values above branches indicate support for the adjacent node based on ML / MP / NJ / BI.

Fig. 6. Maximum likelihood tree of *L. longicaudis* mtDNA CR + *ATP8* + *ND5* lineages identified in this study, based on 1472 bp. Labels are haplotype identification numbers (see Table 5). Values above branches indicate support for the adjacent node based on ML / MP / NJ / BI.

Fig. 7. Median-joining network of *L. longicaudis* mtDNA *ATP8* + *ND5* haplotypes (using 681 bp; all sites containing indels or missing information were excluded). The crossed marks are nucleotide substitutions inferred in that branch. Bars on branches indicate nucleotide substitutions inferred to have occurred between the connected haplotypes. Vertically hatched circles are haplotypes sampled in Brazil; horizontally hatched symbols are haplotypes sampled in French Guiana and Peru; the light grey haplotype (Ll-AN14) was sampled in Colombia; the dark grey one (Ll-AN16) was sampled in Bolivia.

Fig 8. Median-joining network of *L. longicaudis* mtDNA CR + *ATP8* + *ND5* haplotypes (using 1164 bp; all sites containing indels or missing information were excluded). The crossed marks are nucleotide substitutions inferred in that branch. Bars on branches indicate nucleotide substitutions inferred to have occurred between the connected haplotypes. Vertically hatched circles are haplotypes sampled in Brazil; horizontally hatched symbols are haplotypes sampled in French Guiana; the light grey haplotype (Ll-ANC21) was sampled in Colombia; the dark grey haplotype (Ll-ANC24) was sampled in Bolivia and the white one (Ll-ANC25) was sampled in Peru.

Fig. 9. Best population clustering result (k = 2 clusters) in a Bayesian analysis of microsatellite data. Each otter individual is represented by a vertical bar partitioned into dark grey and light grey segments, the lengths of which indicate the probability of membership in each population cluster (parenthesis numbers: (1) Brazil; (2) Northwestern South America). Numbers below to the horizontal axe are sample identification labels (number after “bLlo” in Table 1) of *L. longicaudis* individuals in each area.

Table 1. Samples of the Neotropical otter analyzed in the present study.

ID	Sample	Geographic origin	Source Institution / Contact
bLlo01	muscle	Rio Grande do Sul State, Brazil	G. Bencke and J. M. Reppold
bLlo02	muscle	Santa Catarina State, Brazil	Sapucaia do Sul Zoo
bLlo03	muscle	Rio Grande do Sul State, Brazil	Carnívoros do RS Project
bLlo04[1]	footpad	Rio Grande do Sul State, Brazil	Carnívoros do RS Project
bLlo05	muscle	Gravataí, Rio Grande do Sul State, Brazil	G. L. Gonçalves
bLlo06	muscle	Rio Grande do Sul State, Brazil	P. H. Ott
bLlo07, bLlo08	muscle	Torres, Rio Grande do Sul State, Brazil	P. Colombo
bLlo09	muscle	Jataí, Goiás State, Brazil	CENAP / IBAMA
bLlo10	kidney	Moji-Mirim, São Paulo State, Brazil	CENAP / IBAMA
bLlo11	muscle	Rio Grande do Sul State, Brazil	A. P. Brandt
bLlo12	muscle	Dois Irmãos, Rio Grande do Sul State, Brazil	Prefeitura de Dois Irmãos / I. Fick
bLlo14	muscle	Angra dos Reis, Rio de Janeiro State, Brazil	H. F. Waldemarin
bLlo15	muscle	Guaratiba, Rio de Janeiro State, Brazil	H. F. Waldemarin
bLlo16	muscle	Barra, Rio de Janeiro State, Brazil	H. F. Waldemarin
bLlo17, bLlo18[2]	muscle	Pantanal, Mato Grosso do Sul State, Brazil	H. F. Waldemarin
bLlo19, bLlo20, bLlo21, bLlo22[2], bLlo25, bLlo41	muscle	French Guiana	B. de Thoisy
bLlo23	skin	Maceo, Antioquia, Colombia	D. Arcila
bLlo24	muscle	Rio Grande do Sul State, Brazil	T. R. O. de Freitas
bLlo26[2]	faeces	Eldorado do Sul, Rio Grande do Sul State, Brazil	Sapucaia do Sul Zoo / R. von Hohendorff
bLlo28[2]	hair	Pantanal, Mato Grosso State, Brazil	I. C. Pfeifer
bLlo29	blood	Foz do Iguaçú, Paraná State, Brazil	São Paulo Zoo / K. Kassaro
bLlo30	blood	Belo Horizonte, Minas Gerais State, Brazil	São Paulo Zoo / K. Kassaro
bLlo31	blood	Corumbá, Mato Grosso do Sul State, Brazil	São Paulo Zoo / K. Kassaro
bLlo34	muscle	Blumenau, Santa Catarina State, Brazil	FURB / S. Althoff
bLlo36[2]	faeces	Sumaré, São Paulo State, Brazil	Campinas Zoo / E. Ferraz
bLlo37[2]	faeces	Nova Santa Rita, Rio Grande do Sul State, Brazil	Canoas Mini Zoo / M. Martins
bLlo38	muscle	P.N. Ilha Grande, Paraná State, Brazil	L. Koprowski
bLlo39	skin	Ribeirão Preto, São Paulo State, Brazil	Ribeirão Preto Zoo / M. dos Santos
bLlo42	muscle	Capela de Santana, Rio Grande do Sul State, Brazil	Canoas Mini Zoo / M. Martins
bLlo51[2]	faeces	P.E. da Ilha do Cardoso, São Paulo State, Brazil	E. Nakano
bLlo57	blood	Paranapanema River, Paraná State, Brazil	L. Koprowski
bLlo58	muscle	Osório, Rio Grande do Sul State, Brazil	P. Colombo, C. Zank and G. Volkmer
bLlo60, bLlo61	blood	Recife, Pernambuco State, Brazil	Dois Irmãos Park / A. L. Brito
bLlo64[2]	faeces	Vermelho River, Corumbá, Mato Grosso do Sul State, Brazil	Embrapa-Pantanal / G. Mourão
bLlo66[3]	DNA	French Guiana	K.P. Koepfli
bLlo67[3]	DNA	Bolivia	K.P. Koepfli
bLlo68[3]	DNA	Peru	K.P. Koepfli

[1] samples typed only for microsatellite loci

[2] samples typed only for the mtDNA control region.

[3] samples typed only for mtDNA segments.

Table 2. mtDNA diversity estimates for the Neotropical river otter and related species.

Segment [a]	Length (bp) [b]	N	No. of haplotypes	S [c]	PI [d]	ð [e, g]	d_{xy} (*Lontra canadensis*) [f,g]	d_{xy} (*Amblonyx cinereus*) [f, g]	d_{xy} (*Pteronura brasiliensis*) [f, g]
CR	492 (438)	44	23	27 / 40	15 /19	0.01133 ± 0.00266	0.05173 ± 0.00953	0.06761 ± 0.01135	0.06834 ± 0.01103
ATP8	329 (295)	37	11	16 / 66	1 / 7	0.00394 ± 0.00128	0.18369 ± 0.02257	0.20953 ± 0.02329	0.20412 ± 0.02343
ND5	651 (547)	37	17	36 / 99	11 / 21	0.00732 ± 0.00198	0.10591 ± 0.01478	0.17378 ± 0.01599	0.15148 ± 0.01696
ATP8 + ND5	980 (681)	36	19	49 / 162	14 / 30	0.00587 ± 0.00130	0.14187 ± 0.01302	0.18971 ± 0.015011	0.17344 ± 0.01367
CR + *ATP8 + ND5*	1472 (1164)	35	26	76 / 206	31 / 50	0.00809 ± 0.00127	0.10857 ± 0.00851	0.14691 ± 0.00940	0.13940 ± 0.00904

[a] CR= mtDNA control region; *ATP8* : *ATPase subunit 8* ; *ND5* : *NADH dehydrogenase subunit 5* .

