Universidade Federal do Rio Grande do Sul

Instituto de Biociências

Programa de Pós-Graduação em Genética e Biologia Molecular

HISTÓRIA EVOLUTIVA E ECOLOGIA MOLECULAR DA LONTRA NEOTROPICAL (*LONTRA LONGICAUDIS*) (CARNIVORA: MUSTELIDAE)

Cristine Silveira Trinca

Tese submetida ao Programa de Pós-Graduação em Genética e Biologia Molecular da UFRGS como requisito parcial para a obtenção do grau de Doutor em Ciências (Genética e Biologia Molecular).

Orientador: Prof. Dr. Eduardo Eizirik

Porto Alegre

Março de 2012.

Instituições Colaboradoras

Pontifícia Universidade Católica do Rio Grande do Sul

Faculdade de Biociências

Laboratório de Biologia Genômica e Molecular

Institut Pasteur de la Guyane

Laboratoire des Interactions Virus-Hôtes

Instituto Nacional de Pesquisas da Amazônia – INPA

Laboratório de Mamíferos Aquáticos

Associação Ecológica Ecomarapendi

Projeto Ecolontras

National Cancer Institute

Laboratory of Genomic Diversity

Pontificia Universidad Católica de Chile

Departamento de Ecosistemas y Medio Ambiente

Agente Financiador:

Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq

Bolsa de Doutorado – processo N° 141882/2007-7

Auxílio N° 478385/2007-2

Aos meus pais,

pelo amor e investimento em educação.

Agradecimentos

Agradeço em primeiro lugar aos meus pais, pelo apoio, diálogo e questionamentos constantes. Obrigada por confiarem nas minhas escolhas mesmo quando elas parecem um tanto estranhas, por dividirem comigo as muitas alegrias que tive ao longo deste período, bem como por aguentarem as minhas (muitas) crises existenciais. Agradeço à minha irmã, que mesmo à distância esteve presente e que sempre torce por mim. Vocês três são o meu porto seguro e só por isso eu já me considero uma pessoa feliz e de muita sorte.

Ao Duda, meu orientador, pela oportunidade, amizade, confiança e por ter me ajudado a amadurecer como pesquisadora. Obrigada pela liberdade de autonomia e por proporcionar um ambiente de trabalho com tantos recursos, os quais viabilizaram a execução deste trabalho.

Aos meus colegas (e ex-colegas) e amigos do Genoma, em especial à Tali, Re, Carla, Lisie, Manoel, Fê Pedone, Marina, Taia, Tati, Ana Lúcia, Gabi Fernandez, Henrique, Mirian, Ale e Ane, por fazerem do laboratório um ótimo lugar para trabalhar. Com vocês nenhum trabalho nem nenhum lugar ficam monótonos.

Às queridas Renata Bornholdt e Carla Pires, pelas muitas conversas e grande apoio, especialmente nestes últimos meses.

À Fernanda Pedone e à Taiana Haag, pela troca de experiência no trabalho com DNA fecal.

À Mirian Tsuchiya Jerep e à Gabi Fernandez, que mesmo à distância e cheias de coisas pra fazer, foram super atenciosas me ajudando com projetos futuros.

Ao Manoel L. F. Rodrigues, pela amizade e pelo carinho, e por estar sempre disponível para discutir resultados ou esclarecer dúvidas. Obrigada especialmente por ter compartilhado comigo um dos momentos mais marcantes (e divertidos) de todo este doutorado... Montar a genealogia das lontrinhas foi bem mais legal e eficiente com a tua ajuda!

Ao Felipe G. Grazziotin pela impagável ajuda com as análises demográficas e pelos muitos momentos de café acompanhados de conversas filosóficas e histórias divertidas, os quais se tornavam ainda melhores na presença da Roberta Graboski, que tornou grande parte dos meus

sábados de trabalho muito mais agradáveis (mas um pouco menos produtivos hehe). Eu adorava a tua companhia Beta!

À Camila Jaeger, minha ex-pupila, que apesar de ter se bandiado pra outras áreas da biologia, me ajudou muuuuuuito com extrações de DNA de fezes, PCRs, sequenciamentos e genotipagens.

À Priscilla M. Zamberlan, técnica do laboratório, que assim que chegou ao Genoma já encontrou uma avalanche de placas de genotipagem para injetar.

À Talita Pereira, que é mais do que colega de laboratório, é uma amiga muito querida e 'sócia', por ser essa pessoa especial, sempre disposta a ajudar, com um caráter inquestionável e com a personalidade mais forte que eu já conheci (haha)... Te admiro muito.

À Cladi, que durante muitos anos foi a técnica do laboratório e que me ajudou muito com o trabalho de bancada, mas que além disso, é uma amiga querida que tem um coração enorme. Obrigada por tudo, inclusive por ter me apresentado ao *patchwork*, o qual além de render momentos divertidíssimos ajudou a aliviar o stress durante o final do doutorado.

Ao Ernesto, pelo incentivo e por toda a ajuda no planejamento das coletas em Maquiné e com a edição de figuras e mapas do primeiro capítulo desta tese.

Ao Seu Matias e à Dona Leonira, que sempre nos receberam de forma tão amável durante as saídas a campo e gentilmente nos acolheram quando ficamos presos na Barra do Ouro por causa da enchente.

Ao Ernesto, Fê Pedone e Lucas Silveira pela parceria nas saídas de campo. Sem a ajuda de vocês as coletas teriam sido muito mais difíceis e menos divertidas. Ao tio do Lucas, seu Aymoré, por emprestar sua casa na Barra do Ouro diversas vezes, o que permitiu que fôssemos agraciados com a bela paisagem do amanhecer visto do "alpendre".

À Fabrícia Ferraz, que além de ter sido uma excelente professora de inglês, foi também uma ótima conselheira e se transformou em uma boa amiga.

A Julio, por la persona brillante que eres y por escribir conmigo los capítulos más increíbles de una historia que habla sobretodo sobre el amor, cumplicidad y el diálogo. Gracias por inspirarme y estimularme a crecer no sólo profesionalmente sino también como persona.

A todos os pesquisadores e instituições que colaboraram com a elaboração dos dois primeiros capítulos desta tese através do envio de amostras e sequências de DNA, em especial ao Dr. Fernando C. W. Rosas, Dr. Benoit de Thoisy, Dra. Helen F. Waldemarin e Dra. Juliana de Abreu Vianna. Ao Dr. Jordi Riuz-Olmo pelas dicas e orientações no início do projeto de ecologia molecular, quando eu ainda estava definindo as perguntas e o esquema amostral.

Ao CNPq pela bolsa de doutorado concedida.

À Universidade Federal do Rio Grande do Sul (UFRGS) pelo ensino gratuito e, especialmente ao Programa de Pós-Graduação em Genética e Biologia Molecular.

À Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS) pela excelente infraestrutura.

Ao Vale do rio Maquiné, um lugar tão rico de natureza, que me fez aprender um pouco mais sobre tudo. Os dois anos em que fui periodicamente para lá me renderam muito mais do que centenas de amostras. Além de biologia, aprendi muito sobre a vida de uma forma geral e sobre as complexidades do ser humano.

Por fim, agradeço às lontras, por sua beleza, mistério e encanto... por serem ao mesmo tempo tão fortes e tão frágeis, e por me oferecem um campo de trabalho repleto de desafios e realizações.

Sumário

Resumo

A lontra neotropical (*Lontra longicaudis*) é um carnívoro de médio porte, de hábito semi-aquático e que apresenta ampla distribuição geográfica, ocorrendo do México até o Norte da Argentina. Pouco se sabe sobre esta espécie e, apesar do atual crescente número de estudos, a grande maioria destes é voltada para a análise de dieta e uso de hábitat da espécie, de forma que outros aspectos sobre a sua biologia, ecologia e história evolutiva continuam pouco explorados. Estudos moleculares são escassos devido à dificuldade de obtenção de amostras convencionais, e à ausência de metodologias padronizadas que possibilitem a utilização de amostragem não-invasiva, a qual tem sido amplamente empregada para o estudo de diversas outras espécies de mamíferos.

Neste contexto, os estudos aqui apresentados têm por objetivo, além de padronizar metodologias para os estudos genéticos e ecológicos de *Lontra longicaudis* através da utilização de uma abordagem molecular, fornecer dados inéditos sobre a diversidade genética, padrões de diferenciação populacional, demografia e ecologia desta espécie. Os resultados aqui apresentados são relevantes não apenas para expandir o conhecimento básico sobre esta espécie, mas também para auxiliar na definição do seu atual status de conservação e delinear futuros planos de manejo e conservação para esta espécie e seus habitats.

O capítulo 3 apresenta um estudo dos padrões filogeográficos de *L. longicaudis* através da análise de três segmentos do DNA mitocondrial (região controle, *ATP8/ATP6* e *ND5*), totalizando 1471 pb. Os resultados indicaram uma diversidade genética relativamente alta e elevado índice de diferenciação genética entre as áreas amostradas, o que sugere a existência de (ao menos) unidades de manejo distintas nestas regiões, bem como salienta a necessidade de analisar outras áreas da distribuição geográfica da espécie para obter um entendimento mais aprofundado da sua história evolutiva.

O capítulo 4 aborda a diversidade genética e a estruturação populacional da lontra neotropical através da análise de locos de microssatélite, a fim de complementar a investigação dos padrões filogeográficos da espécie observados no DNA mitocondrial (DNAmt). Alguns padrões similares de estruturação populacional foram observados nestes marcadores, embora a magnitude de diferenciação genética entre as populações estudadas tenha sido consideravelmente menor do

que aquela observada para o DNAmt. Os resultados encontrados sugeriram que este padrão pode ser derivado de fluxo gênico mediado por machos, bem como indicaram que a estruturação populacional de *L. longicaudis* nas regiões estudadas não parece estar associada a bacias hidrográficas.

No capítulo 5 é apresentada a padronização de uma metodologia para a determinação sexual de *L. longicaudis* a partir de amostras de fezes/muco coletadas em campo. Este método é baseado na amplificação conjunta de um loco de microssatélite e um segmento do gene *SRY*. Métodos similares têm sido aplicados a outras espécies de lontras, mas nenhum foi previamente testado em *L. longicaudis*. Os resultados demonstraram que o método proposto é altamente eficiente para identificação do sexo da lontra neotropical utilizando-se DNA de amostras não-invasivas, e pode auxiliar na obtenção de dados ecológicos da espécie na natureza.

O capítulo 6 apresenta o primeiro estudo de ecologia molecular de *L. longicaudis*, o qual foi baseado exclusivamente na análise de DNA extraído a partir de amostras não-invasivas. As amostras foram identificadas a nível individual, e através da uma amostragem sistemática, diversos parâmetros populacionais, os quais até então eram praticamente desconhecidos, foram estimados. Os resultados demonstram a grande potencial da análise de DNA extraído de amostras não-invasivas não apenas para o estudo da dinâmica populacional de *L. longicaudis*, mas também de diversas outras espécies pouco conhecidas de carnívoros neotropicais.

Abstract

The Neotropical otter (*Lontra longicaudis*) is a medium-size semi-aquatic carnivore that presents a wide geographic distribution, ranging from Mexico to Northern Argentina. It is a poorly known otter species, and although the current increase in the number of studies, most of them are directed to diet composition and habitat use, so as many other issues regarding its biology, ecology and evolutionary history still remain virtually absent. Molecular studies involving this otter are few due to the difficulty in obtaining traditional biological samples, and also because standard methods that allow the use of noninvasive sampling for studying this organism are absent, in spite of such methodologies have been widely used for investigating several mammal species around the world.

In the context to address these issues, the studies presented here aimed to standardize methodologies for applying in genetic and ecological studies of *Lontra longicaudis* by using a molecular approach, and to provide new data on genetic diversity, patterns of population structure, demography and ecology of this species. The results presented here are relevant not only to increase the basic knowledge about this otter, but also to help in defining its current conservation status and to contribute for designing future management and conservation plans for this species and its habitats.

Chapter 3 presents a study of the phylogeographic patterns of the *L. longicaudis* revealed by analyzing three segments of the mitochondrial DNA (control region, *ATP8/ATP6* and *ND5*), totaling 1471 bp. Our results indicated a considerably high molecular diversity and a significant genetic partition among the sampled areas, suggesting the observed population structure may represent distinct Management Units (MUs) on those regions. Additionally, these results emphasize the necessity of investigating additional areas of the species distribution to obtain a more comprehensive understanding of its evolutionary history.

Chapter 4 addresses the genetic diversity and the population structure of the Neotropical otter by analyzing microsatellite loci in order to complement the investigation of the phylogeographic patterns observed for the mtDNA presented in chapter 3. Some similarities in patterns of population subdivision were observed, although the magnitude of the genetic differentiation has been considerably lower than those reported for the mtDNA. The results suggested this pattern may be derived from male-biased gene flow, as well as indicated the genetic

structure of *L. longicaudis* populations did not seem to be related to the delimitation of hydrographic basins.

On chapter 5 it is presented a standardization of a molecular sex typing methodology of *L. longicaudis* by using noninvasive samples collected in the field. This method employs a duplex-PCR assay in which a microsatellite is amplified along with a segment of the *SRY* gene. Similar approaches have been applied for other otter species, but none of them have been previously tested in *L. longicaudis*. The results demonstrated the proposed method is highly efficient in gender determining of the Neotropical otter noninvasive samples which may be useful in obtaining ecological information of this organism in the wild.

Finally, chapter 6 presents the first molecular ecology study of *L. longicaudis*, which was exclusively based on DNA analyses of noninvasively collected samples. Samples were identified at individual level, and based on a systematic sampling approach it was possible to estimate several population parameters of this otter, which were virtually unknown. Results demonstrated the great potential of this molecular approach for studying population dynamics of this otter, but also highlighted its usefulness for addressing ecological and demographic issues of other poorly known Neotropical carnivores.

Capítulo 1 – Introdução Geral

1.1. A Biologia e a Genética da Conservação

A Biologia da Conservação é uma ciência multidisciplinar que surgiu como resposta à recente crise na diversidade biológica (Soulé 1985). Preservar a biodiversidade e manter os processos evolutivos que a geraram é uma das principais metas da Biologia da Conservação (Johnson et al. 2001). Diante do ritmo atual e sem precedentes de transformação dos ambientes naturais e das altas taxas de perda de biodiversidade observadas ao redor do mundo, tem sido cada vez mais necessário o desenvolvimento de programas de conservação, os quais utilizam estudos científicos sobre as espécies e ambientes para embasar a elaboração de estratégias e ações práticas, objetivas e eficazes para a conservação e manejo dos recursos naturais (Primack e Rodrigues 2001).

Neste contexto, a manutenção da diversidade genética é considerada um dos níveis fundamentais da conservação da biodiversidade (Frankham et al. 2002). Dentro da ampla área da Biologia da Conservação, a disciplina da Genética da Conservação surgiu como a união da teoria com a prática em genética voltada para a preservação de espécies como entidades dinâmicas capazes de evoluir e lidar com mudanças ambientais, minimizando assim, o risco de extinção das mesmas (Frankham et al. 2002). De uma forma geral, a Genética da Conservação compreende cinco grandes linhas de atuação: i) manejo e reintrodução de populações cativas, e a restauração de comunidades biológicas, ii) descrição e identificação de indivíduos, estrutura genética populacional, parentesco e relações taxonômicas, iii) detecção e predição de efeitos da perda de habitat, fragmentação e isolamento, iv) detecção e predição dos efeitos de hibridação e introgressão, e v) compreensão das relações entre adaptação ou fitness e as características genéticas de indivíduos ou populações (Allendorf e Luikart 2007).

Assim como outras áreas dentro da Biologia da Conservação, a Genética da Conservação é impulsionada pela necessidade de reduzir as taxas de extinção atuais e preservar a biodiversidade (Frankham et al. 2002) na tentativa de evitar o que constituiria o sexto grande evento de extinção em massa na história conhecida da vida na Terra (Leakey e Lewin 1995), o qual seria totalmente diferente dos eventos anteriores pois resultaria, direta ou indiretamente, das atividades de uma única espécie dominante – o ser humano (Beebee e Rowe 2008).

Mais especificamente, a Genética da Conservação tem como principal objetivo entender as relações entre a diversidade genética e a viabilidade das populações (Beebee e Rowe 2008) através da utilização de ferramentas moleculares e conceitos de genética de populações a fim de minimizar efeitos da redução no fluxo gênico, de endocruzamento e perda de diversidade genética, e identificar populações e áreas prioritárias para a conservação.

O emprego de métodos moleculares na área da Biologia da Conservação vem aumentando consideravelmente à medida que se descobrem novas aplicações para o uso de marcadores genéticos, associados ao grande avanço das técnicas laboratoriais e dos métodos analíticos (Frankham et al. 2002, DeSalle e Amato 2004, DeYoung e Honeycutt 2005, Kohn et al. 2006) As técnicas moleculares podem fornecer dados com precisão nunca antes vista e contribuir significativamente para o entendimento de diversos aspectos da biologia das espécies, bem como questões taxonômicas e parâmetros genético-populacionais, os quais servem de base para muitas decisões de manejo e auxiliam na definição de espécies, populações e/ou áreas prioritárias para a conservação.

1.2. Estruturação da diversidade genética e filogeografia

É amplamente aceito que a maioria das espécies não ocorre na forma de uma única população panmítica, mas sabe-se também que há muitos tipos de estruturação populacional na natureza (Beebee e Rowe 2008), seja ela natural ou causada por fatores antrópicos. A ausência de panmixia comumente resulta em uma variabilidade genética distribuída de forma heterogênea ao longo da área de ocorrência de uma espécie. Dentre os fatores que podem gerar este padrão, podemos citar a limitação na capacidade de deslocamento de indivíduos e sua relação com a distância, fidelidade de sítio (filopatria) e a presença de barreiras geográficas (Wright 1978), bem como os atuais níveis de fragmentação da paisagem.

A investigação de padrões de estruturação populacional através de marcadores moleculares talvez seja uma das abordagens mais importantes dentro da Genética da Conservação. Uma das principais inferências resultante de análises de estruturação populacional é a quantidade de fluxo gênico que está ocorrendo e/ou que havia no passado (Beebee e Rowe

2008), pois este é o principal fator componente da estruturação populacional e que permite compreender a extensão em que cada população de uma espécie constitui uma unidade geneticamente distinta (Slatkin 1994).

Métodos moleculares têm comprovado ser uma ferramenta poderosa para revelar eventos históricos complexos que tiveram um papel importante na distribuição atual das espécies e na forma como sua diversidade genética está estruturada. Os padrões históricos de distribuição geográfica da variabilidade genética de uma espécie, bem como os processos que os geraram, são o objeto de interesse dos estudos filogeográficos.

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. A filogeografia constitui uma disciplina que envolve dados sobre genética de populações, ecologia molecular, demografia e geografia histórica, com o objetivo de revelar a história evolutiva de linhagens genealógicas, relacionando-a com sua distribuição geográfica, principalmente em um contexto intraespecífico (Bermingham e Moritz 1998, Avise 2000).

Além da importância na compreensão dos níveis de diversidade genética, dos padrões de distribuição desta diversidade e da identificação de possíveis barreiras ao fluxo gênico em uma espécie, a análise de padrões filogeográficos é também de extrema relevância na identificação de linhagens evolutivas independentes e com distribuição geográfica restrita, bem como de áreas com baixa diversidade genética, ambas potencialmente prioritárias no âmbito de planos de manejo, monitoramento e conservação (Avise 1994, Moritz e Faith 1998). Neste contexto, através de estudos filogeográficos é possível identificarmos 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) (Moritz 1994).