[b] values in parentheses are segment lengths after exclusion of all sites containing gaps or missing information.

[c] S = Segregating (polymorphic) sites; values are given for the *L. longicaudis* data set / data set including *L. longicaudis + L. canadensis* .

[d] PI = Parsimony-informative sites; values are given for the *L. longicaudis* data set / data set including *L. longicaudis* + *L. canadensis* .

[e] ð = nucleotide diversity per site.

[f] dxy= mean nucleotide divergence (p-distance) between *L. longicaudis* haplotypes and selected outgroups.

[g] all sites containig indels or missing information were excluded from this analysis.

Table 3. List of individuals that bear each mtDNA control region haplotype. Also indicated are the absolute frequency in the total sample (Fr) and the geographic distribution of each haplotype.

Haplotype[a]	Individuals	Fr	Country of haplotype occurrence
Ll-C1	bLlo01, bLlo03,bLlo11, bLlo24	4	Brazil
Ll-C2A	bLlo02, bLlo06, bLlo08, bLlo09, bLlo12, bLlo14, bLlo15, bLlo18, bLlo26, bLlo34, bLlo38, bLlo58, bLlo64	13	Brazil
Ll-C2B	bLlo30	1	Brazil
Ll-C3	bLlo05, bLlo39	2	Brazil
Ll-C4	bLlo07	1	Brazil
Ll-C5	bLlo10	1	Brazil
Ll-C6	bLlo16	1	Brazil
Ll-C7	bLlo17	1	Brazil
Ll-C8	bLlo28	1	Brazil
Ll-C9	bLlo29, bLlo37	2	Brazil
Ll-C10	bLlo31	1	Brazil
Ll-C11	bLlo36	1	Brazil
Ll-C12	bLlo42	1	Brazil
Ll-C13	bLlo51	1	Brazil
Ll-C14	bLlo57	1	Brazil
Ll-C15	bLlo60, bLlo61	2	Brazil
Ll-C16	bLlo19, bLlo20	2	French Guiana
Ll-C17	bLlo21, bLlo22	2	French Guiana
Ll-C18	bLlo23	1	Colombia
Ll-C19	bLlo25	1	French Guiana
Ll-C20	bLlo41, bLlo66	2	French Guiana
Ll-C21	bLlo67	1	Bolivia
Ll-C22	bLlo68	1	Peru

[a] Haplotypes with the same number and different letters (e.g. Ll-C2A, 2B) are collapsed into a single haplotype when all sites with missing information or indels are excluded (e.g. Fig.3).

Table 4. List of individuals that bear each mitochondrial DNA *ATP8* + *ND5* haplotype. Also indicated are the absolute frequency on the total sample (Fr) and the geographic distribution of each haplotype.

Haplotype [a]	Individuals	Fr	Country(ies) of haplotype occurrence
Ll-AN1	bLlo01	1	Brazil
Ll-AN2	bLlo02, bLlo14, bLlo15	3	Brazil
Ll-AN3	bLlo03, bLlo24, bLlo31	3	Brazil
Ll-AN4A	bLlo05, bLlo06, bLlo08, bLlo12, bLlo16, bLlo29, bLlo30, bLlo34, bLlo57, bLlo58	10	Brazil
Ll-AN4B	bLlo60, bLlo61	2	Brazil
Ll-AN5	bLlo07	1	Brazil
Ll-AN6	bLlo09, bLlo39	2	Brazil
Ll-AN7	bLlo10	1	Brazil
Ll-AN8	bLlo11	1	Brazil
Ll-AN9	bLlo17	1	Brazil
Ll-AN10	bLlo42	1	Brazil
Ll-AN11A	bLlo19	1	French Guiana
Ll-AN11B	bLlo20	1	French Guiana
Ll-AN12A	bLlo21, bLlo68	2	French Guiana, Peru
Ll-AN12B	bLlo25	1	French Guiana
Ll-AN13	bLlo22	1	French Guiana
Ll-AN14	bLlo23	1	Colombia
Ll-AN15	bLlo41, bLlo66	2	French Guiana
Ll-AN16	bLlo67	1	Bolivia

[a] Haplotypes with the same number and different letters (e.g. Ll-AN4A, 4B) are collapsed into a single haplotype when all sites with missing information or indels are excluded (e.g. Fig.5).

Table 5. List of individuals that bear each mitochondrial DNA CR + *ATP8* + *ND5* haplotype. Also indicated are the absolute frequency on the total sample (Fr) and the geographic distribution of each haplotype.

Haplotype [a]	Individuals	Fr	Country of haplotype occurrence
Ll-ANC1	bLlo01	1	Brazil
Ll-ANC2	bLlo02, bLlo14, bLlo15	3	Brazil
Ll-ANC3	bLlo03, bLlo24	2	Brazil
Ll-ANC4	bLlo05	1	Brazil
Ll-ANC5	bLlo06, bLlo08, bLlo12, bLlo34, bLlo58	5	Brazil
Ll-ANC6	bLlo07	1	Brazil
Ll-ANC7	bLlo09	1	Brazil
Ll-ANC8	bLlo10	1	Brazil
Ll-ANC9	bLlo11	1	Brazil
Ll-ANC10	bLlo16	1	Brazil
Ll-ANC11	bLlo17	1	Brazil
Ll-ANC12	bLlo29	1	Brazil
Ll-ANC13	bLlo30	1	Brazil
Ll-ANC14	bLlo31	1	Brazil
Ll-ANC15	bLlo39	1	Brazil
Ll-ANC16	bLlo42	1	Brazil
Ll-ANC17	bLlo57	1	Brazil
Ll-ANC18	bLlo60, bLlo61	2	Brazil
Ll-ANC19A	bLlo19	1	French Guiana
Ll-ANC19B	bLlo20	1	French Guiana
Ll-ANC20	bLlo21	1	French Guiana
Ll-ANC21	bLlo23	1	Colombia
Ll-ANC22	bLlo25	1	French Guiana
Ll-ANC23	bLlo41, bLlo66	2	French Guiana
Ll-ANC24	bLlo67	1	Bolivia
Ll-ANC25	bLlo68	1	Peru

[a] Haplotypes with the same number and different letters (e.g. Ll-ANC19A, 19B) are collapsed into a single haplotype when all sites with missing information or indels are excluded (e.g. Fig.7).

Table 6. Nucleotide and haplotype diversity observed in the *Lontra longicaudis* mtDNA segments, specified separately for different geographically defined populations.

Segment[a]	Group[b]	Nucleotide diversity (SE)[c]	Haplotype diversity (SE)[c]
CR	Brazil	0.0062 ± 0.0019	0.8217 ± 0.0106
	FG	0.0165 ± 0.0039	0.8571 ± 0.0386
	NW	0.0178 ± 0.0040	0.9333 ± 0.0196
	Total	0.0113 ± 0.0027	0.8911 ± 0.0060
ATP8 + *ND5*	Brazil	0.0019 ± 0.0006	0.7754 ± 0.0153
	FG	0.0058 ± 0.0016	0.9048 ± 0.0389
	NW	0.0094 ± 0.0019	0.9333 ± 0.0196
	Total	0.0059 ± 0.0013	0.8762 ± 0.0077
CR + *ATP8* + *ND5*	Brazil	0.0038 ± 0.0009	0.9538 ± 0.0053
	FG	0.0100 ± 0.0018	0.8667 ± 0.0527
	NW	0.0124 ± 0.0018	0.9444 ± 0.0233
	Total	0.0081 ± 0.0012	0.9714 ± 0.0027

[a] CR: mtDNA control region; *ATP8*: *ATPase subunit 8*; *ND5*: *NADH dehydrogenase subunit 5*.

[b] FG: French Guiana; NW: Northwestern South America (including French Guiana, Peru, Bolivia and Colombia).

[c] All sites containing indels or missing information were excluded from this analysis. SE: standard error.

Table 7. Measures of microsatellite diversity in the two geographic groups of the Neotropical otter investigated in this study.

	Brazil						Northwestern South America					
Locus	N[a]	NEA[b]	SR (bp)[c]	H_e[d]	H_o[e]	HWE[f] (P)	N[a]	NEA[b]	SR (bp)[c]	H_e[d]	H_o[e]	HWE[f] (P)
Lut453	6	3	136-146	0.756	0.704	ns	3	0	138-144	0.648	0.429	ns
Lut701	6	1	180-198	0.680	0.826	ns	7	2	172-198	0.924	0.833	ns
Lut733	7	2	154-178	0.756	0.690	ns	5	0	154-170	0.758	0.714	ns
Lut782	6	2	178-198	0.672	0.517	ns	5	1	182-206	0.803	0.500	ns
RIO06	8	5	261-289	0.729	0.630	ns	3	0	275-281	0.711	0.000	ns
RIO07	8	5	176-194	0.791	0.379	*	3	0	182-186	0.604	0.286	ns
RIO11	11	7	168-188	0.895	0.862	ns	4	0	168-182	0.758	0.429	ns
RIO18	11	7	142-174	0.827	0.462	*	5	1	142-154	0.670	0.429	ns
RIO19	9	5	286-306	0.712	0.571	ns	6	2	290-304	0.747	0.571	ns
RIO20	8	5	261-279	0.845	0.250	*	3	0	265-269	0.545	0.000	ns
Mean	8	4.2	-	0.766	0.589	-	4.4	0.6	-	0.717	0.419	-