As ESUs são constituídas por unidades demográficas intraespecíficas, distintas geograficamente e que se apresentam diferenciadas geneticamente (implicando isolamento histórico) de outras unidades semelhantes contidas na mesma espécie. Dentro da perspectiva da Biologia da Conservação, cada ESU deveria ser protegida e manejada de forma independente para garantir a continuidade de todos os processos evolutivos envolvidos na diferenciação desta(s) unidade(s) populacional(is) (Moritz 1994, Eizirik 1996).

Já as Unidades de Manejo podem estar contidas em ESUs, e são formadas por populações regionais com restrita conexão demográfica entre si, apresentando significativa diferenciação em suas frequências alélicas, mas não necessariamente com diferenciação genética profunda (não implicando em monofilia recíproca no DNA mitocondrial, por exemplo [Moritz 1994]). Entretanto, em uma escala de tempo curta (uma ou poucas gerações), o contato entre estas populações através de migração ou recolonização torna-se escasso, transformando-as em entidades ecológicas e demográficas relativamente separadas, e que deveriam ser manejadas de forma independente ou coordenada (Moritz 1994, Eizirik 1996, Crandall et al. 2000, Fraser e Bernatchez 2001, Frankham et al. 2002). Sendo assim, o foco da definição de MUs é voltado principalmente para a estruturação atual das populações, ao invés de processos históricos geradores de diferenciação genética mais profunda (Moritz 1994).

A aplicação destes conceitos em programas de conservação e manejo, bem como no planejamento de políticas públicas, tem como objetivos manter os processos evolutivos que resultaram na diferenciação populacional natural (que por sua vez podem compreender características adaptativas locais), assim como mitigar efeitos de estruturação genética causada por efeitos antrópicos, os quais podem em última análise, levar à extinção local de populações silvestres.

1.3. Ecologia molecular e o emprego de amostragem não-invasiva em estudos genéticos com carnívoros

Para a conservação efetiva de espécies é muitas vezes importante associar estudos de campo com estudos moleculares. Nesse contexto, a disciplina da Ecologia Molecular compreende a integração de uma grande variedade de tópicos incluindo biologia molecular, ecologia, evolução, biologia comportamental e genética (Beebee e Rowe 2008). De forma resumida, pode-se definir Ecologia Molecular como o emprego de ferramentas moleculares na investigação de diversos aspectos ecológicos e populacionais. Nos últimos anos, esta nova disciplina tem permitido obter informações sobre espécies que até a bem pouco tempo pareciam inacessíveis, contribuindo para

o surgimento de novas perguntas e campos de conhecimento, tanto na área da ecologia quanto da evolução (Eguiarte et al. 2007).

Dentro do escopo da Ecologia Molecular, a utilização de marcadores moleculares tem permitido investigar questões clássicas de genética populacional como definições de populações, identificação de espécies e de populações híbridas. Em uma perspectiva ecológica, a Ecologia Molecular ainda tem possibilitado estudar questões relacionadas à distribuição geográfica de espécies, dinâmica e estrutura populacional, permitindo também investigar dieta, parasitismo, relações simbióticas, sistemas reprodutivos e seleção sexual, bem como avaliar níveis de fluxo gênico entre populações e padrões de dispersão (Eguiarte et al. 2007, Beebee e Rowe 2008).

Neste contexto, em se tratando de grande parte das espécies de carnívoros, um dos maiores impedimentos à condução de estudos desta natureza é a dificuldade de obter amostras suficientes que representem de forma adequada a população de interesse. Este problema atinge especialmente espécies de carnívoros raras e/ou ameaçadas, de comportamento arredio e de hábito noturno/crepuscular, características que as tornam organismos difíceis de detectar e capturar (Johnson et al. 2001). Além disso, carnívoros em geral ocorrem em baixas densidades na natureza e ocupam grandes áreas (às vezes inacessíveis), tornando ainda mais problemática e menos eficiente a obtenção de amostras biológicas através de métodos tradicionais de captura.

Com o intuito de auxiliar na solução deste problema, recentes avanços na área da Biologia Molecular têm permitido o emprego de amostras denominadas ‘não-invasivas’ como importante fonte de DNA para diversas espécies de mamíferos (p.ex. Taberlet et al. 1999, Palomares et al. 2002, Valière et al. 2007). Com o surgimento deste novo campo para pesquisa, diferentes tipos de amostra não-invasiva foram descritas, tais como pelos (Taberlet e Bouvet 1992), fezes (Höss et al. 1992), urina (Valière e Taberlet 2000), penas (Taberlet e Bouvet 1991), regurgitos (Valière et al. 2003) e casca de ovos (Strausberger e Ashley 2001). Atualmente, fezes e pelos compreendem as amostras não-invasivas mais amplamente empregadas no estudo de espécies selvagens de comportamento arredio e difícil detecção (Waits 2004).

Dentre as espécies de carnívoros, análises de DNA fecal têm sido usadas para investigar mustelídeos (Frantz et al. 2003, Kalz et al. 2006), felídeos (Ernest et al. 2000, Janecka et al. 2008) e canídeos (Kohn et al. 1999, Prugh et al. 2005) sem a necessidade de observação direta ou

captura do animal. Além deste tipo de informação básica sobre a presença de diferentes indivíduos e espécies, este tipo de amostra pode ser utilizado para avaliar a diversidade genética (Dallas et al. 2003, Miotto et al. 2011), estrutura populacional, parentesco e tamanho populacional de uma espécie em determinada área (Bellemain et al. 2005, Arrendal et al. 2007, Miotto et al. 2011), além de ser útil também na determinação de espécies simpátricas e de suas presas (Ernest et al. 2000, Farrel et al. 2000), na definição de estruturas sociais e familiares (Kays et al. 2000), na abundância e organização espacial (Hung et al. 2004) e no monitoramento de populações de vida livre (de Barba et al. 2010, Brøseth et al. 2010). Sendo assim, a análise de amostras não-invasivas vem sendo amplamente adotada e tem mostrado ser um método efetivo para obtenção de dados ecológicos, demográficos e populacionais relevantes para o melhor conhecimento de diversas espécies, em muitos casos auxiliando na definição de políticas públicas eficientes para a conservação e manejo de populações naturais.

1.4. Utilização de marcadores moleculares em estudos evolutivos e populacionais

As informações empregadas na área da Genética da Conservação provêm principalmente da análise de polimorfismos encontrado em segmentos de DNA, os quais são exemplos importantes do que se denomina ‘marcadores moleculares’. Diversos tipos de marcadores já foram descritos e aplicados à investigação de aspectos evolutivos, demográficos e populacionais dos mais variados organismos. Atualmente, o DNA mitocondrial e o DNA microssatélite constituem os dois marcadores mais amplamente empregados na condução destes estudos e serão brevemente apresentados a seguir.

1.4.1. O DNA mitocondrial

O DNA mitocondrial (DNAmt) tem sido um marcador molecular amplamente utilizado na avaliação de níveis de diversidade genética bem como no estudo das relações filogenéticas entre diferentes *taxa* e na identificação de subdivisão evolutiva entre unidades populacionais geográficas (Bermingham e Moritz 1998, Avise 2000).

O DNAmt animal está organizado na forma de um genoma circular haplóide de pequeno tamanho (cerca de 17 kb), que está presente em centenas de milhares de cópias dentro das células destes organismos (Ferreira 2001). Em vertebrados, o genoma mitocondrial consiste de 37 genes, dos quais 13 representam sequências codificadoras de proteínas, 22 são genes para tRNA e 2 são genes codificantes de rRNA. O DNAmt ainda compreende a chamada 'região controladora' que contém sequências regulatórias dos processos de duplicação e transcrição do DNA (Graur e Li 2000). A região controladora de vertebrados é também chamada de *D-Loop* (*Displacement Loop*) devido à formação de uma estrutura em fita tripla no início do seu processo de replicação (Brown et al. 1986). Esta região tem sido bastante utilizada em estudos de genética de populações e análises filogeográficas devido ao alto nível de polimorfismo de nucleotídeos observado em suas sequências em relação às sequências dos genes codificadores, os quais tendem a ser mais conservados e por isso classicamente mais utilizados em abordagens filogenéticas (Graur e Li 2000).

De uma forma geral, a grande popularidade do DNA mitocondrial como marcador molecular em estudos filogenéticos se deve principalmente ao fato de que o DNAmt representa a porção do genoma animal mais bem estudada e também porque há evidências de que a taxa de evolução do DNAmt seja 5 a 10 vezes mais rápida do que a observada em locos nucleares de cópia única na maioria das espécies de mamíferos (Baker et al. 1993, Martin e Palumbi 1993, Ballard e Whitlock 2004).

Apesar de ser considerado um único loco genético devido à ausência de recombinação neste genoma, o DNAmt animal apresenta duas características que podem ser vistas em alguns contextos como vantagens sobre os marcadores nucleares: (i) a relação filogenética do DNAmt 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, o que torna este marcador bastante sensível à diferenciação populacional devido a efeitos de deriva genética aleatória (a qual acontece mais rapidamente do que em marcadores nucleares) e *bottleneck* populacional (Neigel e Avise 1986, Beebee e Rowe 2008).

Entretanto, pode haver certa limitação na utilização deste marcador, uma vez que sua análise refere-se apenas à história evolutiva das linhagens maternas das populações estudadas,

não revelando os padrões genealógicos que representam a população como um todo (Hare 2001). Além disso, por evoluir como um loco único, a construção de árvores de genes a partir de qualquer segmento mitocondrial refletirá apenas uma única realização do processo genealógico, limitando assim interpretações genômicas ou demográficas mais gerais (Harlt e Clark 2007). Sendo assim, a utilização deste marcador em conjunto com locos nucleares permite uma investigação mais completa e aprofundada dos padrões de variação genética e estruturação populacional dos organismos.

1.4.2. Microssatélites

Microssatélites consistem de segmentos curtos de DNA (1-6 pares de bases) altamente repetitivos e que são constituídos, em sua maioria, de repetições de mono, tetra ou dinucleotídeos, podendo formar conjuntos de até centenas de repetições por loco, resultando em segmentos altamente polimórficos dispersos pelo genoma de eucariotos (Schlötterer 1998, 2004).

Em geral, microssatélites não estão sujeitos a uma ação muito intensa da seleção natural, o que possibilita a manutenção do seu alto nível de polimorfismo, e os torna compatíveis com os pressupostos requeridos em estudos de genética de populações (Murray 1996). Além disso, outra característica que justifica sua ampla utilização em estudos genéticos é a codominância, a qual permite identificar indivíduos homozigotos e heterozigotos em cada loco (Schlötterer 1998). Esta característica permite traçar o perfil genético de cada indivíduo em um conjunto de locos de microssatélites, o que é de especial interesse para algumas questões em genética de populações e ecologia molecular.

Desta forma, a alta variabilidade observada nos microssatélites, resultante de sua alta taxa de mutação associada à tendência à neutralidade seletiva e herança codominante, permite o emprego deste tipo de marcador em uma ampla variedade de estudos populacionais, tais como: investigação do grau de estruturação genética entre populações, migração e padrões de dispersão (Cegelski et al. 2003, Hájková et al. 2007, Haag et al. 2010), comparação da variação genética entre espécies e populações (Johnson et al. 1999; Wisely et al. 2002), identificação de indivíduos (Ernest et al. 2000), determinação de parentesco e estrutura social (Stenglein et al. 2011) e

estimativas de censo e tamanho efetivo (Bellemain et al. 2005, Curtis et al. 2011). Além disso, o uso de microssatélites é muito útil em estudos sobre história evolutiva e filogeografia (Eizirik et al. 2001, Nyström et al. 2006), bem como na área da genética de paisagem (Pease et al. 2009, Ottaviani et al. 2009).

1.4.2.1. Microssatélites aplicados a estudos baseados em amostragem não-invasiva

Outra característica que faz dos microssatélites o tipo de marcador genético mais utilizado atualmente em estudos populacionais é que é possível utilizá-los em DNAs altamente fragmentados e em baixas concentrações, como aqueles obtidos a partir de amostras não-invasivas e amostras antigas (Bruford e Wayne 1993).

Todavia, a amplificação de microssatélites em amostras com DNA de baixa qualidade/quantidade é trabalhosa e necessita superar três problemas principais: o alto potencial para contaminação, a baixa taxa de sucesso na amplificação do DNA e as altas taxas de erros de genotipagem. Nos últimos anos, métodos laboratoriais e analíticos têm sido desenvolvidos com o objetivo de contribuir para a redução destes problemas. Por exemplo, a extração e amplificação do DNA devem ser realizadas em ambiente separado, livre de DNA de boa qualidade (p.ex. proveniente de amostras de sangue ou tecido) e produtos de PCR, para evitar contaminação; a amplificação de DNA deve visar à obtenção de fragmentos curtos; as amostras utilizadas devem ser o mais frescas possível e múltiplas amplificações devem ser realizadas para cada loco, diminuindo assim os problemas associados a erros de genotipagem (Johnson et al. 2006).

Vários estudos vêm utilizando métodos para detectar e minimizar erros de genotipagem (p.ex. Fernando et al. 2003, Broquet e Petit 2004, Hoffman e Amos 2005, Kalinowski et al. 2006). Estes estudos incluem (i) a adoção de uma abordagem de múltiplos tubos, a qual consiste de múltiplas amplificações independentes para cada amostra e loco; (ii) a comparação do genótipo obtido com aquele a partir de sangue e/ou tecido (quando possível); (iii) a repetição da genotipagem para amostras escolhidas ao acaso; ou (iv) a verificação se mãe e prole compartilham um alelo comum (Gossens et al. 1998, Smith et al. 2006, Hedmark e Ellegren 2006).

1.5. A lontra neotropical

A lontra neotropical (*Lontra longicaudis* [Olfers, 1818]) é um mamífero da ordem Carnivora que integra a família Mustelidae, a qual possui 22 gêneros agrupados em duas subfamílias, Lutrinae e Mustelinae (Wozencraft 2005). Dentro desta família, *Lontra longicaudis* encontra-se dentro da subfamília Lutrinae juntamente com outras 12 espécies de lontras (Koepfli et al. 2008), as quais estão agrupadas em seis gêneros (*Aonyx, Enhydra, Lutrogale*, *Pteronura, Lutra* e *Lontra* - Kruuk 2006) distribuídos por todos os continentes, exceto Austrália e Antártica (Foster-Turley et al. 1990). Além de *Lontra longicaudis*, três outras espécies de lontra ocorrem na região Neotropical: *Lontra felina*, *L. provocax* e *Pteronura brasiliensis*, esta última ocorrendo em simpatria com *L. longicaudis* em algumas áreas de sua distribuição geográfica.

Lontra longicaudis é um animal de médio porte que apresenta o corpo alongado e uma pelagem densa e curta de coloração geral marrom-pardacenta, levemente mais clara na região ventral, especialmente na garganta (Bertonatti e Parera 1994). É uma espécie de hábito semi-aquático e, portanto, apresenta algumas adaptações morfológicas para a locomoção na água, tais como patas apresentando membranas interdigitais, cauda longa levemente achatada na extremidade e grandes vibrissas (Emmons 1990, Silva 1994), as quais têm papel importante na propulsão e direcionamento do animal embaixo d'água, bem como na localização de presas (Cimardi 1996).

Devido ao hábito semi-aquático, a distribuição da lontra neotropical é fortemente associada a corpos d'água, ocorrendo em uma grande variedade de habitats como rios, lagos, pequenos canais e banhados, além de não raramente ser observada em ambientes marinhos associados a cursos d'água doce (p.ex. nos Estados de Santa Catarina e Rio de Janeiro) (Blacher 1987, Mason 1990). Peixes, crustáceos e moluscos são os principais itens que compõem a sua dieta, a qual pode ainda ser complementada por anfíbios, insetos, aves e pequenos mamíferos (Chebez 1999).

A distribuição geográfica de *Lontra longicaudis*, embora ainda bastante controversa e incerta, é descrita como abrangendo uma faixa contínua que inicia na região noroeste do México e estende-se até o norte da Argentina (Chehébar 1990, Chebez 1999, Eisenberg e Redford 1999),

ocorrendo em todo o território brasileiro (Emmons 1990). Desta forma, *L. longicaudis* é reconhecida como sendo a espécie de lontra mais amplamente distribuída na América do Sul (Chehébar 1990).

Estudos sobre a lontra neotropical são ainda poucos e, apesar do notável aumento no número de trabalhos nos últimos anos, estes são, em sua grande maioria, geograficamente restritos e/ou direcionados a registros de ocorrência e distribuição local (Parera 1993, Ramírez-Bravo 2010) e análises da composição da dieta e uso do habitat (*ex.* Helder e De Andrade 1997, Pardini 1998, Pardini e Trajano 1999, Colares e Waldemarin 2000, Waldemarin e Colares 2000, Quadros e Monteiro-Filho 2000, Rheingantz et al. 2011). Além disso, alguns estudos sobre reprodução e fisiologia da espécie foram realizados com a espécie em cativeiro (Colares e Silva 1987, Colares e Best 1991).

A carência de estudos sobre a lontra neotropical deve-se em parte ao fato de que este mustelídeo é difícil de ser visualizado na natureza, pois geralmente possue hábitos noturnos e/ou crepusculares, é tímido e apresenta comportamento arredio (Macdonald 1990), o que dificulta consideravelmente a condução de investigações ecológicas que envolvam a definição de áreas de vida, estruturas familiares, padrões populacionais e de comportamento.

Em termos de *status* de conservação, a lontra neotropical é classificada como “Dados Insuficientes” pela IUCN (World Conservation Union; Waldemarin e Alvarez 2008), e consta no Apêndice I (“Espécies ameaçadas”) da CITES (Convention on the International Trade in Endangered Species of Fauna and Flora), a qual regula o comércio internacional de espécies ameaçadas. No Brasil, esta espécie foi recentemente retirada da lista nacional de espécies ameaçadas (MMA 2008) embora em três Estados da federação (Minas Gerais, Paraná e Rio Grande do Sul) a espécie seja considerada “Vulnerável” (Paraná 1995, Machado et al. 1998, Indrusiak e Eizirik 2003).

No passado, a lontra neotropical, assim como outras espécies de lontra, foi fortemente caçada devido ao alto valor comercial de sua pele no mercado internacional. Atualmente as principais ameaças a esta espécie compreendem (i) a perda e fragmentação do habitat, especialmente no que se refere ao desmatamento da vegetação ripária, poluição das águas por dejetos orgânicos, produtos químicos e pesticidas agrícolas (Macdonald e Mason 1985,

Waldemarin e Alvarez 2008); (ii) constantes conflitos com pescadores e proprietários de criadouros de peixes (Waldemarin e Alvarez 2008); e (iii) atropelamentos em estradas (Macdonald e Mason 1990; Trinca CS, observação pessoal).

Na primeira metade do século 20, estudos taxonômicos tradicionais baseados na variação da forma do rinário propuseram que *L. longicaudis* representasse, na verdade, um complexo de espécies (Cabrera 1957, Harris 1968, Pohle 1920). Posteriormente, foi sugerida que estas diferenças morfológicas representavam apenas variação geográfica dentro da mesma espécie, e que esta poderia assim, ser subdividida em três subespécies: *Lontra longicaudis annectens* (distribuída no México, Nicarágua, Costa Rica, Panamá, Venezuela, Colômbia e Equador), *L. longicaudis enudris* (com ocorrência na Guiana Francesa, Suriname, Trinidad e Tobago e Peru) e *L. longicaudis longicaudis* (com ampla distribuição em quase toda América do Sul, incluindo Brasil e Uruguai) (Larivière 1999, van Zyll de Jong 1972). Todavia, esta subdivisão taxonômica é ainda controversa devido à falta de estudos mais amplos e detalhados sobre a variação morfológica e genética da espécie ao longo de sua distribuição; desta forma, o uso desta classificação infraespecífica é atualmente desencorajado até que estudos aprofundados sejam conduzidos (X^{th} International Otter Colloquium, Hwacheon, Coréia do Sul, 2007).