[a] Number of alleles

[b] Number of exclusive alleles

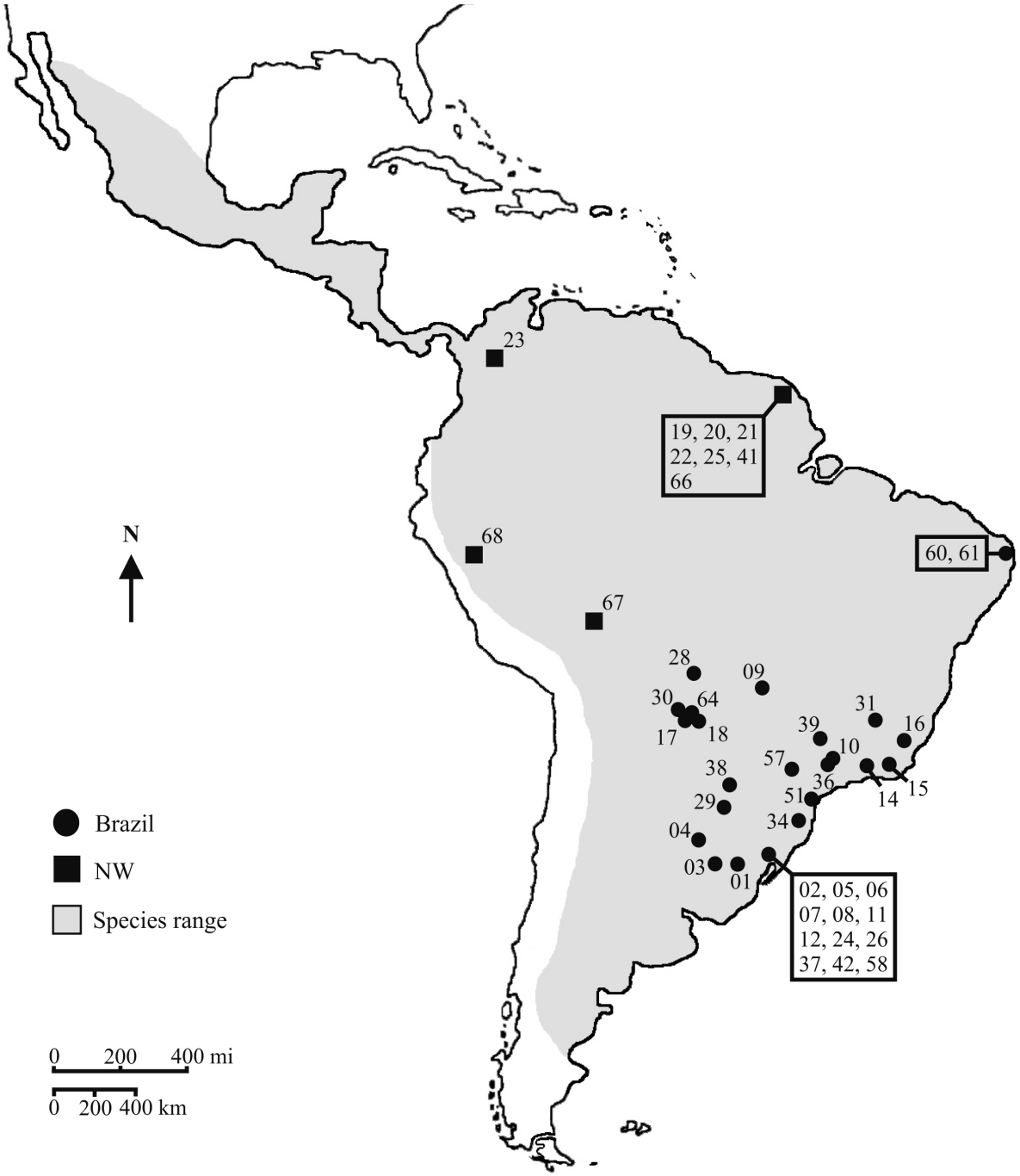
[c] Size range

[d] Expected heterozygosity

[e] Observed heterozygosity

[f] Deviation from Hardy-Weinberg Equilibrium

[*] Significant after Bonferroni adjustment ($p < 0.001$)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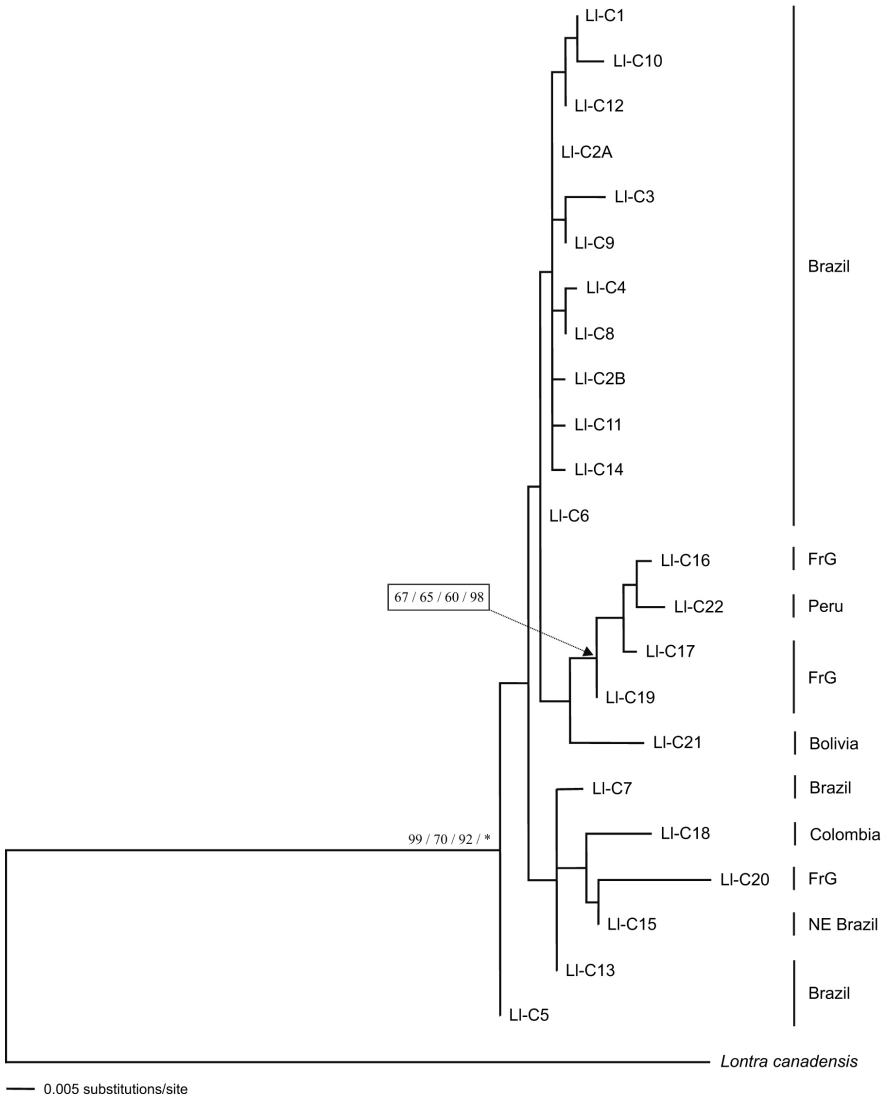
Brazil
NW
Species range
0 200 400 mi
0 200 400 km
23
19, 20, 21
22, 25, 41
66
68
60, 61
67
28
09
30
64
17
18
31
16
39
10
57
38
36
51
14
15
29
34
04
03
01
02, 05, 06
07, 08, 11
12, 24, 26
37, 42, 58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LI-C12
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LI-C4
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LI-C2B
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LI-C14
LI-C6
LI-C16
LI-C22
LI-C17
LI-C19
LI-C21
LI-C7
LI-C18
LI-C20
LI-C15
LI-C13
LI-C5
67 / 65 / 60 / 98
99 / 70 / 92 / *
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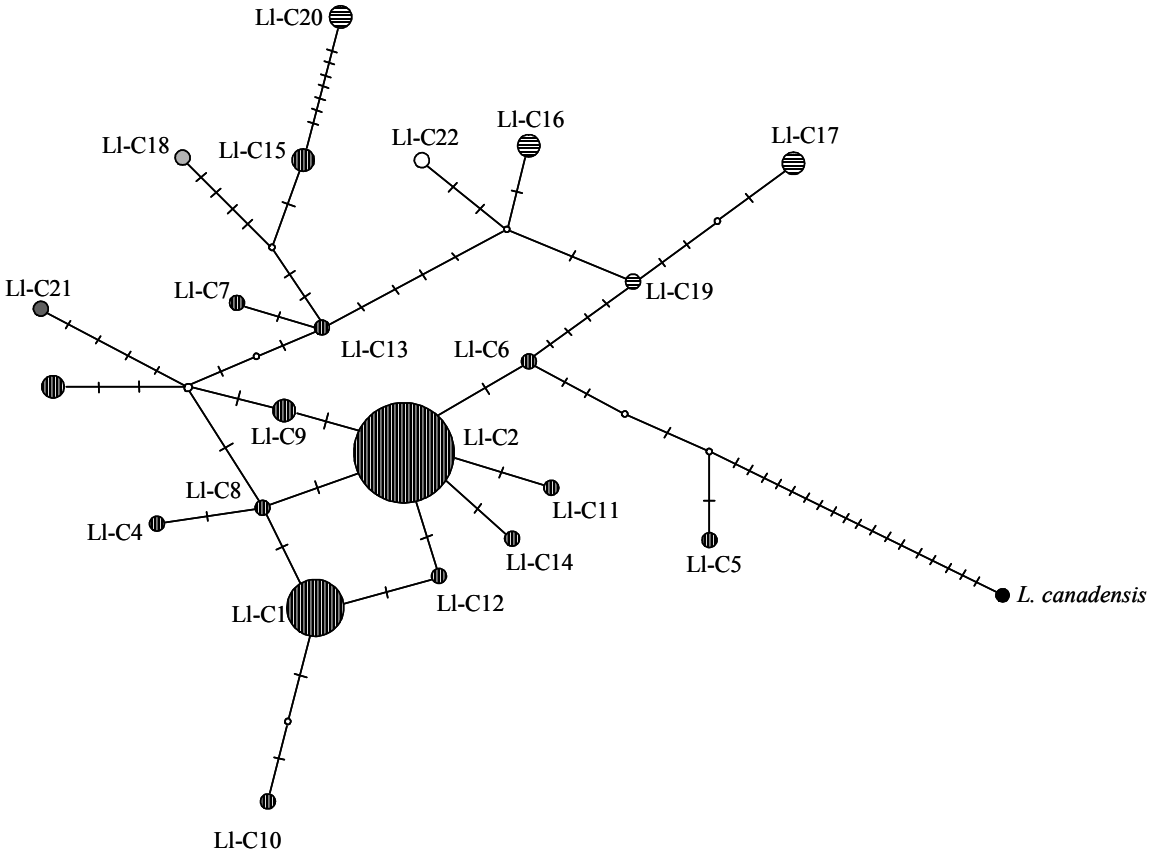
FrG
Peru
FrG
Bolivia
Brazil
Colombia
FrG
NE Brazil
Brazil
Lontra canadensis
0.005 substitutions/site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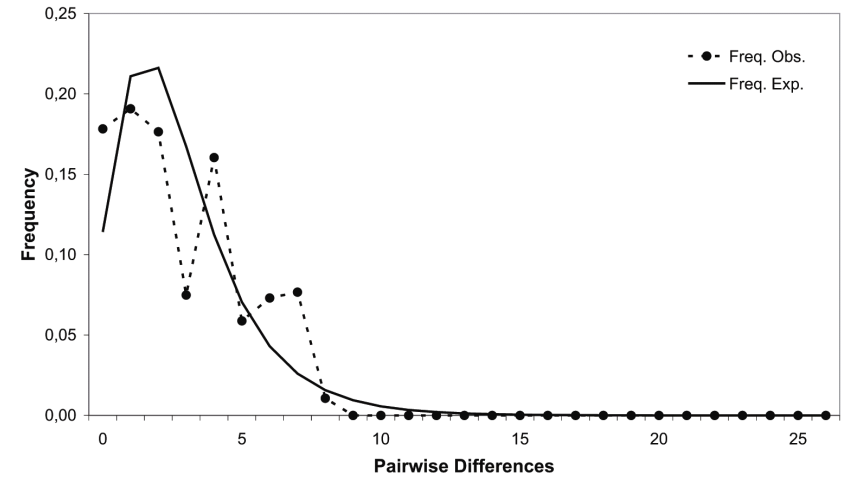
Ll-C6
Ll-C9
Ll-C2
Ll-C8
Ll-C11
Ll-C4
Ll-C14
Ll-C5
L. canadensis
Ll-C1
Ll-C12
Ll-C10

(A)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0,15
0,10
0,05
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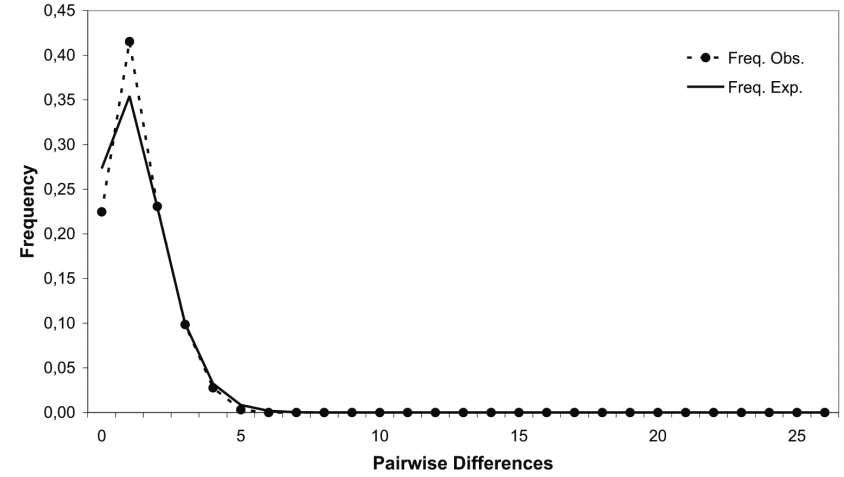
5
10
15
20
25
Pairwise Differences
Freq. Obs.
Freq. Exp.

(B)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Frequency
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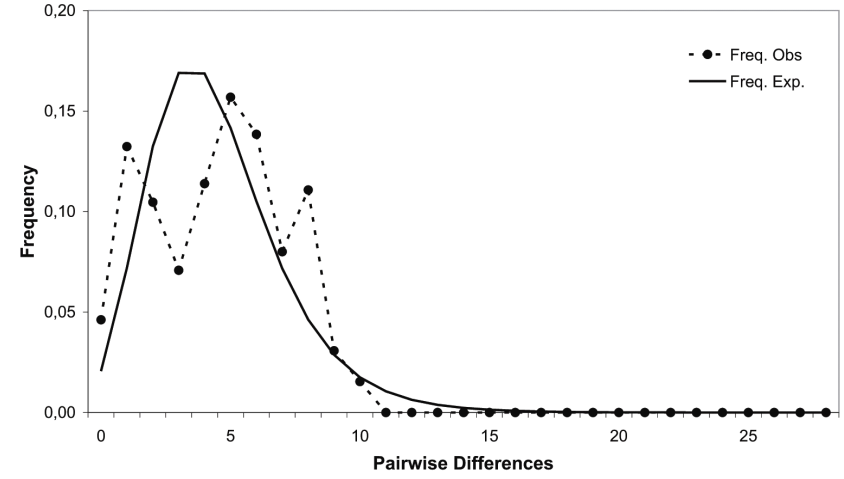
10
15
20
25
Pairwise Differences
Freq. Obs.
Freq. Exp.