1.6. Ferramentas moleculares aplicadas ao estudo de mustelídeos

Nos últimos anos, métodos moleculares têm sido empregados no estudo de diversas espécies de mustelídeos, especialmente em se tratando da análise de polimorfismos em segmentos de DNA mitocondrial e microssatélites. No âmbito do vasto campo em que as ferramentas moleculares podem ser aplicadas, estas têm sido úteis na identificação de espécies simpátricas de mustelídeos (Riddle et al. 2003, Gómez-Moliner et al. 2004, Pilot et al. 2007) e na investigação de padrões filogeográficos e análises de diferenciação populacional de texugos (*Meles meles*; Marmi et al. 2006), arminhos (*Mustela erminea*; Fleming e Cook 2002), doninhas (*Mustela putorius*; Pertoldi et al. 2006) e martas (*Martes americana* - Small et al. 2003, *Mustela lutreola* – Michaux et al. 2005). Além disso, métodos moleculares têm sido utilizados na investigação de aspectos demográficos e ecológicos como estimativas de tamanho de censo e

dinâmica populacional (Hedmark et al. 2004, Lynch e Brown 2006, Dalerum et al. 2007, Dugdale et al. 2008, Brøseth et al. 2010, Mullins et al. 2010).

Dentre as 13 espécies de lontra, a grande maioria dos estudos envolvendo análises moleculares tem sido direcionada para a lontra euro-asiática (*Lutra lutra*), tendo sido voltados para a investigação de sua história evolutiva, estruturação populacional e estimativa de parâmetros demográficos e ecológicos (p. ex. Dallas et al. 2000, Ketmaier e Bernardini 2005, Stanton et al. 2009, Mucci et al. 2010, Lanski et al. 2008, Ferrando et al. 2008).

A lontra do mar (*Enhydra Lutris*) é outra espécie de lontra bastante estudada através de análises moleculares, especialmente devido à necessidade de investigar o efeito da caça severa à qual esta espécie foi submetida durante os séculos 18 e 19. Os estudos genéticos publicados sobre esta lontra focam-se principalmente em estimativas da ocorrência de efeitos gargalo-de-garrafa populacionais e seus impactos nos níveis atuais de diversidade genética desta espécie (Larson et al. 2002, Zagrebel'nyi et al. 2008, Larson et al. 2009).

Além disso, alguns estudos genéticos foram desenvolvidos com outras quatro espécies de lontra com distribuição predominantemente alopátrica no Novo Mundo: a lontra de rio norte-americana (*Lontra canadensis)*, a lontra marinha (*L. felina*), a lontra de rio sul-americana (*L. provocax*) e a ariranha ou lontra gigante (*Pteronura brasiliensis*). Estes estudos foram voltados principalmente para análises de variabilidade genética, estruturação populacional e história evolutiva das espécies (Garcia et al. 2007, Latch et al. 2008, Valqui et al. 2010, Vianna et al. 2010, Vianna et al. 2011, Pickles et al. 2011, 2012) embora questões relacionadas à socialidade e relações de parentesco também já tenham sido abordadas para a lontra de rio norte-americana (Blundell et al. 2004).

1.6.1. O uso de métodos moleculares no estudo da lontra neotropical

Estudos genéticos incluindo *Lontra longicaudis* publicados até o momento são escassos e, na sua maioria, são direcionados a análises filogenéticas da família Mustelidae e/ou da subfamília Lutrinae, não abordando quaisquer aspectos específicos desta lontra em particular (van Zyll de Jong 1987, Koepfli & Wayne 1998, Marmi et al. 2004, Koepfli e Wayne 2003, Koepfli et al. 2008).

Até o momento, apenas dois estudos foram publicados com foco específico na investigação de parâmetros genéticos e populacionais de *L. longicaudis* (Trinca et al. 2007 [ver Anexo], Weber et al. 2009). Trinca et al. (2007) reportaram a primeira estimativa de diversidade genética para esta espécie através da análise de variabilidade em um segmento de aproximadamente 500 pb da região controle do DNA mitocondrial, e investigaram a presença de estruturação genética entre as regiões sul e sudeste do Brasil. Uma alta diversidade haplotípica foi observada nesta espécie, bem como ausência de estruturação populacional entre as regiões amostradas. Os resultados obtidos sugeriram a ocorrência fluxo gênico entre as populações de lontra desta porção da sua distribuição geográfica, mediado pela conectividade entre as microbacias hidrográficas destas regiões. Por outro lado, Weber et al. (2009) investigaram a existência de diferenciação genética entre duas localidades no sul do estado do Rio Grande do Sul, Brasil, através da utilização de locos de microssatélite amplificados a partir de DNA extraído de fezes coletadas em campo. Os autores relataram a presença de estruturação genética entre as áreas estudadas, sugerindo a existência de duas populações distintas, restritas a sistemas aquáticos distintos. A partir destes resultados iniciais, diversas questões podem ser levantadas e estudos delineados a fim de caracterizar a variabilidade genética desta espécie, bem como sua distribuição geográfica. A presente tese de doutorado tem como intuito abordar algumas destas questões, contribuindo para o conhecimento acerca da história evolutiva e estrutura populacional desta espécie.

Capítulo 2 – Objetivos

Objetivo Geral

O objetivo geral deste trabalho é investigar a história demográfica e a estrutura e dinâmica populacional de *Lontra longicaudis* através de análises genéticas realizadas em diferentes escalas espaço-temporais.

Objetivos Específicos

- Estimar a diversidade genética da lontra neotropical ao longo de sua área de ocorrência na região Neotropical através da análise de segmentos de DNA mitocondrial e microssatélites;

- Investigar a existência de estruturação geográfica da diversidade genética presente nestes marcadores entre as regiões amostradas;

- Estimar o tempo de divergência entre as linhagens mitocondriais de *L. longicaudis*, bem como entre as demais espécies do gênero que ocorrem na América do Sul, e realizar inferências sobre os processos históricos atuantes na diversificação destes organismos;

- Estabelecer e padronizar um protocolo de amplificação e genotipagem para determinação sexual de amostras de *L. longicaudis*, aplicável a amostras de baixa qualidade/quantidade de DNA (como o caso de amostras não-invasivas);

- investigar aspectos ecológicos e demográficos de *L. longicaudis* através da análise de amostras não-invasivas coletadas em campo, a fim de estimar tamanho mínimo populacional, razão sexual, organização espacial e relações de parentesco em uma população de lontras na região do Vale do rio Maquiné, no Rio Grande do Sul, Brasil.

Capítulo 3 – Artigo I

Phylogeography and demographic history of the Neotropical otter (*Lontra longicaudis*)

Phylogeography and demographic history of the Neotropical otter (*Lontra longicaudis*)

CRISTINE S. TRINCA, BENOIT DE THOISY, FERNANDO C. W. ROSAS, HELEN F. WALDEMARIN, KLAUS-PETER KOEPFLI, JULIANA A. VIANNA, EDUARDO EIZIRIK

Artigo aceito para publicação na revista *Journal of Heredity*

Phylogeography and demographic history of the Neotropical otter (*Lontra longicaudis*)

CRISTINE S. TRINCA[1,2], BENOIT DE THOISY[3,4], FERNANDO C. W. ROSAS[5], HELEN F. WALDEMARIN[6], KLAUS-PETER KOEPFLI[7], JULIANA A. VIANNA[8], EDUARDO EIZIRIK[2,9]

[1] Departamento de Genética, Universidade Federal do Rio Grande do Sul, Avenida Bento Gonçalves, 9500, prédio 43323, Porto Alegre, RS 91501-970, Brazil.

[2] Faculdade de Biociências, PUCRS, Avenida Ipiranga, 6681, prédio 12C, sala 172, Porto Alegre, RS 90619-900, Brazil.

[3] Kwata NGO, 16 Avenue Pasteur, F-97300, Cayenne, French Guiana.

[4] Laboratoire des Interactions Virus-Hôtes, Institut Pasteur de la Guyane, 23 Avenue Pasteur, F-97300, Cayenne, French Guiana.

[5] Instituto Nacional de Pesquisas da Amazônia – INPA, Avenida André Araújo, 2936, Manaus, AM 69060-001, Brazil.

[6] Projeto Ecolontras/Associação Ecológica Ecomarapendi, Rua Paissandu 362, Rio de Janeiro, RJ 22210-080, Brazil.

[7] Laboratory of Genomic Diversity, National Cancer Institute, Frederick, MD 21702, USA.

[8] Departamento de Ecosistemas y Medio Ambiente, Pontificia Universidad Católica de Chile, Av. Vicuña MacKenna 4860, Santiago, Chile.

[9] Instituto Pró-Carnívoros, Av. Horácio Neto, 1030, casa 10, Atibaia, SP 12945-010, Brazil.

Corresponding authors:

Cristine S. Trinca and Eduardo Eizirik

Emails: cristine.trinca@gmail.com and eduardo.eizirik@pucrs.br

Running title: Neotropical otter phylogeography

Abstract

The Neotropical otter (*Lontra longicaudis*) is a medium-sized semi-aquatic carnivore with a broad distribution in the Neotropical region. 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 a large portion of its geographic range by analyzing 1471 base pairs (bp) of mitochondrial DNA from 52 individuals. Our results indicate that *L. longicaudis* presents high levels of genetic diversity, and a consistent phylogeographic pattern suggesting the existence of at least four distinct evolutionary lineages in South America. The observed phylogeographic partitions are partially congruent with the subspecies classification previously proposed for this species. Coalescence-based analyses indicate that Neotropical otter mtDNA lineages have shared a rather recent common ancestor, *ca.* 0.5 MYA, and have subsequently diversified into the observed phylogroups. A consistent scenario of recent population expansion was identified in Eastern South America based on several complementary analyses of historical demography. The results obtained here provide novel insights on the evolutionary history of this largely unknown Neotropical mustelid, and should be useful to design conservation and management policies on behalf of this species and its habitats.

Keywords: biogeography, population structure, divergence time, Bayesian skyline plot, taxonomy, conservation

Introduction

The Neotropical otter (*Lontra longicaudis*) is a relatively common carnivore species with a broad distribution, ranging from Mexico to northern Argentina (Chehébar 1990). In the past humans made heavy use of these otters for skins, as their pelts were very much in demand in the international market, especially 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 about wildlife conservation and the enforcement of CITES designation for the Neotropical otter by Latin American countries (Chehébar 1990). However, this mustelid has been subjected to several other threats throughout its range, such as habitat loss and fragmentation, water pollution, road-killing and direct persecution in retaliation for its supposed predation on fish stocks (Macdonald & Mason 1990).Like other otter species, it is among the first species to decline and disappear when the aquatic environment is degraded, as it plays a top predator role in local food chains (Foster-Turley et al. 1990). Therefore it has been a particularly important focus for conservation efforts due to its potential role as an indicator of healthy aquatic environments. In spite of its relevance and conservation concern, very little is still known about this species, which has led the IUCN to categorize it as “Data Deficient” (Waldemarin and Alvarez 2008). Although recent studies have addressed some ecological aspects of this otter, such as diet and habitat use (Chemes et al. 2010, Gallo-Reynoso et al. 2008, Kasper et al. 2008), other issues remain unexplored, such as population structure, current demography and intra-specific evolutionary history.

Interestingly, traditional taxonomic studies have proposed that *L. longicaudis* might in fact be a species complex, based on variation in the rhinarium shape (Cabrera 1957, Harris 1968, Pohle 1920). Subsequent analyses suggested that those forms were conspecific, but could be geographically subdivided into three subspecies: *L. longicaudis annectens* (occurring in Mexico, Nicaragua, Costa Rica, Panama, Colombia, Venezuela and Ecuador), *L. longicaudis enudris* (distributed through French Guiana, Suriname, Trinidad and Peru) and *L. longicaudis longicaudis* (distributed through most of South America, including Brazil and Uruguay) (Larivière 1999, van Zyll de Jong 1972). However, such intra-specific taxonomic subdivision remains controversial, as no comprehensive revision of this species’ morphological variation has so far been performed.

Furthermore, recent observations have brought back into focus the hypothesis that this otter may in fact represent an assemblage of distinct species, which may or may not correspond to the proposed subspecies (Xth International Otter Colloquium, South Korea, 2007). Given this controversy and the currently scarce knowledge on variation across the species' geographic range, the use of any infra-specific classification has been discouraged until more detailed studies are conducted, which was deemed a high priority by the community of otter researchers (Xth International Otter Colloquium, South Korea, 2007).

In the context of defining species-level boundaries or intra-specific units, a useful approach is to investigate phylogeographic patterns of molecular variation (Avise 2000). So far only one study has addressed the genetic diversity and geographic differentiation of *L. longicaudis* populations (Trinca et al. 2007). That study employed mitochondrial DNA (mtDNA) control region sequences to survey the variation present in otters from southern and southeastern Brazil, and reported high levels of haplotype diversity, low nucleotide variation and no signal of genetic structuring in those regions. However, given the focus of that study on a small portion of the otter's extensive geographic distribution, no comprehensive assessment of its overall phylogeographic structure and evolutionary history could be attempted.

In this study we aimed to expand the investigation of *L. longicaudis* mitochondrial diversity by employing three different segments of the mtDNA to characterize a broad geographic sample spanning most of the species' range in South America. On the basis of this information we investigated the phylogeographic structure and demographic history of Neotropical otter populations, including an evaluation of whether inferred historical partitions coincided with previously proposed subspecies. Since our focal species is a semi-aquatic mammal, we also compared the observed phylogeographic patterns to some of those reported for terrestrial or aquatic taxa on a similar continental scale, aiming to test whether different life histories and dispersal capabilities have yielded contrasting or congruent subdivisions as reflected in current mtDNA diversity.

105 **Materials and Methods**

106 ***Sample collection***

107 Biological samples were collected from 52 Neotropical otters across a large portion of the
108 species' range (Fig. 1; Supplementary Table S1). Blood or faecal samples were obtained from
109 captive individuals with known geographic origin; muscle tissue and skin samples were collected
110 opportunistically by collaborators from animals found dead (e.g. road-killed); and additional faecal
111 samples were also collected by field researchers working in different areas. Blood samples were
112 preserved in a salt saturated solution (100mM Tris, 100mM EDTA, 2% SDS); tissue, faecal and
113 skin samples were preserved in 96% ethanol. All samples were stored at -20°C prior to DNA
114 extraction. Samples of South American River Otter (*Lontra provocax*), Marine Otter (*L. felina*),
115 North American River Otter (*L. canadensis*), Sea Otter (*Enhydra Lutris*), Asian Small-Clawed Otter
116 (*Aonyx cinereus*), and Giant Otter (*Pteronura brasiliensis*) were also sequenced, so as to be used
117 as outgroups in some of the analyses. For two of the mtDNA segments, we employed previously
118 published sequences of *L. provocax* and *L. felina* (accession numbers GQ843782, GQ843800,
119 GQ843819 and GQ843803).
120
121 ***Generation of sequence data***

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

128 Three segments of the mtDNA genome were amplified by the Polymerase Chain Reaction
129 (PCR) using primers developed or adapted for improved performance in carnivores: (I) ~550 bp of
130 the 5' portion of the mtDNA control region (CR), containing the first hypervariable segment, using
131 primers MTLPRO2 and CCR-DR1 (Tchaicka et al. 2007); (II) a ~400 bp segment including the
132 entire *ATP8* gene and part of the *ATP6* gene using primers ATP8-DF1 and ATP6-DR1 (Trigo et al.

2008); and (III) a ~750 bp segment of the *ND5* gene using primers ND5-DF1 and ND5-DR1 (Trigo et al. 2008).

Because fecal samples tend to present degraded DNA, hampering the sequencing of long fragments, in such cases we used the control region primers reported by Trinca et al. (2007), which divide our target segment into three shorter, overlapping fragments (each one with *ca.* 250 bp). Each 20ul PCR reaction contained 1-2 ul of empirically diluted template DNA, 1x PCR Buffer (Invitrogen), 2.0 - 2.5 mM $MgCl_2$, 200 uM dNTPs, 0.2uM of each primer and 0.5 unit of *Platinum* Taq DNA Polymerase (Invitrogen). The PCR conditions were the same for the three mitochondrial segments, and began with one step of 94°C for 3 min, 10 cycles (*Touchdown*) of 94°C for 45s, 60-51°C for 45s (-1°C per cycle), 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. The PCR conditions for amplification of DNA from faeces were similar as above, except for 5 cycles (*Touchdown*) of 94°C for 45s, 55-51°C for 45s, 72°C for 1.5 min followed by 40 cycles of 94°C for 45s, 50°C for 45s, 72°C for 1.5 min and final extension of 72°C for 30 min. Products were visualized on a 1% agarose gel stained with GelRed 10x (Biothium) and purified either by precipitation methods with Polyethyleneglycol-8000 or amonium acetate, or by employing the enzymes Exonuclease I and Shrimp Alkaline Phosphatase. Purified PCR products were sequenced using the *DYEnamic ET Dye Terminator Sequencing Kit* (GE Healthcare), and analyzed in a MegaBACE 1000 automated sequencer (GE Healthcare). Sequences were deposited in GenBank under accession numbers JQ038804 – JQ038869.

Sequence analyses

Sequences were visually checked and manually corrected using CHROMAS 2.0 (http://www.technelysium.com.au/chromas.html) or FinchTV 1.4.0 (www.geospiza.com/Products/finchtv.shtml) and aligned with the CLUSTALW algorithm implemented in MEGA 4 (Tamura et al. 2007). The alignment of each mtDNA segment was checked and edited by eye separately. Statistics such as nucleotide (π) and haplotype (*h*) diversity were computed using MEGA, DnaSP v.5 (Librado & Rozas 2009) and ARLEQUIN 3.01 (Excoffier et al. 2005).

Phylogenetic analyses were performed separately for two mtDNA data sets: (i) full concatenation of all segments (CR+*ATP8*/*ATP6+ND5*) (Data Set 1 [DS1]) and (ii) concatenation of the three coding fragments (*ATP8*/*ATP6+ND5*) (Data Set 2 [DS2]). The data sets were assessed for the best-fitting model of nucleotide substitution using the Akaike Information Criterion (AIC) as implemented in Modeltest 3.7 (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 tree-bisection-reconnection (TBR) branch-swapping method; (ii) maximum parsimony (MP) using heuristic searches with 50 replicates of random taxon addition and TBR branch-swapping; and (iii) distance-based, using the NJ algorithm and ML genetic distances. Support of internal branches for all of the above methods was evaluated with 100 nonparametric bootstrap replicates. Additionally, we employed a Bayesian Inference (BI) approach with MrBayes 3.1 (Huelsenbeck & Ronquist 2001), using two independent Markov Chain Monte Carlo (MCMC) runs, each containing four Metropolis-coupled chains (one cold and three heated) for 1 million generations. Trees were sampled every 100 generations, discarding the first 2,500 trees as burn-in. Convergence was considered satisfactory when the average standard deviation of split frequencies between parallel runs was lower than 0.05. Finally, we also assessed the relationships among haplotypes using the relaxed phylogenetics approach implemented in BEAST 1.6.0 (Drummond & Rambaut 2007), in parallel with the divergence dating analyses. In all cases, trees were rooted using *Lontra provocax* and *L. felina* as outgroups.