(C)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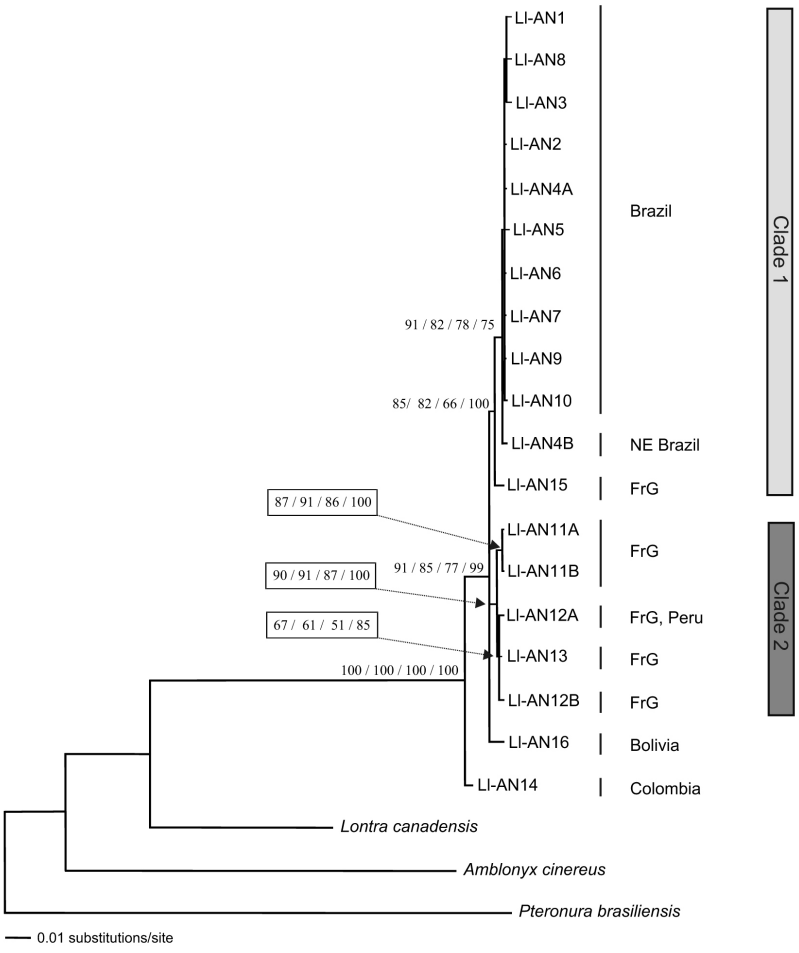
0,05
0,00
Frequency
0
5
10
15
20
25
Pairwise Differences
Freq. Obs
Freq. Exp.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LI-AN15
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LI-AN11B
LI-AN12A
LI-AN13
LI-AN12B
LI-AN16
LI-AN14
Brazil
NE Brazil
FrG
FrG
FrG, Peru
FrG
FrG
Bolivia
Colombia
Clade 1
Clade 2
91 / 82 / 78 / 75
85/ 82 / 66 / 100
87 / 91 / 86 / 100
91 / 85 / 77 / 99
90 / 91 / 87 / 100
67 / 61 / 51 / 85
100 / 100 / 100 / 100
Lontra canadensis
Amblonyx cinereus
Pteronura brasiliensis
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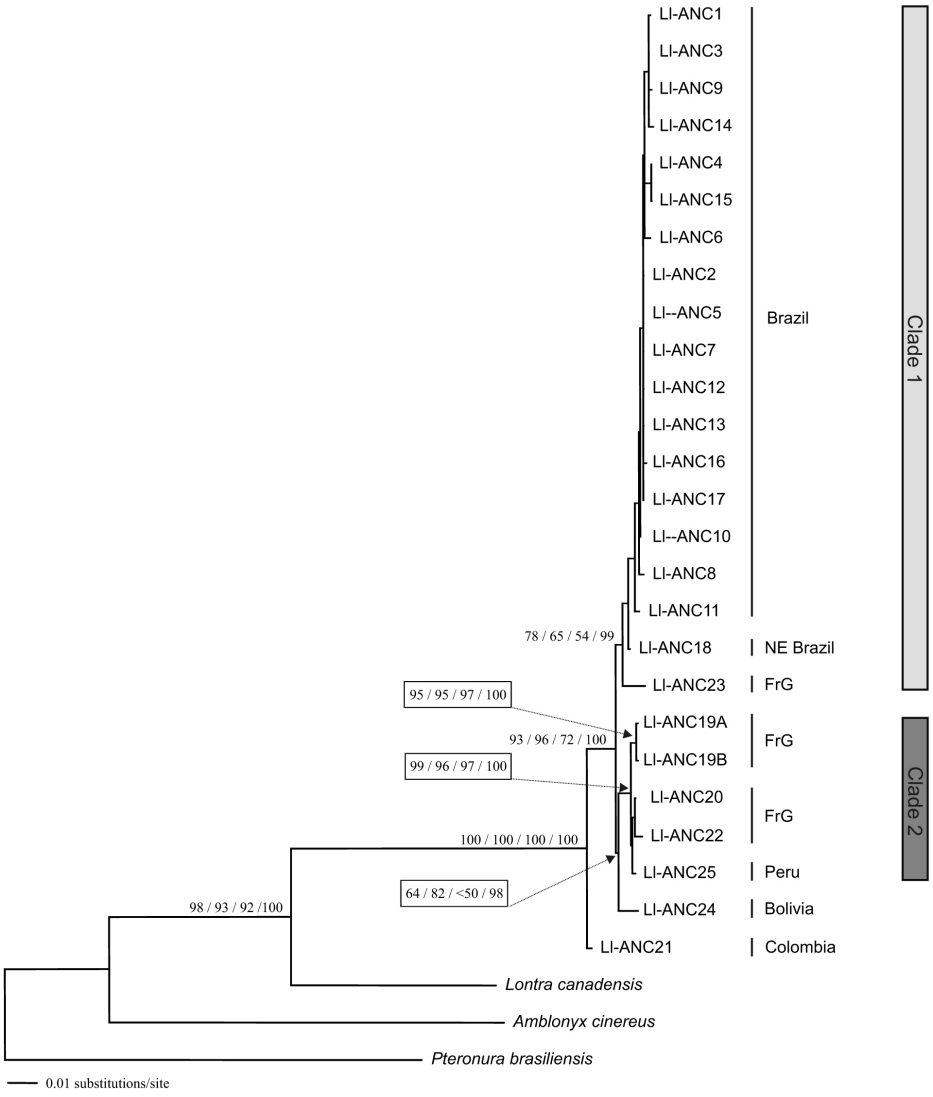
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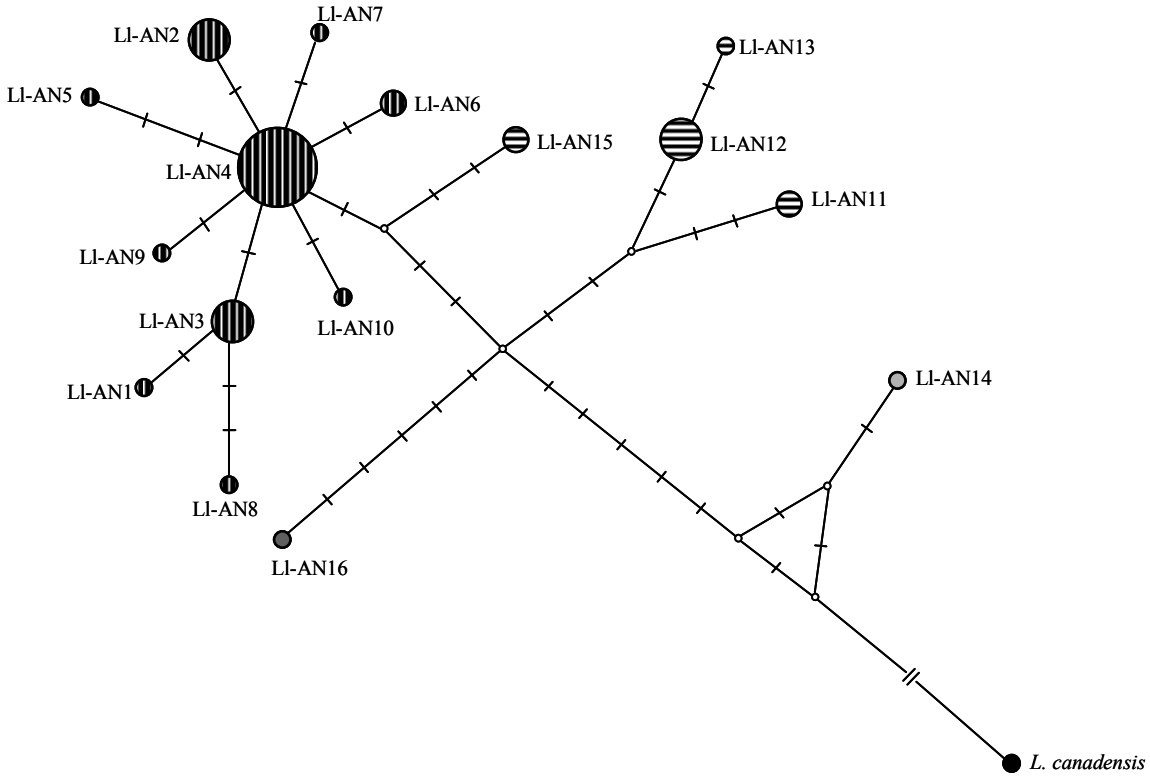
LI-ANC6
LI-ANC2
LI--ANC5
LI-ANC7
LI-ANC12
LI-ANC13
LI-ANC16
LI-ANC17
LI--ANC10
LI-ANC8
LI-ANC11
Brazil
78 / 65 / 54 / 99
LI-ANC18
NE Brazil
LI-ANC23
FrG
95 / 95 / 97 / 100
LI-ANC19A
93 / 96 / 72 / 100
LI-ANC19B
FrG
99 / 96 / 97 / 100
LI-ANC20
LI-ANC22
FrG
100 / 100 / 100 / 100
LI-ANC25
Peru
64 / 82 / <50 / 98
LI-ANC24
Bolivia
98 / 93 / 92 /100
LI-ANC21
Colombia
Lontra canadensis
Amblonyx cinereus
Pteronura brasiliensis
0.01 substitutions/site
Clade 1
Clade 2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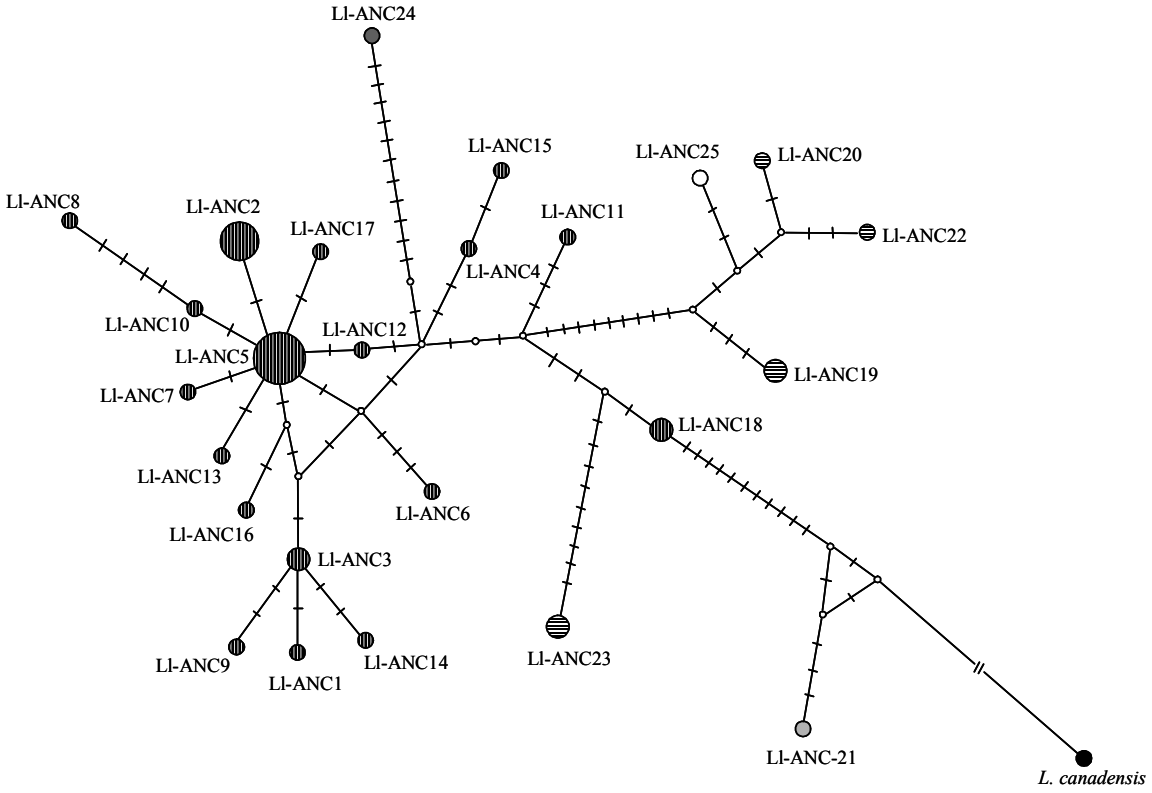
Ll-AN16
L. canadensis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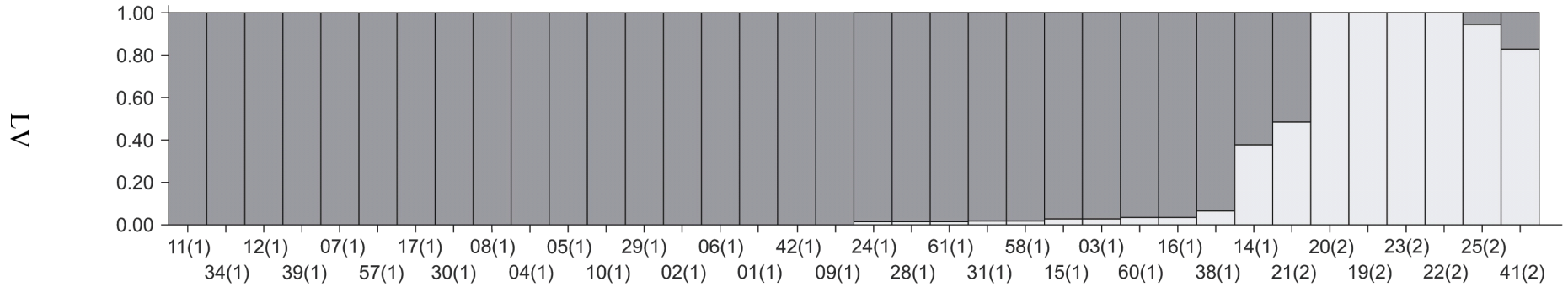
Ll-ANC1
Ll-ANC-21
L. canadensis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0.40
0.20
0.00
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34(1)
12(1)
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07(1)
57(1)
17(1)
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08(1)
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29(1)
02(1)
06(1)
01(1)
42(1)
09(1)
24(1)
28(1)
61(1)
31(1)
58(1)
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16(1)
38(1)
14(1)
21(2)
20(2)
19(2)
23(2)
22(2)
25(2)
41(2)

Author Information Box

This project is part of Cristine S. Trinca's M. Sc. thesis at the Graduate Program in Zoology of the Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Brazil, where she is advised by Dr. Eduardo Eizirik. Her research interests are focused on population genetics, ecology, phylogeography and conservation of Neotropical otters. Dr. Helen Waldemarin is an otter biologist interested in ecology, behavior and conservation of Neotropical populations of this group. Dr. Klaus-Peter Koepfli works on carnivore phylogenetics, evolutionary biology and biogeography. Dr. Benoit de Thoisy is a conservation biologist interested in diverse issues pertaining to genetics and ecology of South American taxa. Dr. Eizirik is an evolutionary biologist focusing most of his research on phylogenetics, conservation genetics and molecular ecology of Neotropical carnivores.

Capítulo III – Discussão Geral

Os resultados obtidos no presente trabalho evidenciam a existência de padrões filogeográficos claros em *Lontra longicaudis*. A partir das análises filogenéticas e populacionais realizadas, são discutidas algumas hipóteses a respeito da história demográfica desta espécie, bem como sugestões para incorporar estas informações no planejamento de estratégias adequadas para a conservação e manejo da mesma. Inferências sobre os padrões de variabilidade nos segmentos de mtDNA utilizados no estudo foram realizadas, avaliando-se seu desempenho como marcadores para estudos evolutivos recentes em mustelídeos. Estes aspectos foram abordados na discussão do artigo precedente. Sendo assim, neste espaço, será feita uma breve revisão dos mesmos, salientando alguns aspectos e implicações adicionais.

O presente trabalho é o primeiro a caracterizar a diversidade genética e a estruturação geográfica observada em populações naturais de *L. longicaudis*, uma vez que os únicos estudos até o momento incluindo esta espécie são análises filogenéticas da família Mustelidae e da Subfamília Lutrinae em geral (Van Zyll de Jong 1987; Koepfli & Wayne 1998, 2003; Marmi *et al*. 2004), não abordando qualquer aspecto genético e/ou populacional da lontra Neotropical em particular. Alguns estudos desta natureza foram realizados até o momento para outras espécies de mustelídeos (p.ex. Mucci *et al*. 1999; Kyle & Strobeck 2001; Fleming & Cook 2002; Blundell *et al*. 2002; Broquet *et al*. 2006; Pertoldi *et al*. 2006), o que permite comparações iniciais com os resultados aqui obtidos. Estas comparações indicam que a lontra Neotropical apresenta níveis relativamente altos de diversidade genética nos segmentos mitocondriais empregados, bem como alta variabilidade nos locos de microssatélite analisados.

A região controladora têm sido bastante empregada em estudos evolutivos e demográficos de diversas espécies de mamíferos, revelando padrões filogeográficos claros em diversas espécies de vertebrados (Sivasundar *et al.* 2001; Márquez *et al*. 2006; Tchaicka *et al*. 2007). Entretanto, este segmento, apesar de bastante variável, não apresentou boa resolução na identificação de padrões de estruturação geográfica e história evolutiva de *Lontra longicaudis*. Nota-se que apesar de relativamente variável em nível intra-específico, quando utilizado para comparações interespecíficas, sua variabilidade é bastante reduzida, dando claras indicações de saturação. Entretanto, ainda assim, este segmento mostrou-se mais variável do que em *Lutra lutra,* em que diversos estudos encontraram uma diversidade nucleotídica e haplotípica bastante reduzida em diversas regiões da Europa (Mucci *et al*. 1999; Effenberger & Suchentrunk 1999; Cassens *et al*. 2000; Pérez-Haro *et al*. 2005).

Comparações com outros estudos realizados com diversas espécies de carnívoros sugerem moderados níveis de variabilidade genética no DNA mitocondrial de *Lontra longicaudis*. A diversidade haplotípica geral encontrada para a região controladora do mtDNA é relativamente alta (h_{Conc}= 0.8911 ± 0.0060), em comparação, por exemplo, com *Mustela lutreola* (*h* = 0.469 e 0.939,

para duas populações da Europa; Michaux *et al*. 2005), *Enhydra lutris* (*h* = 0.412; Larson *et al*. 2002) e *Lutra lutra* (*h* = 0.16 ± 0.06; Ferrando *et al*. 2004). A diversidade nucleotídica encontrada neste conjunto de dados é moderada (π = 0.0113 ± 0.0027) comparada com estimativas obtidas para outros mustelídeos, como por exemplo, *Lutra lutra* (π = 0.0006; Ferrando *et al*. 2004), e *Mustela lutreola* (π = 0.012 ± 0.058 e 0.0012 ± 0.088 para cada uma das duas populações analisadas). Em contrapartida, uma menor diversidade é observada quando comparada a *Enhydra lutris* (π = 0.098 ± 0.029; Larson *et al*. 2002).