Given the evidence for saturation in the control region, divergence times for inferred phylogeographic partitions were estimated for DS2 only. Moreover, to further minimize the effects of saturation, we limited this analysis to genus *Lontra*, thus excluding any more distant outgroups. Divergence dates were estimated with BEAST, applying molecular calibrations reported in a previous phylogenetic study of the Mustelidae (Koepfli et al. 2008) for two different divergence points in the subfamily Lutrinae: i) base of the genus *Lontra*; ii) *Lontra longicaudis vs. L. felina* + *L. provocax*. The age of these nodes (corresponding to nodes 14 and 15 in Koepfli et al. [2008], respectively) was assumed to lie within the credibility intervals reported in that study: i) 1.6 – 5.2 million years ago (MYA) and ii) 0.5 – 3.2 MYA, respectively. To apply these molecular calibrations

191 in a conservative fashion, we used these minimum and maximum ages as boundaries in a uniform
192 prior distribution for the node age. We employed two distinct strategies to model nucleotide
193 substitution in our data set: (i) estimating the best-fit model for each segment using Modeltest; and
194 (ii) assuming a segment-specific SRD06 model (Shapiro et al. 2006), in which the 1st and 2nd codon
195 positions were grouped, and the 3rd codon position was placed in a separate category. In every
196 case, we assumed an uncorrelated lognormal relaxed molecular clock, and a tree prior based on a
197 Yule process. The MCMC procedure was run for 10-100 million generations, with samples taken
198 every 1,000-10,000 steps. Results were analyzed with the program Tracer v. 1.5 (Rambaut &
199 Drummond 2007) removing the initial 1-10 million steps (10% of each run) as burn-in.
200 In addition to the phylogeny-based approaches, haplotype networks were constructed
201 using the median-joining approach (Bandelt et al. 1999) implemented in Network 4.5.1.0
202 (www.fluxus-engineering.com) to depict phylogenetic, geographic, and potential ancestor-
203 descendent relationships among the sequences. Population structure analyses were performed
204 assuming broad geographic units based on the observed phylogeographic pattern. The testing of
205 additional, alternative scenarios of geographic subdivision could not be fully performed with this
206 approach due to limitations of sample size for some of the included areas which are represented by
207 only one to few individuals. As measures of differentiation among populations, we estimated
208 fixation indices (F_{ST}) (Wright 1965), using an Analysis of Molecular Variance (AMOVA) approach
209 (Excoffier et al. 1992) implemented in ARLEQUIN. Finally, we tested the null hypothesis of no
210 correlation between geographic and genetic distances using a Mantel test (Mantel 1967) as
211 implemented in AIS 1.0 (Miller 2005), with statistical significance assessed from 1,000 random
212 permutations.
213 To investigate the historical demography of *L. longicaudis*, we used DnaSP and
214 ARLEQUIN to perform neutrality tests, namely Tajima's D, Fu and Li's F* & D*, and Fu's Fs (Tajima
215 1989, Fu & Li 1993, Fu 1997), and also to conduct Mismatch Distribution Analyses (Rogers &
216 Harpending 1992). In addition, we used BEAST to generate Bayesian Skyline plots (BSP), which
217 allow an assessment of historical patterns of change in N_e over time. For this intra-specific analysis,
218 we assumed a strict molecular clock and a piecewise-constant Bayesian skyline tree prior. The
219 substitution model and evolutionary rate for each segment were derived from the initial BEAST run

that provided the best fit to the data set (see Results). We employed a segment-specific clock rate, which was entered as a range of values reflecting the 95% HPD interval resulting from the best-fitting initial BEAST run.

Results

A 516 base-pair (bp) fragment of the control region (CR) was sequenced for 51 *L. longicaudis* individuals. Due to ambiguous alignment in one hypervariable segment, 25 sites of the CR were excluded from all further analyses, yielding a final data set of 491 bp for this fragment. Sequences of the *ATP8*/*ATP6* fragment (329 bp, including164 bp of *ATP8* and 165 of *ATP6*, with an overlap of 40bp) and the *ND5* fragment (651 bp) were obtained for 52 Neotropical otters each. Outgroups were sequenced for these mtDNA regions yielding the same sequence length, except for *Aonyx cinereus*, whose control region segment was 1 bp longer than the remaining individuals. All segments were concatenated for use in various analyses, leading to a combined alignment containing 1472 bp (1471 bp when excluding the *Aonyx* sequence).

For all three segments, haplotype (gene) diversity was moderate to high, while nucleotide diversity was low to moderate (Tables 1 and 2). The CR was the most variable segment, but showed evidence of saturation when other otter species were included in the comparisons (see Table 1 and Fig. 2). This led us to conduct separate analyses with and without this fragment. Alignments containing concatenations of the full data set (DS1; n = 51) or the two coding fragments (DS2; n = 52) led to the observation of 37 and 24 unique haplotypes, respectively (Supplementary Table S2). The TIM + I + G and the TrN + G nucleotide substitution models were found to provide the best fit to DS1 (-lnL = 4888.1147) and DS2 (-lnL = 1986.4604), respectively, and were applied in all subsequent model-based phylogenetic analyses.

Phylogenetic trees produced with all different methods were consistent with respect to major topological features, with mostly subtle differences in nodal support. Support for major groups was strong with all methods, indicating robust resolution of the main clades (Fig. 3). A clear phylogeographic pattern could be discerned, with two major clades retrieved by all analyses (Fig. 3 and Supplementary Figure S1) and supported by robust bootstrap values (>75% with DS1 and >88% with DS2) and Bayesian posterior probabilities of 1.0 for both DS1 and DS2. Clade 1

249 contained almost all sequences sampled in Brazil, along with two sequences from French Guiana
250 (bLlo41 and bLlo66). Clade 2 contained all other samples from French Guiana, along with most
251 individuals from the Brazilian Amazon and one sample from Peru.

252 In addition to these two main clades, two other distinct haplotypes were observed in the
253 phylogenetic analysis, each of them represented by a single sample in this study. One of them was
254 the haplotype found in Bolivia (Ll-ANC33 and Ll-AN18 in DS1 and DS2, respectively), whose exact
255 placement was not identical with the two data sets: in DS1, it was the sister-group of Clade 2,
256 whereas in DS2 it was positioned in a trichotomy with respect to Clades 1 and 2 (Fig. 3 and
257 Supplementary Figure S1). This suggests that Bolivia may contain a third phylogeographic lineage
258 separate from Clades 1 and 2, whose exact relationships should be further investigated with
259 additional sampling. Furthermore, the individual from Colombia (bLlo23) contained a very distinct
260 haplotype (Ll-ANC35 and Ll-AN13 in DS1 and DS2, respectively) presenting at least 17 mutation
261 steps relative to any other haplotype and being positioned as the most basal lineage of all *L.*
262 *longicaudis*.

263 An analysis of phylogenetic and geographic structure within Clade 1 revealed some further
264 patterns. The samples from French Guiana (individuals bLlo41 and bLlo66 – see Supplementary
265 Table S2 for haplotype numbers) were consistently placed in basal positions. With DS1 they
266 grouped with one haplotype from northeastern Brazil (Ll-ANC17), while with DS2 they were the
267 sister-group to all other samples (see Supplementary Figure S1). In the latter case, the haplotype
268 from northeastern Brazil (Ll-AN16) was the next one to diverge, and thus remained at a relatively
269 basal position. All other haplotypes (representing a broad sample of individuals from across
270 eastern, central and southern Brazil) clustered in a single internal clade, which was robustly
271 supported by most analyses. Its internal phylogeny exhibited very short branches, little structure
272 and no evidence of geographic substructure, suggestive of a recent population expansion. On the
273 basis of these results, we defined a geographic group named Eastern South America (ESA),
274 containing most samples from Clade 1 and no haploytpe from Clade 2 (see Fig. 1). A second group
275 named ‘Amazonia’ was defined as the geographically-delimited sample set containing all
276 sequences from Clade 2 plus three haplotypes from Clade 1 (see Fig. 1 and Supplementary Table
277 S2).

The median-joining network produced with the full concatenation (DS1) was not entirely efficient at resolving the relationships among haplotypes (see Supplementary Figure S2), probably due to saturated mutation sites in the control region, which may have led to reticulation and a high number of median vectors. Nevertheless, a star-shaped pattern could be observed in the set of ESA haplotypes. In contrast, the haplotype network produced with DS2 depicted a clear phylogeographic pattern, with at least 7 mutational steps separating the samples belonging to Clades 1 and 2 (Fig. 4). The ESA haplotypes, contained within Clade 1, exhibited a star-shaped pattern with several localized lineages connected by short branches to a more common, widespread sequence. Again, individuals bLlo41 and bLlo66 (see Supplementary Table S2 for haplotype identification) were positioned near this group instead of being associated with other samples from French Guiana or the Brazilian Amazon. This analysis also showed Clade 2 as a separate group, which comprised most haplotypes from French Guiana, one sample from Peru and almost all animals from the Brazilian Amazon (see Fig. 4). Interestingly, one Amazonian haplotype (Ll-AN21) deviated from this pattern by nesting within Clade 1, directly connected to its central sequence.

Given these results and the availability of denser sampling on a regional scale, we focused subsequent geography-based analyses on the two broad phylogeographic units defined above (ESA *vs.* Amazonia), which were assessed in terms of their genetic diversity and differentiation. The AMOVA results indicated that 62% - 72% (DS1 and DS2, respectively) of the observed genetic variability (excluding the samples from Bolivia and Colombia) corresponded to differences between these populations. The F_{ST} between these two geographic groups was very high (0.63, p = 0.000; and 0.72, p = 0.000, for DS1 and DS2, respectively), as expected given their almost perfect reciprocal monophyly. Finally, the Mantel test did not reveal any significant relationship between genetic and geographic distances when the entire sample was compared (r = 0.19, P = 0.98), or when Clade 1 (r = 0.28, P = 0.96) or Clade 2 (r = -0.12, P = 0.31) were analyzed separately.

Initial rounds of divergence dating analyses with BEAST were focused on establishing the best-fit model of nucleotide substitution for each segment (in this case we treated *ATP8* and *ATP6* as distinct segments, including the overlapping 40 bp only in the latter; additionally, the initial 3 bp of the *ATP8* fragment were excluded so as to exactly match the coding region of this genetic

307 locus). The AIC assessment with Modeltest indicated that HKY+G provided the best fit to *ATP8*,
308 HKY+I to *ATP6*, and GTR+I to *ND5*. Although the analysis employing such models provided a good
309 fit to the data, we observed that a considerable improvement (*i.e.* significantly higher posterior
310 probability) could be attained with the use of the codon-based SDR06 model. We therefore
311 employed this approach in all subsequent analyses with BEAST, including divergence dating and
312 Bayesian skyline plots. The mean divergence time between *L. longicaudis* and *L. felina* + *L.*
313 *provocax* was estimated to be 1.15 MYA (95% highest posterior density [HPD]: 0.53 – 1.99 MYA),
314 while the mean coalescence of all *L. longicaudis* haplotypes (Time to the Most Recent Common
315 Ancestor [TMRCA]) was estimated at 0.58 MYA (95% HPD: 0.22 – 1.07 MYA). Furthermore, the
316 age of haplotype coalescence within each of the two main *L. longicaudis* internal clades (Clades 1
317 and 2) was estimated at 0.28 MYA (95% HPD: 0.097 - 0.52 MYA) and 0.19 MYA (95% HPD: 0.056
318 – 0.38 MYA), respectively. The divergence time between *L. felina* and *L. provocax* was estimated
319 to have occurred *ca.* 0.28 MYA (95% HPD: 0.076 – 0.59 MYA).

320 To test the hypothesis of a recent population expansion in *L. longicaudis*, we performed
321 mismatch distribution analyses and neutrality tests for each of the mtDNA haplogroups. Mismatch
322 distribution analyses revealed some cases of smooth unimodal patterns, indicative of a population
323 expansion following a genetic bottleneck (Fig. 5). This was especially the case for Clade 1 (and
324 even more so its ESA subgroup) when assessed with DS2. Clade 2 did not show such a clear
325 pattern, but did exhibit a unimodal distribution when assessed with DS1 (Fig. 5). These results
326 were congruent with those obtained with the neutrality tests (Table 3), which yielded negative
327 values for all the assessed groups, consistent with the inference of historical population expansion.
328 Significantly negative values were observed in all tests for the full sample of *L. longicaudis* when
329 assessed with DS2 (but only with Fu's Fs when using DS1). The strongest signal for population
330 expansion (significantly negative values across most or all tests) was observed for Clade 1 and
331 ESA when assessed with DS2, with a trend for stronger departure from the neutral model when the
332 analysis was restricted to the latter, geographically defined, set of sequences.

333 The Bayesian skyline plots were consistent with a scenario of population growth in Clade 1
334 (Fig. 6A), with a clear signal of demographic expansion starting *ca.* 0.025 MYA. In contrast, there

335 was only a modest trend suggesting gradual population increase in Clade 2, but no marked signal
336 of recent demographic expansion.
337
338 **Discussion**
339 ***Genetic Diversity***
340 As observed in a previous study based on a smaller data set (Trinca et al. 2007), the
341 present results support the view that the Neotropical otter presents moderate genetic diversity in its
342 mtDNA control region (CR). Levels of polymorphism in this segment were similar to those reported
343 for other otters, such as *Lontra felina* and *Pteronura brasiliensis* (Garcia et al. 2007, Valqui et al.
344 2010, Vianna et al. 2010), and also comparable to those observed in other carnivores such as
345 jaguars (Eizirik et al. 2001) and crab-eating foxes (Tchaicka et al. 2007). However, diversity in *L.*
346 *longicaudis* tended to be higher than that found for *Lontra provocax* in the Argentinean Patagonia
347 (Centrón et al. 2008), and for *Lutra lutra* in Europe (Cassens et al. 2000; Effenberger &
348 Suchentrunk 1999; Mucci et al. 1999; Ferrando et al. 2004, Pérez-Haro et al. 2005; Stanton et al.
349 2009). In contrast, Larson et al. (2002) reported an about eight-fold higher estimate of nucleotide
350 diversity for the sea otter *Enhydra lutris*.
351 In addition to the CR results, our data sets allowed an assessment of diversity levels in the
352 mitochondrial genes *ATP8*/*ATP6* and *ND5* (Tables 1 and 2). The observed variability in the
353 *ATP8*/*ATP6* and *ND5* segments could not be directly compared to other otters, due to the absence
354 of polymorphism data from additional species. Nevertheless, data from two small Neotropical felids
355 (*Leopardus tigrinus* and *L. geoffroyi*) reveal a somewhat lower diversity in both segments (Trigo et
356 al. 2008; T. Trigo, pers. comm.) relative to *L. longicaudis*, suggesting that the latter species harbors
357 considerable variability in these fragments, albeit lower than that observed in the CR.
358 In spite of its high diversity relative to other mtDNA segments, our analyses suggest that the
359 control region may not be the best mitochondrial marker for phylogeographic studies in *L.*
360 *longicaudis* and perhaps other related species. Variable sites in this segment seem to be saturated
361 even at the intra-specific level, leading to a lower signal-to-noise ratio than observed for the other
362 two fragments (Fig. 2). Conversely, the *ND5* segment used here seems to be very informative and

less prone to saturation at recent levels, which has also been observed for other carnivores studied by our group (unpublished data).

Phylogeographic Structure

The most evident pattern provided by the mtDNA data was the deep partition between two broad groups of populations, separated in a northwest-southeast fashion, in addition to divergent lineages in Bolivia and Colombia (see Figs. 1, 3 and 4). The two main phylogeographic groups (ESA and Amazonia) were strongly supported by all analyses, including phylogenies, haplotype networks and AMOVA. Overall, our results indicate that *L. longicaudis* is composed by at least four geographically structured phylogroups: (i) Colombia; (ii) Bolivia; (iii) Amazonia, encompassing all samples from the Amazon basin (except Bolivia) and French Guiana; and (iv) Eastern South America, containing several other drainages. It may be noted that Colombia and Bolivia were represented by a single sample each (whose genetic divergence from the other individuals indicates deep phylogeographic partitions involving these areas), so that further sampling is needed to ascertain their evolutionary uniqueness.

Interestingly, the separation of the Colombian haplotype from any other *L. longicaudis* implies at least 17 mutational steps, which is considerably deeper than the divergence estimated between *Lontra felina* and *L. provocax* (12 mutational steps). This substantial differentiation is also reflected in the estimates of divergence times, with the separation between the Colombian sample and the remaining *L. longicaudis* inferred to have been 0.575 MYA, while that between *L. felina* and *L. provocax* would have occurred ca. 0.28 MYA (see Vianna et al. [2010] for a different but overlapping estimate of the divergence age for this node). This Colombian sample originated from the Magdalena river valley, which is separated from other regions on either side by the Cordillera Central and the Cordillera Oriental, respectively. The deep genetic divergence observed in this region relative to the remaining samples could be due to fact that the Magdalena river flows northward into the Caribbean Sea (Eisenberg & Redford 1999), possibly hampering the genetic connectivity between the two sides of the Cordillera Oriental. In addition to the interest in unraveling the biogeographic processes shaping this evolutionary distinction, such a deep divergence could imply that a taxonomic revision in this group may indeed be warranted.

392 The almost complete reciprocal monophyly of the two better-sampled phylogroups
393 (Amazonia and ESA) is remarkable, with only three individuals (bLlo41, bLlo66 and bLlo72) found
394 in a geographic region inconsistent with their phylogenetic placement (French Guiana and Brazilian
395 Amazon). The position of these three samples in Clade 1 is intriguing, and raises two alternative
396 hypotheses, namely relictual sister-group relationship due to ancestral colonization *vs.* secondary
397 gene flow between the two regions (Avise 2000). The observed phylogenetic pattern supports the
398 former hypothesis for bLlo41 and bLlo66 (which are solidly placed at the base of Clade 1), and the
399 latter for bLlo72 (which is clearly positioned within Clade 1). We may thus postulate that the ESA
400 group (almost completely bearing Clade 1 haplotypes) derives from a recent episode of
401 colonization from the north of South America, so that northern populations are paraphyletic with
402 respect to more southerly ones. Another observation that is consistent with this inference is the
403 position of the single haplotype (sampled in two different individuals) from the Brazilian northeast,
404 which is the most basal of all Brazilian lineages within Clade 1 (e.g. LI-ANC19 in Fig. 3; see also
405 DS2 tree in Supplementary Figure S1). This hypothesis should be further tested with additional
406 sampling in northern Brazil and adjacent areas.
407 Within Clades 1 and 2, there was little evidence of geographic structure, with no regional
408 clustering of haplotypes or clear associations to river basins (Figs. 3 and 4). The apparent lack of
409 genetic structure within Clade 1 and Clade 2 could derive from extensive gene flow among
410 populations in those areas and/or from a recent origin of each set of populations from a common
411 ancestral gene pool. Our divergence date estimates indicate that both clades have a considerably
412 recent coalescence (0.28 MYA for Clade 1 and 0.19 MYA for Clade 2), suggesting that this may be
413 an important component underlying the observed lack of internal geographic structure.
414 The phylogeographic patterns observed here are largely congruent with the results
415 reported by Hubert & Renno (2006) for South American characid fishes, which indicated that
416 different drainages such as the Amazon and Paraná comprised distinct species pools. In the case
417 of river dolphins, molecular analyses allowed the recognition of two distinct mtDNA lineages from
418 the Bolivian Amazon and the Colombian Orinoco/Colombian Amazon, supporting the recognition of
419 two Evolutionarily Significant Units (ESUs) in those areas (Banguera-Hinestroza et al. 2002). Also,
420 a phylogeographic pattern similar to those observed here was reported by Camargo et al. (2006)

421 for the Neotropical frog *Leptodactylus fuscus*, a species that occupies open habitats and river
422 edges. In that species, three well-supported clades were observed, one from central and northern
423 South America (including most of the Amazon basin), another from Bolivia and the third comprising
424 almost the entire territory of the Brazilian shield and Argentina. Those clades did not seem to
425 overlap geographically, supporting the hypothesis of *L. fuscus* being a "species complex"
426 (Camargo et al. 2006). Such congruent patterns in different taxa are suggestive of common
427 underlying processes leading to regional population isolation and differentiation, likely influenced by
428 the history of river basins and its impact on associated species.