Johnson *et al*. (1999) utilizaram os genes *ATP8* e *ND5* na investigação de padrões filogeográficos de quatro espécies de pequenos felinos neotropicais. A diversidade nucleotídica (π) encontrada variou de 0.0029 (*Oncifelis guigna*) a 0.0126 apresentando grande número de sítios polimórficos (em média 36, exceto para *O. guigna*). Os valores encontrados para *L. longicaudis* podem ser considerados altos (36 sítios polimórficos e π = 0.0080 ± 0.0015).

Todavia, comparações com outras espécies de carnívoros, especialmente mustelídeos, sobre a variabilidade dos segmentos dos genes *ATP8* e *ND5* não puderam ser realizadas, pois os estudos realizados até o momento não utilizaram estas regiões do mtDNA.

Quando a diversidade genética é comparada entre os segmentos empregados, a região controladora é mais variável do que os genes *ATP8* e *ND5*, entretanto, em comparações interespecíficas, estes dois apresentam-se muito mais informativos. Isto também fica evidente na análise das árvores filogenéticas, as quais foram claras em demonstrar sinais filogeográficos, com exceção daquela construída somente com a porção hipervariável I da região controladora (Fig. 2). Isto sugere que este segmento esteja saturado de mutações, perdendo assim poder informativo. Este resultado é interessante do ponto de vista experimental e analítico, uma vez que a região controladora tem sido muito utilizada para investigar padrões filogeográficos com diversas espécies de vertebrados. Caso o padrão da região controladora observado para *Lontra longicaudis* venha a ser corroborado em outras espécies, é possível que a escolha preferencial deste segmento como portador de sinais filogeográficos seja questionada.

Em contrapartida, os genes *ATP8* e *ND5* apresentaram alta diversidade intra- e interespecífica, sendo que devido ao tamanho do segmento e número de sítios polimórficos e informativos para as análises dentro e entre espécies, o gene *ND5* parece, neste caso, ser o mais indicado para análises filogeográficas.

Com relação aos locos de microssatélite, estes se mostraram bastante variáveis, com grande número de alelos, comparados às espécies para os quais foram descritos (*Lontra canadensis*, Beheler *et al.* 2004, 2005; *Lutra lutra*, Dallas & Piertney 1998). Beheler *et al*. (2004, 2005) encontraram, em geral, menor número de alelos e maior heterozigosidade em *L. canadensis* em relação ao observado para *L. longicaudis*. Este fato, porém, pode ser um efeito do tamanho

amostral analisado para *L. longicaudis*, o qual pode não ter sido totalmente representativo da variabilidade nestes locos na espécie em questão. Dallas & Piertney (1998) encontraram números semelhantes de alelos, todavia, em menor nível de heterozigosidade do que o observado em *L. longicaudis*.

Estudos direcionados à análise e estruturação da variabilidade genética de populações naturais de *Lutra lutra* foram realizados em diversas regiões da Europa e Ásia (Dallas *et al.* 2002; Randi *et al.* 2003; Hung *et al.* 2004), os quais utilizaram alguns dos mesmos locos de microssatélite empregados neste estudo. De um modo geral, estes locos demonstraram-se tão ou mais variáveis em *Lontra longicaudis* do que em *Lutra lutra*, indicando que os marcadores selecionados são eficientes para a investigação de parâmetros populacionais naquela espécie.

As análises de mtDNA revelaram a existência de um padrão filogeográfico claro, sem compartilhamento de haplótipos entre as regiões. Este padrão é necessariamente decorrente de um extenso período de isolamento entre as populações ancestrais (Avise *et al*. 1987). Os resultados obtidos indicam a existência de quatro entidades filogeográficas, duas delas sustentadas por um conjunto amostral robusto e as outras duas representadas por apenas um indivíduo cada. Os dois maiores grupos de linhagens de mtDNA são representados pelo Clado 1, o qual compreende todas as linhagens brasileiras e uma proveniente da Guiana Francesa, e o Clado 2 incluindo todos os outros haplótipos encontrados na Guiana Francesa e Peru.

As duas outras linhagens de mtDNA são provenientes da Bolívia e da Colômbia. Coletado na Cordilheira central dos Andes, o indivíduo proveniente da Colômbia revelou conter a linhagem mais divergente, sugerindo que a Cordilheira dos Andes pode ser uma barreira ao fluxo gênico entre os indivíduos localizados a Leste e Oeste da mesma. Contudo, o tempo de divergência entre esta linhagem e o restante, parece bastante recente (644 mil anos atrás), indicando que o episódio de isolamento deste grupo foi posterior ao surgimento desta provável barreira.

O tempo de divergência entre *L. longicaudis* e *L. canadensis* datado foi cerca de 4.16 Ma. A divergência média entre o haplótipo Colombiano e as outras linhagens foi estimada em 0.644 Ma, enquanto que a separação da linhagem encontrada na Bolívia e dos Clados 1 e 2 foi datada em aproximadamente 450 mil anos. Estes dois clados apresentaram uma alopatria quase completa, exceto por um haplótipo geograficamente proveniente da Guiana Francesa incluído no Clado 1, predominantemente brasileiro. Este dado indica haver alguma conectividade (ao menos ancestral) entre as populações destas duas regiões, onde o sentido de colonização seria Norte–Sul, tendo em vista as posições dos haplótipos da Guiana Francesa e do Nordeste brasileiro (basal entre todas as seqüências do Brasil) no Clado 1. De especial interesse é a datação do tempo de divergência na base do Clado 1 (separação entre o haplótipo da Guiana e o grupo brasileiro de linhagens), a qual foi estimada em 0.422 Ma. A coalescência das linhagens brasileiras foi datada em cerca de 196

Ma. Além disso, os haplótipos da Guiana Francesa demonstram algum grau de sub-estruturação indicado pelas análises filogenéticas, com tempo de divergência datado em aproximadamente 176 mil anos atrás.

Apesar de Colômbia e Bolívia serem, cada uma, representadas por apenas um indivíduo, sua posição consistente nas várias análises e o alto grau de apoio aos principais ramos das árvores filogenéticas indica que há uma alta probabilidade de haver entidades demográficas distintas nestas regiões.

Resultados obtidos através das análises dos locos de microssatélite concordam com aqueles revelados pelo mtDNA, indicando haver clara separação entre o Brasil e o Noroeste da América do Sul (apoiado pelo alto valor de F_{ST} e pelos testes de associação). Entretanto, devido ao pequeno tamanho amostral, não foi possível testar cenários mais refinados de estruturação geográfica da variabilidade genética destes marcadores. Fluxo gênico no sentido Norte-Sul também pode ser inferido, revelado pelos testes de associação, os quais indicaram haver um indivíduo com localização geográfica na Guiana Francesa e que apresentou maior similaridade de alelos com as amostras brasileiras.

Entretanto, estudos mais aprofundados e com maior tamanho amostral devem ser conduzidos em diferentes escalas geográficas, a fim de desvendar padrões ainda desconhecidos e também confirmar se estas subdivisões devem ser interpretadas como Unidades de Manejo (MUs) ou Unidades Evolutivamente Significativas (ESUs), merecendo considerações próprias em iniciativas de conservação.

É importante definir quais populações devem ser consideradas separadamente para fins de conservação, de modo a não interferir na distribuição geográfica da diversidade genética formada através dos processos históricos. Esforços isolados voltados para a conservação da espécie estão em desenvolvimento, abordando especialmente aspectos ecológicos, enquanto persiste uma carência significativa de informações sobre a composição genética das populações naturais de *Lontra longicaudis*. A identificação detalhada de unidades populacionais com diferenciação genética e/ou demográfica ao longo da distribuição da espécie é extremamente importante para uma avaliação precisa da representatividade das áreas protegidas, e o desenho adequado de estratégias de conservação.

Capítulo IV - Referências Bibliográficas

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