429 Finally, some aspects of the *L. longicaudis* phylogeographic structure are comparable to
430 those previously reported for a related and largely sympatric species, the giant river otter *Pteronura*
431 *brasiliensis* (Garcia et al. 2007). In both cases, there is evidence of significant genetic structure on
432 a broad geographic scale. On the other hand, both species show evidence of long-range historical
433 connectivity among regions, leading to some haplotype sharing between different biomes (in the
434 case of *Pteronura*) or relatedness among haplotypes sampled in very distant areas (in *L.*
435 *longicaudis*). These observations (still restricted to matrilineal markers and possibly even more
436 visible when male lineages are surveyed) are likely a result of these species' semi-aquatic habits
437 and consequent dispersal capabilities within and among drainages.

438

439 ***Demographic History***

440 When intra-specific levels of molecular variation were assessed for *L. longicaudis*, we
441 observed that the ESA group presented lower nucleotide and haplotype diversity than the
442 Amazonian group, a pattern which was consistent across all data partitions (Table 2). Given the
443 larger sample size available for ESA relative to Amazonia, this observation may in fact be a
444 reflection of contrasting demographic histories between these two regions. Such pattern prompted
445 us to investigate in more detail the population history of each region, based on multiple
446 complementary approaches.

447 The network analysis placed haplotype LI-AN04 at a central position in the ESA portion of
448 Clade 1, with its high frequency also suggesting an ancestral status within this lineage. The pattern
449 observed with the mtDNA phylogenies and haplotype networks is consistent with a recent

450 population expansion in ESA, with most haplotypes from this region differing from each other by
451 only one or two mutational steps, connected in a star-shaped fashion (e.g. Fig. 4). This inference is
452 also supported by the mismatch distribution analyses (Fig. 5) and the neutrality tests (Table 3)
453 performed for Clade 1 (which contains all ESA lineages). The mismatch distribution results for this
454 clade based on DS2 (less noise in terms of phylogenetic signal, with less homoplasy than inferred
455 for the control region) fit particularly well the expected pattern under a sudden demographic
456 expansion (Rogers & Harpending 1992), with a single, smooth prominent peak. Although there was
457 also some suggestion of recent demographic expansion in Clade 2 (especially when DS1 was
458 analyzed – see Fig. 5), it was not as conclusive as that observed for Clade 1. The same conclusion
459 emerges from an inspection of the neutrality test results (Table 3), which showed consistent
460 support for a demographic expansion in Clade 1 (and ESA within it), and only modest support for
461 an equivalent process in Clade 2 (significant only for Fu's Fs estimated with DS1).
462 A congruent pattern was also observed in the Bayesian skyline plot analyses (Fig. 6), with
463 Clade 1 exhibiting a much clearer signal of recent population expansion than Clade 2. An
464 interesting inference derived from this approach was the estimation of the time frame for this
465 demographic expansion affecting Clade 1. Our results suggest that Clade 1 lineages remained
466 rather stable in effective population size until *ca.* 25,000 years ago, when they underwent a
467 substantial (approximately 10-fold) demographic increase.
468
469 ***Taxonomy***
470 The taxonomy of *L. longicaudis* has been the target of recent discussion, since
471 morphological variation in the rhinarium (which is often argued to underlie species or subspecies
472 differentiation in otters - Larivière 1999), has been reported by researchers from different parts of
473 the species' distribution (X^{th} International Otter Colloquium, South Korea, 2007). Although the
474 variation in rhinarium shape has not yet been thoroughly characterized across the species' range,
475 preventing a more complete assessment of the putative subspecies, our phylogeographic results
476 demonstrate considerable agreement with the proposed geographic range of these infra-specific
477 partitions. The ESA phylogroup largely agrees with the proposed range of *L. longicaudis*
478 *longicaudis*, presumed to occur in most of South America. The Amazonian phylogroup may

479 represent the subspecies *L. longicaudis enudris*, believed to occur in French Guiana, Suriname,
480 Trinidad and Peru. Finally, the divergent Colombian lineage could be considered to be suggestive
481 of historical differentiation of the subpecies *L. longicaudis annectens*, which would be restricted to
482 Central America and northwestern South America (Larivière 1999). A possible discrepancy
483 between the proposed subspecific scheme and our phylogeographic results is the indication that
484 Bolivia contains a fourth major mtDNA lineage in *L. longicaudis*, suggesting that further sampling
485 may reveal additional evolutionary partitions in this otter species.

486 However, any intra-specific taxonomic decision on the Neotropical otter is premature at this
487 point, given the still modest geographic sampling across the species' range, and the breadth of the
488 present data set. Although our phylogeographic results largely match the currently proposed
489 subspecies, additional analyses should be conducted employing both molecular and morphological
490 approaches, so as to further ascertain and refine the observed geographic partitions. Molecular
491 analyses should include nuclear markers, which would complement the matrilineal mtDNA
492 sequences analyzed here. Likewise, more in-depth morphological assessments should include a
493 broad suite of characters (e.g. cranial measurements), in order to complement and test the
494 perceived differentiation based on rhinarium shape.

495

496 ***Implications for conservation***

497 The results presented here have important implications for the conservation and
498 management of this species in the wild and in captivity as well as its habitats. In spite of being
499 represented in this study by a single sample each, the divergent lineages observed in Colombia
500 and Bolivia suggest that these areas may be sufficiently differentiated to indicate their provisional
501 recognition as distinct evolutionary entities. Given the limited sampling, we do not propose that they
502 be treated as fully recognized Evolutionarily Significant Units (ESUs), but rather point out that they
503 should be a priority for further study in this regard.

504 The main phylogeographic groups identified here (ESA and Amazonia) indicate that these
505 regions have likely been historically isolated, at least with respect to female lineages. However,
506 some historical female connectivity between these areas may also be inferred from our data, as
507 illustrated by sample bLlo72, which was collected in the Brazilian Amazon but harbors an mtDNA

haplotype contained in Clade 1. Nevertheless, the genetic differentiation detected between these two regions was substantial, indicating little gene flow, and consequently they may be viewed as distinct ESUs or at least Management Units (MUs) (Moritz et al. 1994). Each of these groups is widespread and associated with broad watersheds and diverse ecoregional units (Olson et al. 1998), likely justifying specific efforts in terms of conservation and management actions. In parallel, to further assess the demographic distinctiveness of these units, additional analyses should be performed including independently evolving markers such as nuclear loci with bi-parental inheritance as well as Y-chromosome loci, so as to investigate the existence of male-based gene flow in this species. Furthermore, additional demographic units may exist in the Neotropical otter, and will potentially emerge as more detailed studies are performed on the basis of expanded geographic sampling. Nevertheless, the results from this study are compelling in the context of demonstrating that this species presents substantial phylogeographic structure, opening up new research avenues targeting its demographic history and dispersal patterns at multiple spatial scales.

Funding

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq, Brazil (grant number 478385/2007-2). Data collection from French Guiana samples was performed in the context of the SPECIES project, which was funded by WWF Network; European Funds (FEDER); Fonds Français pour l'Environnement Mondial (FFEM); and the French Ministry of Higher Education and Research.

Acknowledgments

The authors would like to thank all the institutions and people listed in Supplementary Table S1, who generously provided the biological samples used in this study. We also thank Cladinara R. Sarturi for technical assistance and suggestions, Felipe G. Grazziotin for valuable support in demographic analysis and Luiz E. Costa-Schmidt for the assistance with tables and figures.

536 **References**

537 Avise JC. 2000. *Phylogeography – The History and Formation of Species*. Harvard University
538 Press, Cambridge, Massachusetts.

539 Bandelt HJ, Forster P, Röhl A. 1999. Median-joining networks for inferring intraspecific
540 phylogenies. Mol Biol and Evol. 16:37 48.

541 Banguera-Hinestroza E, Cárdenas H, Ruíz-Garcia M, Marmontel M, Gaitán E, Vásquez R, García-
542 Vallejo F. 2002. Molecular Identification of Evolutionary Significant Units in the Amazon River
543 Dolphin *Inia* sp. (Cetacea: Iniidae). J Hered. 93:312-322.

544 Cabrera A. 1957. Catálogo de los mamíferos de América del Sur. I (Metatheria-Unguiculata-
545 Carnivora). Revista del Museo Argentino de Ciências Naturales "Bernardino Rivadavia" e
546 Instituto Nacional de Investigación de las Ciências Naturales, Ciências Zoológicas. 4:1-307.

547 Camargo A, de Sá RO, Heyer WR. 2006. Phylogenetic analyses of mtDNA sequences reveal three
548 cryptic lineages in the widespread neotropical frog *Leptodactylus fuscus* (Schneider, 1799)
549 (Anura, Leptodactylidae). Biol J Lin Soc. 87:325–341.

550 Cassens I, Tiedemann R, Suchentrunk F, Hartl GB. 2000. Mitochondrial DNA variation in the
551 European otter (*Lutra lutra*) and the use of spatial autocorrelation analysis in conservation. J
552 Hered. 91:31-41.

553 Centrón D, Ramirez B, Fasola L, MacDonald DW, Chehébar C, Schiavini A, Cassini MH. 2008.
554 Diversity of mtDNA in Southern River Otter (*Lontra provocax*) from Argentinean Patagonia. J
555 Hered. 99:198-201.

556 Chehébar CE. 1990. Action Plan from Latin American Otters. In: *Otters: A Plan for their*
557 *Conservation* (eds. Foster-Turley P, Macdonald S, Mason C), pp. 64-73. IUCN Otter Specialist
558 Group, Kelvin Press, Inc., Illinois, USA.

559 Chemes SB, Giraudo AR, Gil G. 2010. Dieta de *Lontra longicaudis* (Carnivora, Mustelidae) en el
560 Parque Nacional El Rey (Salta, Argentina) y su comparación con otras poblaciones de la
561 cuenca del Paraná. Mastozoología Neotropical. 17:19-29.

562 Drummond A, Rambaut A. 2007. BEAST: Bayesian evolutionary analysis by sampling trees. BMC
563 Evol Biol, 7:214.

564 Effenberger S, Suchentrunk F. 1999. RFLP analyses of the mitochondrial DNA of otters (*Lutra*
565 *lutra*) from Europe – implications for conservation of a flagship species. Biol Conserv. 90:229-
566 234.
567 Eisenberg JF, Redford KH. 1999. Mammals of the Neotropics, Vol. 3. The Central Tropics:
568 Ecuador, Peru, Bolivia, Brazil. Chicago, University of Chicago Press.
569 Eizirik E, Kim J, Menotti-Raymond M, Crawshaw Jr PG, O'Brien SJ, Johnson WE. 2001.
570 Phylogeography, population history e conservation of jaguars (*Panthera onca*, Mammalia,
571 Felidae). Mol Ecol. 10:65-79.
572 Excoffier L, Smouse P, Quattro J. 1992. Analysis of Molecular Variance inferred from metric
573 distances among DNA haplotypes: application to human mitochondrial DNA restriction data.
574 Genetics. 131:479-491.
575 Excoffier L, Laval G, Schneider S. 2005. Arlequin ver. 3.0: An integrated software package for
576 population genetics data analysis. Evol Bioinform Online. 1:47-50.
577 Ferrando A, Ponsà M, Marmi J, Domingo-Roura X. 2004. Eurasian otters, *Lutra lutra*, have a
578 dominant mtDNA haplotype from the Iberian Peninsula to Scandinavia. J Hered, 95:430-435.
579 Foster-Turley P, Macdonald S, Mason CF. 1990. Otters: An action plan for their conservation.
580 IUCN/SSC Otter Specialist Group, Kelvin Press, Inc., Illinois, USA.
581 Fu YX. 1997. Statistical test of neutrality of mutations against population growth, hitchhiking and
582 background selection. Genetics. 147:915-925.
583 Fu YX, Li WH. 1993. Statistical tests of neutrality of mutations. Genetics. 133:693-709.
584 Gallo-Reynoso JP, Ramos-Rosas NN, Rangel-Aguilar O. 2008. Aquatic bird predation by
585 neotropical river otter (*Lontra longicaudis annectens)*, at Río Yaqui, Sonora, México. Revista
586 Mexicana de Biodiversidad, 79: 275- 279.
587 Garcia DM, Marmontel M, Rosas FCW, Santos FR. 2007. Conservation genetics of the giant otter
588 (*Pteronura brasiliensis* (Zimmerman, 1780)) (Carnivora, Mustelidae). Braz J Biol. 67:819-827.
589 Harris CJ. 1968. Otters: A study of the recent Lutrinae. Weinfield and Nicholson, London, 397 pp.
590 Hubert N, Renno JF. 2006. Historical biogeography of South American freshwater fishes. J.
591 Biogeogr. 33:1414–1436.

592 Huelsenbeck JP, Ronquist F. 2001. MRBAYES: Bayesian inference of phylogeny. Bioinformatics.
593 17:754-755.
594 Kasper CB, Bastazini VAG, Salvi J, Grillo HCZ. 2008. Trophic ecology and the use of shelters and
595 latrines by the Neotropical otter (*Lontra longicaudis*) in the Taquari Valley, Southern Brazil.
596 Iheringia, Sér. Zool. 98:469-474.
597 Koepfli KP, Deere KA, Slater GJ, Begg C, Begg K, Grassman L, Lucherini M, Veron G, Wayne R.
598 2008. Multigene phylogeny of the Mustelidae: Resolving relationships, tempo and
599 biogeographic history of a mammalian adaptive radiation. BMC Biol. 6:10.
600 Larivière S. 1999. *Lontra longicaudis*. Mamm Species. 609:1-5.
601 Larson S, Jameson R, Bodkin J, Staedler M, Bentzen P. 2002. Microsatellite DNA and
602 mitochondrial dna variation in remnant and translocated sea otter (*Enhydra lutris*) populations. J
603 Mammal. 83:893–906.
604 Librado P, Rozas J. 2009. DnaSP v5: A software for comprehensive analysis of DNA
605 polymorphism data. Bioinformatics. 25:1451-1452.
606 Macdonald SM, Mason CF. 1990. Threats. In*: Otters: An Action Plan for their Conservation* (eds.
607 Foster-Turley P, Macdonald S, Mason C), pp. 11-14. IUCN Otter Specialist Group, Kelvin Press,
608 Inc., Illinois, USA.
609 Mantel N. 1967. The detection of disease clustering and a generalized regression approach.
610 Cancer Research. 27:209-220.
611 Miller MP. 2005. ALLELES IN SPACE: Computer software for the joint analysis of interindividual
612 spatial and genetic information. J Hered. 96:722-724.
613 Moritz C. 1994. Defining “Evolutionary Significant Units” for conservation. Trends Ecol Evol. 9:373-
614 375.
615 Mucci N, Pertoldi C, Madsen AB, Loeschcke V, Randi E. 1999. Extremely low mitochondrial DNA
616 control-region sequence variation in the otter *Lutra lutra* population of Denmark. Hereditas.
617 130:331-336.
618 Olson DM, Dinerstein E, Canevari P, Davidson I, Castro G, Morisset V, Abell R, Toledo E. 1998.
619 Freshwater Biodiversity of Latin America and the Caribbean: A Conservation Assessment.
620 Washington (DC): Biodiversity Support Program.

621 Pérez-Haro M, Viñas J, Mañas F, Batet A, Ruiz-Olmo J, Pla C. 2005. Genetic variability in the
622 complete mitochondrial control region of the Eurasian Otter (*Lutra lutra*) in the Iberian
623 Peninsula. Biol J Linn Soc. 86:397-403.
624 Pohle H, 1920. Die Unterfamilie der Lutrinae. Eine systematisch-tiergeographische Studie an dem
625 Material der Berliner Messen. Archiv für Naturgeschichte. 85: 1-247.
626 Posada D, Crandall KA. 1998. MODELTEST: testing the model of DNA substitution. Bioinf Appl
627 Notes. 14:817-818.
628 Rambaut A, Drummond AJ. 2007. Tracer Analysis Tool Version 1.4, Available from
629 http://beast.bio.ed.ac.uk/Tracer. University of Oxford, Oxford, UK.
630 Rogers AR, Harpending HC. 1992. Population growth makes waves in the distribution of pairwise
631 genetic differences. Mol Biol Evol. 9:552-569.
632 Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular Cloning, 2nd edn. Cold Spring Harbor
633 Laboratory Press, New York.
634 Shapiro B, Rambaut A, Drummond AJ. 2006. Choosing appropriate substitution models for the
635 phylogenetic analysis of protein-coding sequences. Mol Biol Evol. 23:7-9.
636 Stanton DWG, Hobbs GI, Chadwick EA, Slater FM, Bruford MW. 2009. Mitochondrial genetic
637 diversity and structure of the European otter (*Lutra lutra*) in Britain. Conserv Genet. 10:733 –
638 737.
639 Swofford DL. 2002. PAUP*. Phylogenetic Analysis Using Parsimony (*and Others Methods),
640 Version 4. Sinauer Associates, Suderland, Massachusetts.
641 Tajima F. 1989. Statistical methods to test for nucleotide mutation hypothesis by DNA
642 polymorphism. Genetics. 123:585-595.
643 Tamura K, Dudley J, Nei M, Kumar S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis
644 (MEGA) software version 4.0. Mol Biol Evol. 24:1596-1599.
645 Tchaicka L, Eizirik E, De Oliveira TG, Cândido Jr JF, Freitas TRO. 2007.
646 Phylogeography and population history of the crab-eating fox (*Cerdocyon thous*).
647 Mol Ecol. 16:819–838.
648 Trinca CS, Waldemarin HF, Eizirik E. 2007. Genetic diversity of the Neotropical otter (*Lontra
649 longicaudis* Olfers, 1818) in Southern and Southeastern Brazil. Braz J Biol 67:813-818.

650 Trigo TC, Freitas TRO, Kunzler G, Cardoso L, Silva JCR, Johnson WE, O'Brien SJ, Bonatto SL,
651 Eizirik E. 2008. Inter-species hybridization among Neotropical cats of the genus *Leopardus*, and
652 evidence for an introgressive hybrid zone between *L. geoffroyi* and *L. tigrinus* in southern Brazil.
653 Mol Ecol. 17:4317-4333.
654 Valqui J, Günther BH, Zachos FE. 2010. Non-invasive genetic analysis reveals high levels of
655 mtDNA variability in the endangered South-American marine otter (*Lontra felina*). Conserv
656 Genet. 11:2067–2072.
657 van Zyll de Jong CG. 1972. A systematic review of the Nearctic and Neotropical river otters (Genus
658 *Lontra*, Mustelidae, Carnivora). Life Sci Contrib Roy Ontario Mus. 80:1-104.
659 Vianna JA, Ayerdi P, Medina-Vogel G, Mangel JC, Zeballos H, Apaza M, Faugeron S. 2010.
660 Phylogeography of the marine otter (*Lontra felina*): historical and contemporary factors
661 determining its distribution. J Hered. 101:676-689.
662 Waldemarin HF, Alvarez R (2008) *Lontra longicaudis*. In: IUCN 2011. IUCN Red List of Threatened
663 Species. Version 2011.2. <www.iucnredlist.org>. Downloaded on 10 February 2012.
664 Wright S. 1965. The interpretation of population structure by *F*-statistics with special regard to
665 systems of mating. Evolution. 19:395-420.
666

667 **Table Legends**

668 Table 1. mtDNA diversity estimates for the Neotropical river otter and related species.

669

670 Table 2. Nucleotide and gene diversity observed in the *Lontra longicaudis* mtDNA segments
671 (specified separately for the two different geographic groups: Eastern South America [ESA] and
672 Amazonia).

673

674 Table 3. Neutrality test results for the two data sets used in this study (DS1 and DS2), as well as
675 different phylogenetic or geographic sub-groups (see text and Fig. 1). Numbers highlighted in bold
676 are significant for $\alpha < 0.05$.

677

678 **Figure Captions**

679 Fig 1. Map depicting the currently assumed geographic distribution (*shaded area*) of the
680 Neotropical otter (modified from
681 http://www.otterspecialistgroup.org/Species/Lontra_longicaudis.html), with sample collection sites.
682 Numbers next to the collection sites are sample identification labels (number after "bLlo" in
683 Supplementary Table S1) of *L. longicaudis* individuals in each area. Boxes indicate individuals from
684 the same region. White squares are indicative of exchanged haplotypes between the two defined
685 geographic groups (see Results). Dotted ellipses represent the geographically defined population
686 groups (Eastern South America and Amazonia).

687

688 Fig. 2. Graph depicting an analysis of saturation at the three mtDNA segments employed in the
689 present study. Values in the Y axis represent nucleotide diversity (π) for *L. longicaudis* or mean p-
690 distance (D_{xy}) for all pairwise comparisons relating Neotropical otter sequences with those of each
691 of several related otter species. The sequence of species placement on the X-axis reflects their
692 evolutionary divergence relative to *L. longicaudis*, but is not strictly positioned on a timescale (see
693 Koepfli et al. [2008] for divergence date estimates among these species).

694

695 Fig. 3. Maximum likelihood tree of *L. longicaudis* mtDNA haplotypes identified in this study, based
696 on 1471 bp of concatenated CR +*ATP8*/*ATP6* + *ND5* sequences (DS1). Labels are haplotype
697 identification numbers (see Supplementary Table S2). Values in the branches indicate support for
698 the adjacent node based on ML / MP / NJ / BI. The asterisk represents nodal support < 60%.
699
700 Fig. 4. Median-joining network of *L. longicaudis* mtDNA haplotypes based on concatenated
701 *ATP8*/*ATP6 + ND5* sequences (DS2; 681 bp were used; all sites containing indels or missing
702 information were excluded). Cross marks are nucleotide substitutions inferred to have occurred on
703 each branch. Circles correspond to haplotypes, whose frequency in our sample is indicated by the
704 circle size. Geographic origins of haplotypes are as follows: red (vertical lines in the print version):
705 eastern South America (ESA); blue (horizontal lines in the print version): French Guiana; yellow
706 (cross-hatched in the print version): Brazilian Amazon; light green (diagonally hatched in the print
707 version): Peru; light grey: Bolivia; black: Colombia; white: outgroups.
708
709 Fig. 5. Mismatch distribution analysis of the two data sets employed in this study (DS1 [A] and DS2
710 [B]) for Clade 1 (A1, B1) and Clade 2 (A2, B2). The continuous line indicates the observed
711 frequency of pairwise differences among haplotypes, while the dotted line depicts the expected
712 frequency under a sudden population expansion model.
713
714 Fig. 6. Bayesian Skyline Plots (BSPs) generated with BEAST 1.6.0 based on Data Set 2
715 (*ATP8*/ATP6 + *ND5* segments). Separate analyses are shown for Clades 1 (A) and 2 (B). The
716 analysis employed segment-specific substitution rates which were previously estimated with
717 BEAST in parallel to divergence time calculations (see text for details).
718
719 **Supplementary Material**
720 Table S1. Samples analyzed in the present study.
721
722 Table S2. List of mitochondrial DNA haplotypes included in Data Set 1 (DS1 – containing CR +
723 *ATP8*/*ATP6* + *ND5*) and DS2 (containing *ATP8*/*ATP6* + *ND5*). For each concatenated haplotype,

724 the individual haplotype for each included segment is indicated, along with its respective GenBank
725 accession number. The individuals carrying each haplotype and their overall geographic
726 distribution are also listed (see Supplementary Table S1 for more details on the geographic origin
727 of each individual).
728
729 Fig. S1. Maximum likelihood tree of *L. longicaudis* mtDNA *ATP8*/*ATP6* + *ND5* lineages identified in
730 this study. Labels are haplotype identification numbers (see Supplementary Table S2). Values
731 above and below branches indicate support for the adjacent node based on ML / MP / NJ / BI.
732
733 Fig. S2. Median-joining network of *L. longicaudis* mtDNA CR + *ATP8*/*ATP6 + ND5* haplotypes
734 (using 1164 bp; all sites containing indels or missing information were excluded). Bars on branches
735 indicate nucleotide substitutions inferred to have occurred between the connected haplotypes. Red
736 circles are haplotypes sampled in Brazil; blue circles are haplotypes sampled in French Guiana; the
737 yellow circles represent haplotypes from Brazilian Amazon; light green are representative of
738 Peruvian haplotype while dark green circle corresponds to the sample from Bolivia and the light
739 blue represents the haplotype from Colombia. Grey circles are the two selected outgroup species.

Table 1. mtDNA diversity estimates for the Neotropical river otter and related species.

Segment [a]	Length (bp) [b]	N	No. of haplotypes	S [c]	PI [d]	π [e]	d_{xy} (*Lontra felina*) [f]	d_{xy} (*Lontra provocax*) [f]	d_{xy} (*Lontra canadensis*) [f]	d_{xy} (*Pteronura brasiliensis*) [f]
CR	491 (438)	51	29	29 / 37	24 / 32	0.01149 ± 0.00270	0.03484 ± 0.00702	0.03763 ± 0.00776	0.05296 ± 0.00950	0.08611 ± 0.01124
ATP8/*ATP6*	329 (295)	52	15	20 / 38	6 / 24	0.00432 ± 0.00142	0.06572 ± 0.01344	0.06263 ± 0.01292	0.17632 ± 0.01968	0.20043 ± 0.02052
ND5	651 (651)	52	19	37 / 64	14 / 38	0.00741 ± 0.00177	0.05217 ± 0.00765	0.05122 ± 0.00778	0.12242 ± 0.01142	0.17166 ± 0.01410
ATP8/*ATP6* + *ND5*	980 (925)	52	24	57 / 102	20 / 62	0.00639 ± 0.00129	0.05666 ± 0.00723	0.05500 ± 0.00677	0.14026 ± 0.01081	0.18117 ± 0.01170
CR + *ATP8*/*ATP6* + *ND5*	1471 (1337)	51	37	86 / 139	44 / 94	0.00814 ± 0.00131	0.04942 ± 0.00548	0.04922 ± 0.00522	0.11125 ± 0.00743	0.14967 ± 0.00963

[a] CR= mtDNA control region; *ATP8*/*ATP6*: *ATPase subunit 8*/*ATPase subunit 6*; *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. felina* + *L. provocax*.

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

[e] π = nucleotide diversity per site.

[f] d_{xy} = mean nucleotide divergence (p-distance) between *L. longicaudis* sequences and selected outgroups.

Table 2. Nucleotide and gene diversity observed in the *Lontra longicaudis* mtDNA segments (specified separately for two different geographic groups: Eastern South America [ESA] and Amazonia).

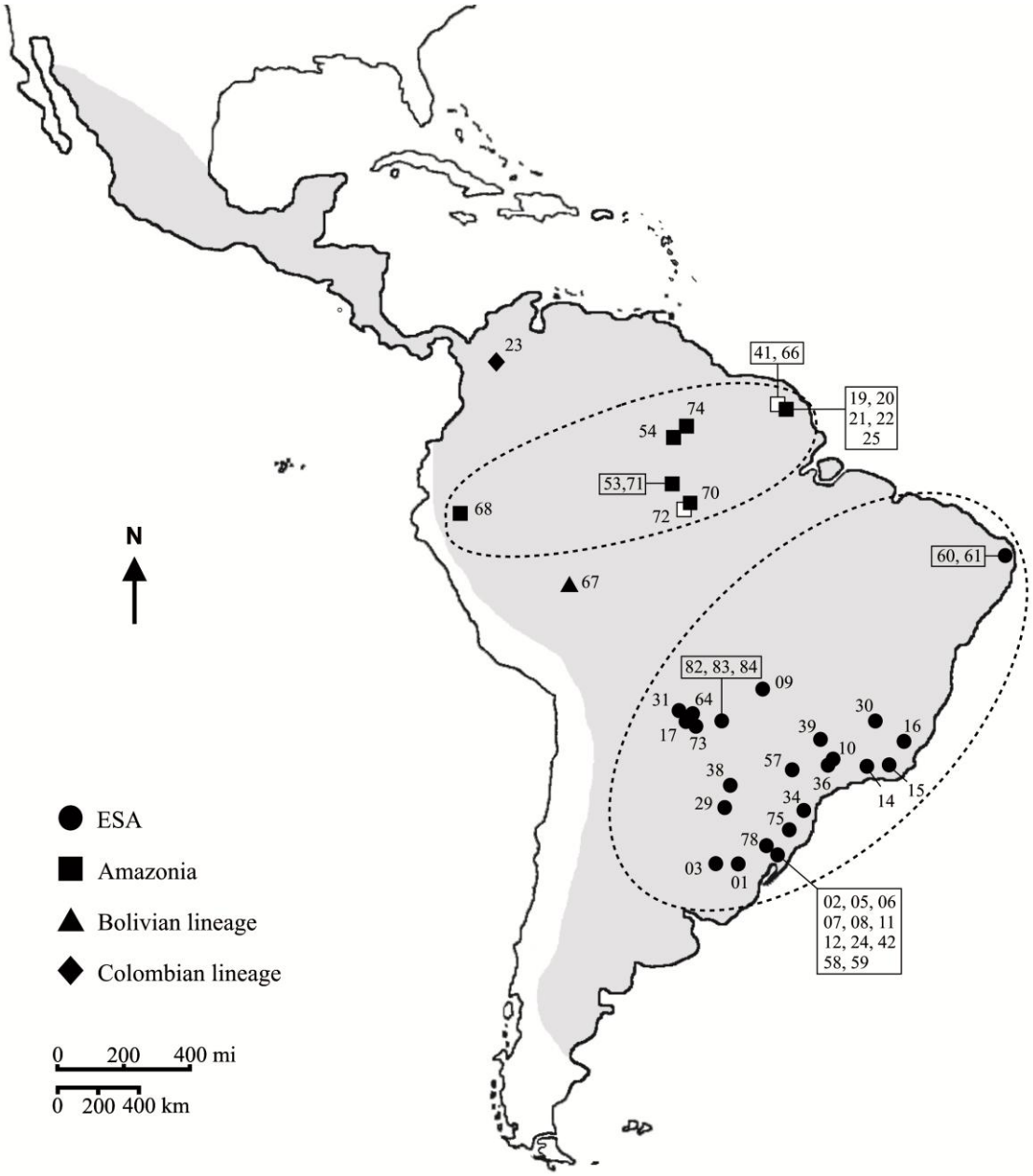
Segment	Geographic group	Nucleotide diversity (SE)[a]	Gene diversity (SE)[a]
CR	ESA	0.00620 ± 0.00180	0.8187 ± 0.00849
	Amazonia	0.01210 ± 0.00258	0.9487 ± 0.00592
	Total	0.01149 ± 0.00270	0.9043 ± 0.00477
ATP8/*ATP6* + *ND5*	ESA	0.00153 ± 0.00040	0.7873 ± 0.00896
	Amazonia	0.00586 ± 0.00124	0.8791 ± 0.01093
	Total	0.00639 ± 0.00129	0.8906 ± 0.00486
CR + *ATP8*/*ATP6* + *ND5*	ESA	0.00310 ± 0.00070	0.9361 ± 0.00408
	Amazonia	0.00822 ± 0.00133	0.9890 ± 0.00439
	Total	0.00814 ± 0.00131	0.9694 ± 0.00210

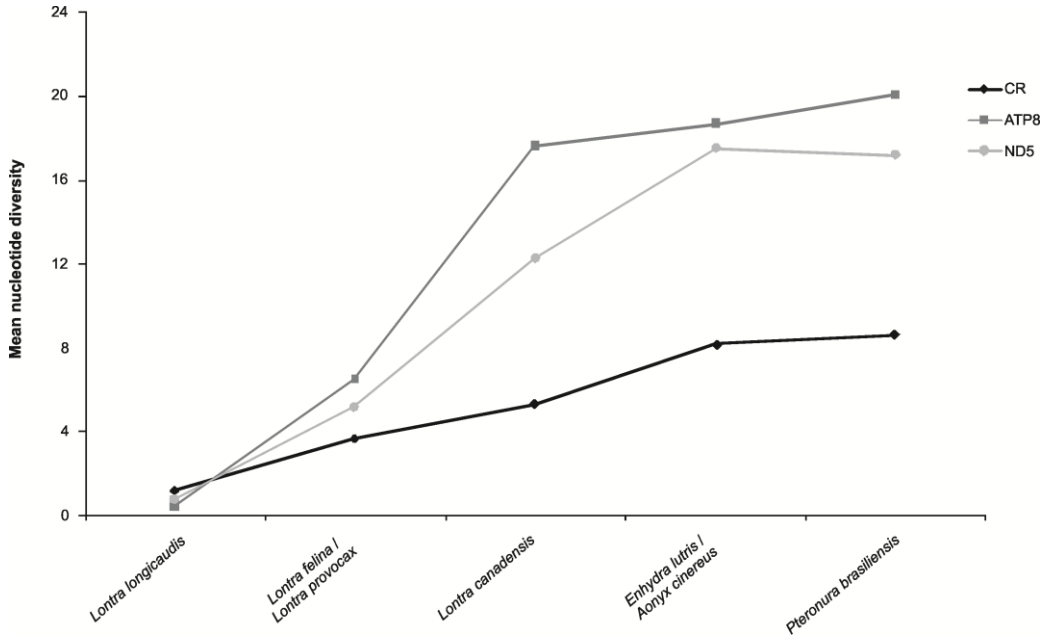
[a] calculated using *p*-distances.

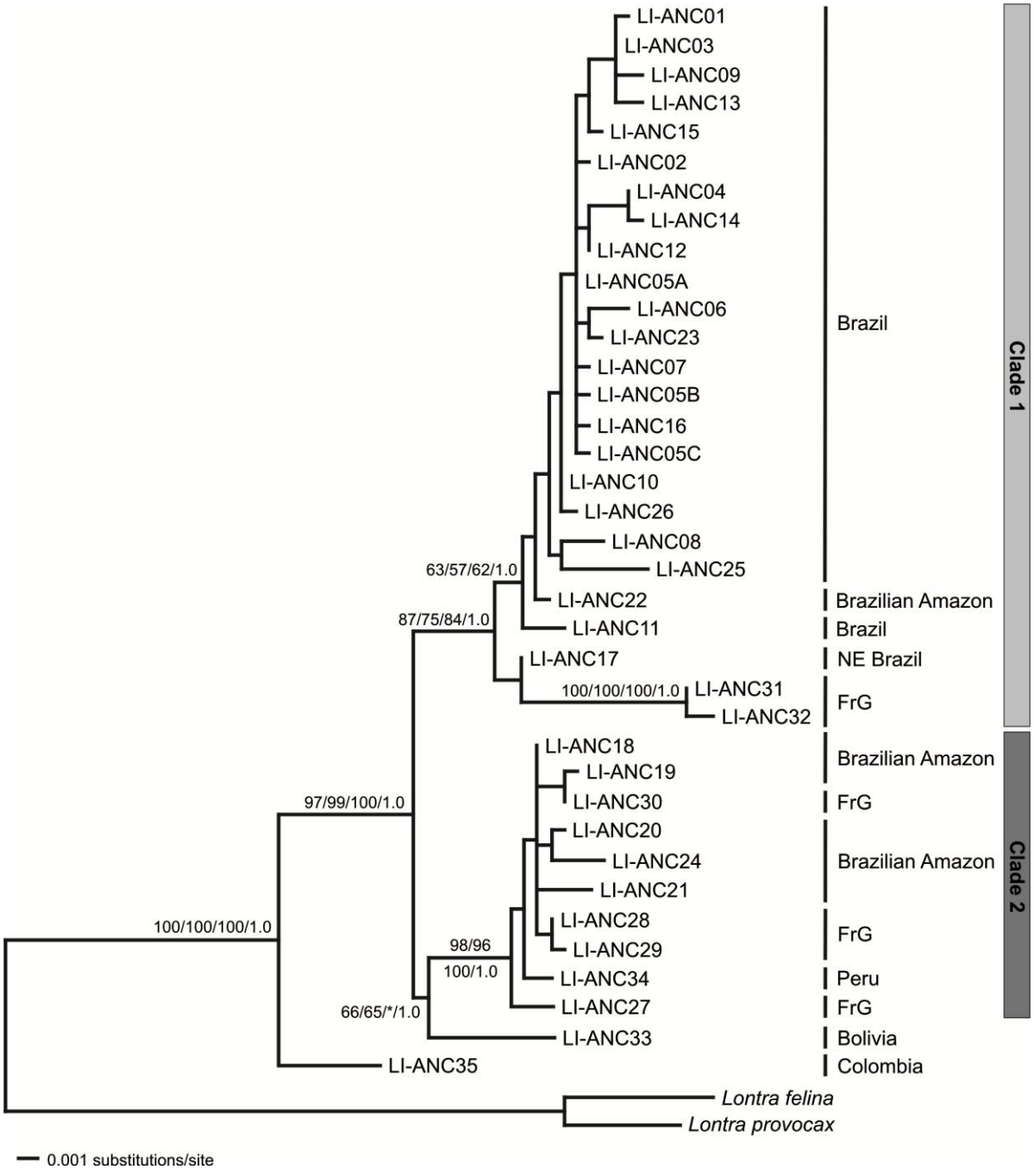
Table 3. Neutrality test results for the two data sets used in this study (DS1 and DS2), as well as different phylogenetic or geographic sub-groups (see text and Fig. 1). Numbers highlighted in bold are significant for $\alpha < 0.05$.

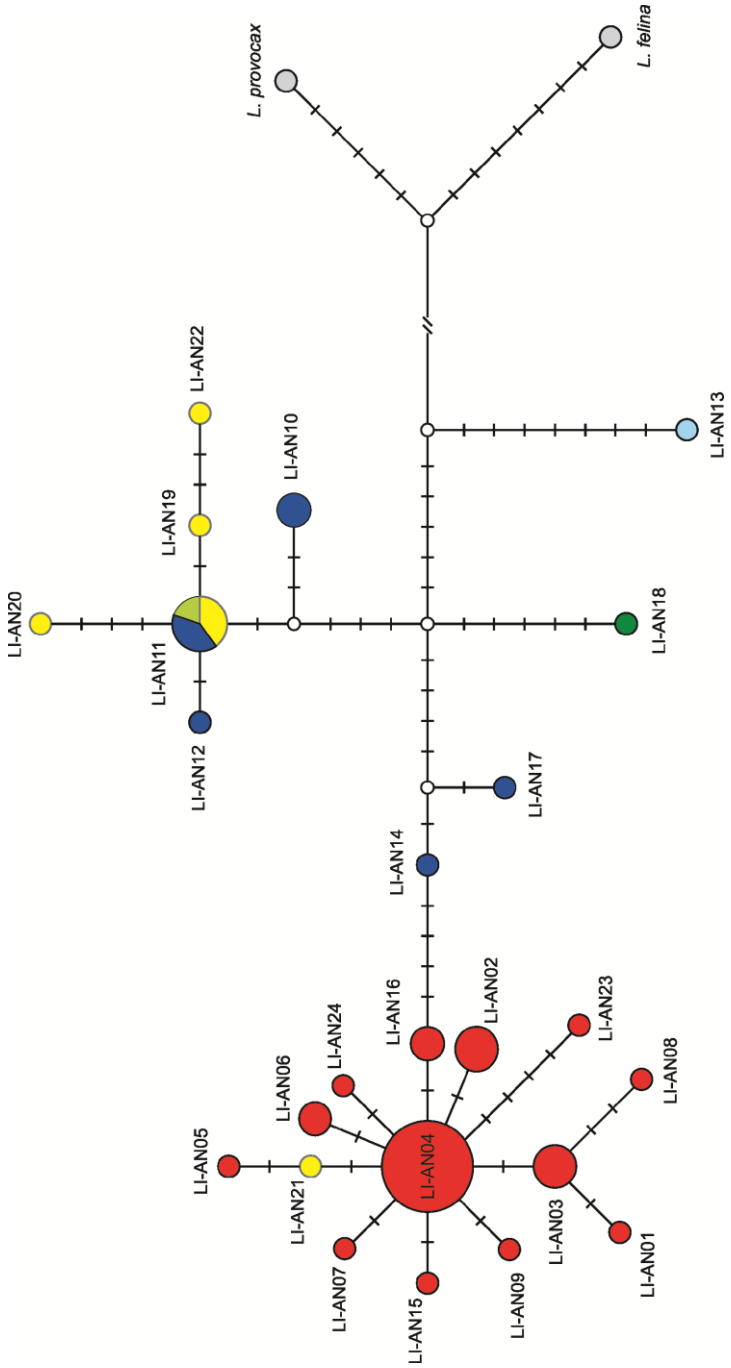
		Fu's Fs	Fu & Li's F	Fu & Li's D	Tajima's D
DS1	All Samples	**-12.869 (p = 0.000)**	**-2.310 (p > 0.05)**	**-2.337 (p > 0.05)**	**-1.254 (p > 0.10)**
	Clade 1[a]	**-10.155 (p = 0.000)**	**-1.741 (p > 0.10)**	**-1.401 (p > 0.10)**	**-1.596 (p > 0.05)**
	ESA[b]	**-10.916 (p = 0.000)**	**-2.306 (p > 0.05)**	**-2.149 (p > 0.05)**	**-1.552 (p > 0.10)**
	Clade 2	**-4.288 (p = 0.012)**	**-0.726 (p > 0.10)**	**-0.514 (p > 0.10)**	**-1.008 (p > 0.10)**
DS2	All Samples	**-7.003 (p = 0.001)**	**-3.523 (p < 0.02)**	**-3.694 (p < 0.02)**	**-2.013 (p < 0.05)**
	Clade 1[a]	**-9.654 (p = 0.000)**	**-2.751 (p < 0.05)**	**-2.410 (p > 0.05)**	**-2.156 (p < 0.05)**
	ESA[b]	**-9.981 (p = 0.000)**	**-3.189 (p < 0.05)**	**-2.987 (p < 0.05)**	**-2.124 (p < 0.05)**
	Clade 2	**-1.088 (p = 0.162)**	**-1.089 (p > 0.10)**	**-0.862 (p > 0.10)**	**-1.264 (p > 0.10)**

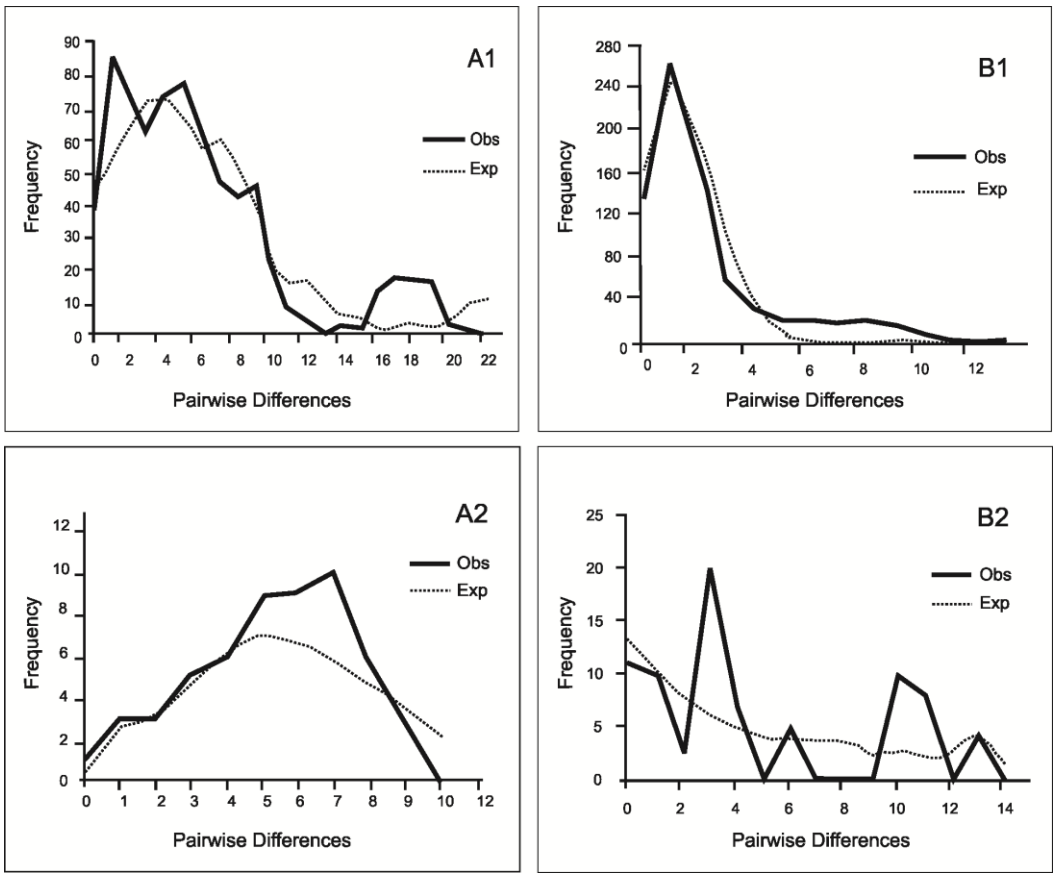
[a, b] as defined in Figs. 1 and 3.


N
ESA
Amazonia
Bolivian lineage
Colombian lineage
0 200 400 mi
0 200 400 km
23
41, 66
19, 20
21, 22
25
74
54
53,71
70
72
68
67
60, 61
82, 83, 84
09
31
64
17
73
30
16
39
10
57
38
36
15
14
34
29
75
78
03
01
02, 05, 06
07, 08, 11
12, 24, 42
58, 59

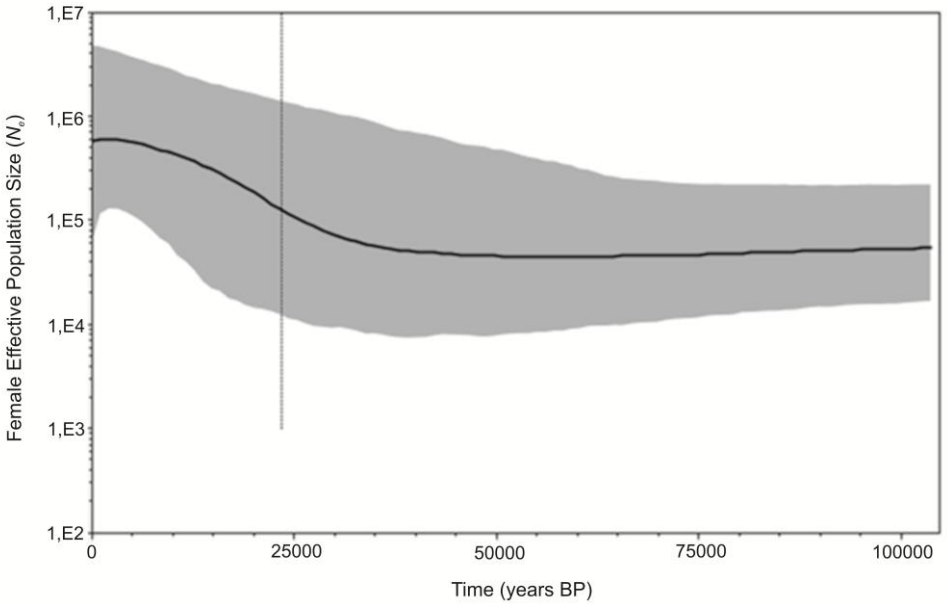

Mean nucleotide diversity
24
20
16
12
8
4
0
CR
ATP8
ND5
Lontra longicaudis
Lontra felina / Lontra provocax
Lontra canadensis
Enhydra lutris / Aonyx cinereus
Pteronura brasiliensis


LI-ANC01
LI-ANC03
LI-ANC09
LI-ANC13
LI-ANC15
LI-ANC02
LI-ANC04
LI-ANC14
LI-ANC12
LI-ANC05A
LI-ANC06
LI-ANC23
LI-ANC07
LI-ANC05B
LI-ANC16
LI-ANC05C
LI-ANC10
LI-ANC26
LI-ANC08
LI-ANC25
LI-ANC22
LI-ANC11
LI-ANC17
LI-ANC31
LI-ANC32
LI-ANC18
LI-ANC19
LI-ANC30
LI-ANC20
LI-ANC24
LI-ANC21
LI-ANC28
LI-ANC29
LI-ANC34
LI-ANC27
LI-ANC33
LI-ANC35
Lontra felina
Lontra provocax
Brazil
Brazilian Amazon
Brazil
NE Brazil
FrG
Brazilian Amazon
FrG
Brazilian Amazon
FrG
Peru
FrG
Bolivia
Colombia
Clade 1
Clade 2
63/57/62/1.0
87/75/84/1.0
100/100/100/1.0
97/99/100/1.0
100/100/100/1.0
98/96
100/1.0
66/65/*/1.0
0.001 substitutions/site


L. provocax
L. felina
LI-AN22
LI-AN10
LI-AN13
LI-AN19
LI-AN20
LI-AN11
LI-AN18
LI-AN12
LI-AN17
LI-AN14
LI-AN16
LI-AN02
LI-AN24
LI-AN23
LI-AN06
LI-AN08
LI-AN05
LI-AN04
LI-AN21
LI-AN03
LI-AN01
LI-AN07
LI-AN09
LI-AN15


A1
Obs
Exp
Frequency
Pairwise Differences
B1
Obs
Exp
Frequency
Pairwise Differences
A2
Obs
Exp
Frequency
Pairwise Differences
B2
Obs
Exp
Frequency
Pairwise Differences

A)


1,E7
1,E6
1,E5
1,E4
1,E3
1,E2
Female Effective Population Size (N_e)
0
25000
50000
75000
100000
Time (years BP)

B)

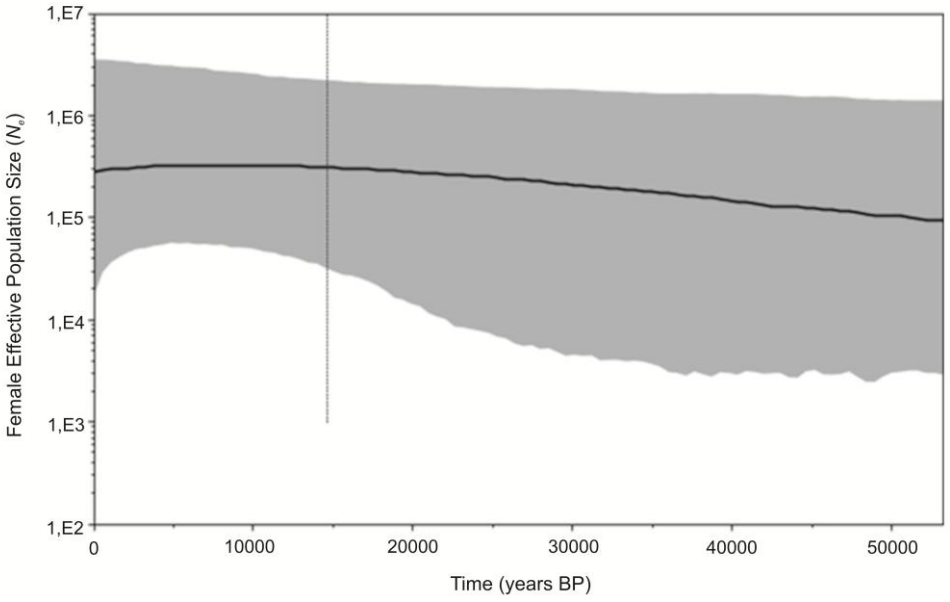

1,E7
1,E6
1,E5
1,E4
1,E3
1,E2
Female Effective Population Size (N_e)
0
10000
20000
30000
40000
50000
Time (years BP)

Table S1. Samples analyzed in the present study.

ID	Sample	Geographic origin	Geographic position	Source Institution/Contact
bLlo01	muscle	Rio Grande do Sul State, Brazil	30°16'47.84"S 53° 7'11.48"W	G. Bencke and J. R. Marinho
bLlo02	muscle	Santa Catarina State, Brazil	-	Sapucaia do Sul Zoo
bLlo03	muscle	Rio Grande do Sul State, Brazil	30°14'41.52"S 54°48'44.54"W	Carnívoros do RS Project
bLlo05	muscle	Gravataí, Rio Grande do Sul State, Brazil	29°57'11.24"S 50°59'28.47"W	G. L. Gonçalves
bLlo06	muscle	Rio Grande do Sul State, Brazil	30° 7'22.80"S 50°36'19.80"W	P. H. Ott
bLlo07, bLlo08	muscle	Torres, Rio Grande do Sul State, Brazil	29°37'2.88"S 49°57'21.18"W	P. Colombo
bLlo09	muscle	Jataí, Goiania State, Brazil	17°52'33.25"S 51°43'17.19"W	CENAP / IBAMA
bLlo10	kidney	Mogi-Mirim, São Paulo State, Brazil	22°25'55.16"S 46°57'29.36"W	CENAP / IBAMA
bLlo11	muscle	Rio Grande do Sul State, Brazil	30° 1'51.28"S 51°19'25.93"W	A. P. Brandt
bLlo12	muscle	Dois Irmãos, Rio Grande do Sul State, Brazil	29°34'53.37"S 51° 5'38.70"W	Prefeitura de Dois Irmãos / I. Fick
bLlo14	muscle	Angra dos Reis, Rio de Janeiro State, Brazil	23° 1'39.26"S 44°33'49.49"W	H. F. Waldemarin
bLlo15	muscle	Guaratiba, Rio de Janeiro State, Brazil	23° 0'21.59"S 43°35'47.35"W	H. F. Waldemarin
bLlo16	muscle	Barra, Rio de Janeiro State, Brazil	21° 7'10.11"S 41°43'24.18"W	H. F. Waldemarin
bLlo17	muscle	Pantanal, Mato Grosso do Sul State, Brazil	19°39'16.73"S 56°24'13.28"W	H. F. Waldemarin
bLlo19 - bLlo22, bLlo25, bLlo41	muscle	Cayenne region, French Guiana	4°51'13.74"N 52°27'21.59"W	B. de Thoisy
bLlo23	skin	Maceo, Antioquia, Colombia	6°33'25.19"N 74°50'23.98"W	D. Arcila
bLlo24	muscle	Rio Grande do Sul State, Brazil	29°55'59.62"S50°15'15.67"W	T. R. O. de Freitas
bLlo29	blood	Foz do Iguaçu, Paraná State, Brazil	25°32'23.15"S 54°35'28.20"W	São Paulo Zoo / K. Kassaro
bLlo30	blood	Belo Horizonte, Minas Gerais State, Brazil	19°45'19.84"S 43°55'3.39"W	São Paulo Zoo / K. Kassaro
bLlo31	blood	Corumbá, Mato Grosso do Sul State, Brazil	19° 0'28.09"S 57°39'8.29"W	São Paulo Zoo / K. Kassaro
bLlo34	muscle	Blumenau, Santa Catarina State, Brazil	26°55'4.49"S 49° 4'35.59"W	FURB / S. Althoff
bLlo36	faeces	Sumaré, São Paulo State, Brazil	22°49'19.12"S 47°15'35.82"W	Campinas Zoo / E. Ferraz
bLlo38	muscle	P.N. Ilha Grande, Paraná State, Brazil	23°25'14.97"S 53°48'35.56"W	L. Koprowski
bLlo39	skin	Ribeirão Preto, São Paulo State, Brazil	21° 8'56.12"S 47°48'14.73"W	Ribeirão Preto Zoo / M. dos Santos
bLlo42	muscle	Capela de Santana, Rio Grande do Sul State, Brazil	29°40'49.71"S 51°18'31.79"W	Canoas Mini Zoo / M. Martins
bLlo53	muscle	Novo Ayrão, Amazonas State, Brazil	2°37'36.74"S 60°56'35.58"W	LMA / F. C. W. Rosas
bLlo54	muscle	Roraima State, Brazil	2°18'50.51"N 61°19'28.54"W	LMA / F. C. W. Rosas
bLlo57	blood	Paranapanema River, Paraná State, Brazil	23° 6'9.28"S 49°47'11.59"W	L. Koprowski
bLlo58	muscle	Osório, Rio Grande do Sul State, Brazil	29°53'15.83"S 50°16'29.35"W	P. Colombo, C. Zank and G. Volkmer
bLlo59	faeces	Rio Grande do Sul State, Brazil	29°59'50.64"S 50°10'3.62"W	Ceclimar / S. B. Nakashima
bLlo60, bLlo61	blood	Recife, Pernanbuco State, Brazil	8° 2'42.89"S 34°54'8.10"W	Dois Irmãos Zoo / A. L. Brito
bLlo64	faeces	Vermelho River, Mato Grosso do Sul State, Brazil	19°36'54.35"S 56°55'20.80"W	Embrapa-Pantanal / G. Mourão
bLlo66	DNA	Cayenne region, French Guiana	4°51'13.74"N 52°27'21.59"W	K.P. Koepfli

Table S1. Continued.

ID	Sample	Geographic origin	Geographic position	Source Institution/Contact
bLlo67	DNA	Pando Department, Bolivia	10°46'44.42"S 67° 0'46.21"W	LSUMZ / K. P. Koepfli
bLlo68	DNA	Kagka River, Amazonas Department, Peru	4°22'59.98"S 78°11'48.01"W	MVZ / K. P. Koepfli
bLlo70, bLlo72	muscle	Careiro, Amazonas State, Brazil	3°48'43.64"S 60°20'43.89"W	LMA/ F. C. W. Rosas
bLlo71	muscle	Anavilhanas, Amazonas State, Brazil	2°30'1.25"S 60°43'37.87"W	LMA / F. C. W. Rosas
bLlo73	muscle	Pantanal, Mato Grosso do Sul State, Brazil	-	PUC-MG / F. R. dos Santos
bLlo74	muscle	Boa Vista, Roraima State, Brazil	2°52'23.82"N60°40'34.35"W	LMA/ Fernando C. W. Rosas
bLlo75	muscle	Urubici, Santa Catarina State, Brazil	28° 1'2.55"S 49°35'52.34"W	C. Castilho and L. G. M. de Sá
bLlo78[1]	muscle	Triunfo, Rio Grande do Sul State, Brazil	29°48'19.66"S 51°31'13.14"W	FZB-RS / M. Jardim
bLlo82, bLlo83, bLlo84	faeces	Correntoso River, Mato Grosso do Sul State, Brazil	19°29'12.98"S 55°37'12.60"W	UNIDERP / M. Muanis
Lontra felina	DNA	Quintay, Chile	33°11'37.09"S 71°41'50.18"W	PUC – Chile / J. A. Vianna
Lontra provocax	DNA	Queule River, Chile	39°12'00"S 72°55'00"W	PUC – Chile / J. A. Vianna
Lontra canadensis	liver	Florida, USA	-	LGD-NCI-NIH / M. Roelke
Aonyx cinereus	muscle	unknown	-	LGD-NCI-NIH
Enhydra lutris	muscle	Alaska,USA	-	LGD-NCI-NIH
Pteronura brasiliensis	muscle	Corumbá, Mato Grosso do Sul State, Brazil	19°36'54.35"S 56°55'20.80"W	H. F. Waldemanrin

[1] sample included only in DS2.

Table S2. List of individuals that presented each mitochondrial DNA haplotype for Data Set 1 (DS1) containing CR + *ATP8*/*ATP6* + *ND5*; and DS2, containing *ATP8*/*ATP6* + *ND5*. The geographic distribution of each haplotype is also indicated (see Supplementary Table S1 for more details on the geographic origin of each individual).

Haplotype (DS1)[a]	Haplotype (DS2)[a]	Haplotype composition[b]	Individuals	GenBank accession numbers	Country of haplotype occurrence
Ll-ANC1	Ll-AN1	A1, N1, C1	bLlo01	JQ038849, JQ038826, EU251949	Brazil
Ll-ANC2	Ll-AN2	A2, N2, C2	bLlo02, bLlo14, bLlo15, bLlo75	JQ038850, JQ038827, EU251950	Brazil
Ll-ANC3	Ll-AN3	A1, N3, C1	bLlo03, bLlo24, bLlo59	JQ038849, JQ038828, EU251949	Brazil
Ll-ANC4	Ll-AN4	A2, N3, C3	bLlo05	JQ038850, JQ038828, EU251952	Brazil
Ll-ANC5A	Ll-AN4	A2, N3, C2	bLlo06, bLlo08, bLlo12, bLlo30, bLlo34, bLlo38, bLlo58, bLlo64	JQ038850, JQ038828, EU251950	Brazil
Ll-ANC5B	Ll-AN4	A2, N3, C8	bLlo36	JQ038850, JQ038828, EU251957	Brazil
Ll-ANC5C	Ll-AN4	A2, N3, C30	bLlo83	JQ038850, JQ038828, JQ038822	Brazil
Ll-ANC6	Ll-AN5	A3, N3, C4	bLlo07	JQ038851, JQ038828, EU251953	Brazil
Ll-ANC7	Ll-AN6	A2, N4, C2	bLlo09	JQ038850, JQ038829, EU251950	Brazil
Ll-ANC8	Ll-AN7	A4, N5, C5	bLlo10	JQ038852, JQ038830, EU251954	Brazil
Ll-ANC9	Ll-AN8	A1, N6, C1	bLlo11	JQ038849, JQ038831, EU251949	Brazil
Ll-ANC10	Ll-AN4	A2, N3, C6	bLlo16	JQ038850, JQ038828, EU251955	Brazil
Ll-ANC11	Ll-AN9	A5, N3, C12	bLlo17	JQ038853, JQ038828, JQ038804	Brazil
Ll-ANC12	Ll-AN4	A2, N3, C7	bLlo29	JQ038850, JQ038828, EU251956	Brazil
Ll-ANC13	Ll-AN3	A1, N3, C17	bLlo31	JQ038849, JQ038828, JQ038809	Brazil
Ll-ANC14	Ll-AN6	A2, N4, C3	bLlo39	JQ038850, JQ038829, EU251952	Brazil
Ll-ANC15	Ll-AN15	A2, N12, C9	bLlo42	JQ038850, JQ038837, EU251958	Brazil
Ll-ANC16	Ll-AN4	A2, N3, C11	bLlo57	JQ038850, JQ038828, EU251960	Brazil
Ll-ANC17	Ll-AN16	A2, N13, C21	bLlo60, bLlo61	JQ038850, JQ038838, JQ038813	Brazil
Ll-ANC18	Ll-AN11	A7, N8, C19	bLlo53	JQ038855, JQ038833, JQ038811	Brazil
Ll-ANC19	Ll-AN11	A7, N8, C20	bLlo54	JQ038855, JQ038833, JQ038812	Brazil
Ll-ANC20	Ll-AN19	A7, N16, C24	bLlo70	JQ038855, JQ038841, JQ038816	Brazil

[a] Haplotypes with the same number and different letters (Ll-ANC5A, 5B and 5C) are collapsed into a single haplotype when all sites with missing information or indels are excluded (e.g. Fig.2).

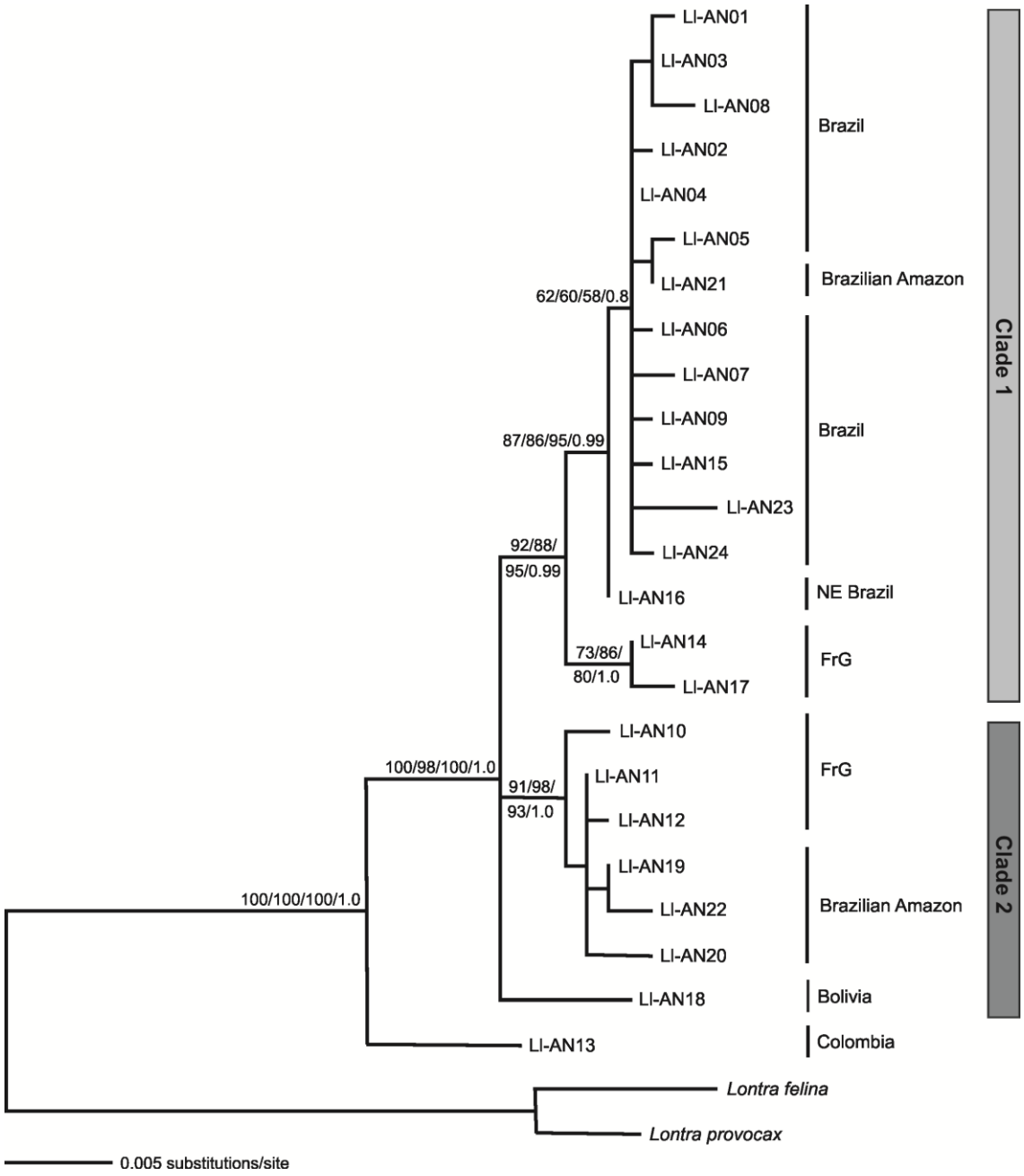
[b] Haplotypes for each mtDNA segment are numbered as in the respective GenBank entries (accession numbers JQ038804-JQ038869); e.g. Ll-A1 for the first *ATP8/ATP6* haplotype. Control region haplotypes C1 – C11 correspond to haplotypes 1-11 previously published by Trinca et al. 2007 (GenBank accession numbers EU251949-EU251960).

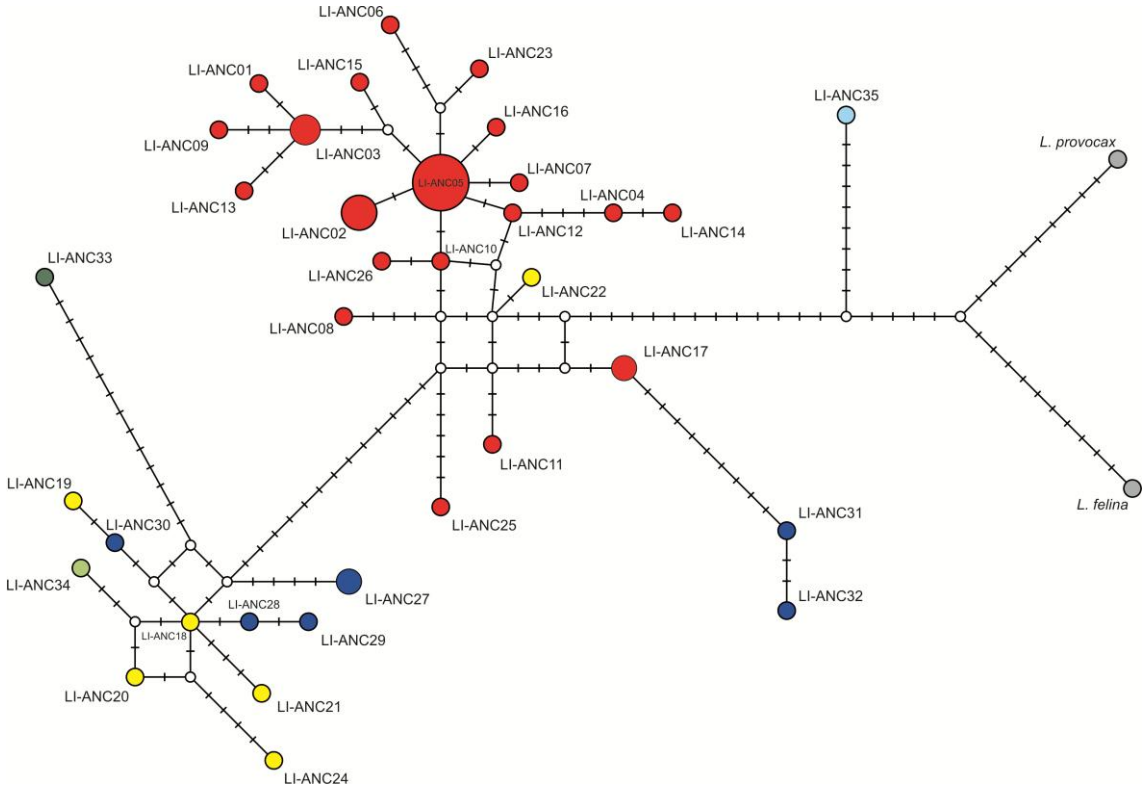
Table S2. Continued.

Haplotype (DS1)[a]	Haplotype (DS2)[a]	Haplotype composition[b]	Individuals	GenBank accession numbers	Country of haplotype occurrence
Ll-ANC21	Ll-AN20	A11, N17, C25	bLlo71	JQ038859, JQ038842, JQ038817	Brazil
Ll-ANC22	Ll-AN21	A12, N3, C26	bLlo72	JQ038860, JQ038828, JQ038818	Brazil
Ll-ANC23	Ll-AN4	A2, N3, C27	bLlo73	JQ038850, JQ038828, JQ038819	Brazil
Ll-ANC24	Ll-AN22	A13, N18, C28	bLlo74	JQ038861, JQ038843, JQ038820	Brazil
-	Ll-AN4	A2, N3	bLlo78	JQ038850, JQ038828	Brazil
Ll-ANC25	Ll-AN23	A14, N19, C29	bLlo82	JQ038862, JQ038844, JQ038821	Brazil
Ll-ANC26	Ll-AN24	A15, N3, C6	bLlo84	JQ038863, JQ038828, EU251955	Brazil
Ll-ANC27	Ll-AN10	A6, N7, C13	bLlo19, bLlo20	JQ038854, JQ038832, JQ038805	French Guiana
Ll-ANC28	Ll-AN11	A7, N8, C14	bLlo21	JQ038855, JQ038833, JQ038806	French Guiana
Ll-ANC29	Ll-AN12	A7, N9, C14	bLlo22	JQ038855, JQ038834, JQ038806	French Guiana
Ll-ANC30	Ll-AN11	A7, N8, C16	bLlo25	JQ038855, JQ038833, JQ038808	French Guiana
Ll-ANC31	Ll-AN14	A9, N11, C18	bLlo41	JQ038857, JQ038836, JQ038810	French Guiana
Ll-ANC32	Ll-AN17	A9, N14, C18	bLlo66	JQ038857, JQ038839, JQ038810	French Guiana
Ll-ANC33	Ll-AN18	A10, N15, C22	bLlo67	JQ038858, JQ038840, JQ038814	Bolivia
Ll-ANC34	Ll-AN11	A7, N8, C23	bLlo68	JQ038855, JQ038833, JQ038815	Peru
Ll-ANC35	Ll-AN13	A8, N10, C15	bLlo23	JQ038856, JQ038835, JQ038807	Colombia

[a] Haplotypes with the same number and different letters (Ll-ANC5A, 5B and 5C) are collapsed into a single haplotype when all sites with missing information or indels are excluded (e.g. Fig.2).

[b] Haplotypes for each mtDNA segment are numbered as in the respective GenBank entries (accession numbers JQ038804-JQ038869); e.g. Ll-A1 for the first *ATP8/ATP6* haplotype. Control region haplotypes C1 – C11 correspond to haplotypes 1-11 previously published by Trinca et al. 2007 (GenBank accession numbers EU251949-EU251960).


LI-AN01
LI-AN03
LI-AN08
LI-AN02
LI-AN04
LI-AN05
LI-AN21
LI-AN06
LI-AN07
LI-AN09
LI-AN15
LI-AN23
LI-AN24
LI-AN16
LI-AN14
LI-AN17
LI-AN10
LI-AN11
LI-AN12
LI-AN19
LI-AN22
LI-AN20
LI-AN18
LI-AN13
Brazil
Brazilian Amazon
Brazil
NE Brazil
FrG
FrG
Brazilian Amazon
Bolivia
Colombia
Clade 1
Clade 2
62/60/58/0.8
87/86/95/0.99
92/88/
95/0.99
73/86/
80/1.0
100/98/100/1.0
91/98/
93/1.0
100/100/100/1.0
Lontra felina
Lontra provocax
0.005 substitutions/site


LI-ANC06
LI-ANC23
LI-ANC01
LI-ANC15
LI-ANC16
LI-ANC09
LI-ANC03
LI-ANC07
LI-ANC05
LI-ANC13
LI-ANC04
LI-ANC02
LI-ANC12
LI-ANC14
LI-ANC10
LI-ANC33
LI-ANC26
LI-ANC22
LI-ANC08
LI-ANC17
LI-ANC11
LI-ANC19
LI-ANC25
LI-ANC30
LI-ANC34
LI-ANC27
LI-ANC28
LI-ANC29
LI-ANC18
LI-ANC20
LI-ANC21
LI-ANC24
LI-ANC35
L. provocax
L. felina
LI-ANC31
LI-ANC32

Capítulo 4 – Artigo II

Genetic diversity and population structure of the Neotropical otter, *Lontra longicaudis* (Carnivora, Mustelidae)

Genetic diversity and population structure of the Neotropical otter, *Lontra longicaudis* (Carnivora, Mustelidae)

Cristine S. Trinca, Benoit de Thoisy, Fernando C. W. Rosas, Eduardo Eizirik

Manuscrito em preparação a ser submetido à revista *Conservation Genetics*

Genetic diversity and population structure of the Neotropical otter, *Lontra longicaudis* (Carnivora, Mustelidae)

Cristine S. Trinca[1, 2], Benoit de Thoisy[3,4], Fernando C. W. Rosas[5], Eduardo Eizirik[2,6]

[1] Departamento de Genética, Universidade Federal do Rio Grande do Sul, Avenida Bento Gonçalves, 9500, prédio 43323, Porto Alegre, RS 91501-970, Brazil.

[2] Faculdade de Biociências, PUCRS, Avenida Ipiranga, 6681, prédio 12C, sala 172, Porto Alegre, RS 90619-900, Brazil.

[3] Kwata NGO, 16 Avenue Pasteur, F-97300, Cayenne, French Guiana.

[4] Laboratoire des Interactions Virus-Hôtes, Institut Pasteur de la Guyane, 23 Avenue Pasteur, F-97300, Cayenne, French Guiana.

[5] Instituto Nacional de Pesquisas da Amazônia – INPA, Avenida André Araújo, 2936, Manaus, AM 69060-001, Brazil.

[6] Instituto Pró-Carnívoros, Av. Horácio Neto, 1030, casa 10, Atibaia, SP 12945-010, Brazil.

Corresponding author:

Cristine S. Trinca

Running title: Neotropical otter population structure

Keywords: molecular diversity, nuclear DNA structuring, gene flow, microsatellite, carnivore, conservation genetics

Abstract

To assess levels of genetic diversity and investigate patterns of population structure of the Neotropical otter, *Lontra longicaudis*, we analyzed 10 microsatellite loci in samples encompassing a large portion of its range in South America. All populations presented moderate to high levels of genetic polymorphism, and overall low but significant levels of genetic differentiation was observed among the sampled areas. Although at a lower scale, the patterns of population structure observed with the microsatellites were congruent with those previously reported for mitochondrial DNA analyses, with the French Guiana population exhibiting the most pronounced differentiation from the other analyzed areas. Data presented here will be useful for conservation and management purposes, and may be used as a baseline for directing future local and regional scale population genetic investigations focusing on this otter.

Introduction

Genetic subdivision is a very common feature of natural populations, and it is likely that very few species behave as a single panmitic population, where mating and individual movements are uniform throughout their range (Beebee and Rowe 2008). A wide variety of nonmutually exclusive agents such as environmental barriers, historical processes and differences in life histories may shape population genetic structuring, which may result in different patterns of genetic structure in wild populations (Balloux and Lugon-Molin 2002). Also, it is common that individual dispersal capabilities are lower than the size of the species range, so that populations may also become genetically distinct by the effect isolation by distance (Balloux and Lugon-Molin 2002).

Understanding how the genetic diversity of a species is structured across its constituent populations is important in order to reach a better understanding of historical and current patterns of isolation and levels of gene flow among them (Avise 2004). Such data are useful for revealing population dynamics, as well as detecting isolated and/or threatened populations. Additionally, such information helps clarify if a given pattern of genetic subdivision is a natural feature of the species, being a result of natural geographic barriers and/or normal behavior, or caused by landscape discontinuities induced by habitat fragmentation (Mace et al. 1996, Frankham et al. 2002).

In this context, microsatellites have been widely used to investigate levels of population subdivision and fine-scale population dynamics for a broad variety of organisms (e.g de Thoisy et al. 2006, Biondo et al. 2011), as well to define target populations for conservation purposes and evaluate the success of reintroduction programs (Ferrando et al. 2008, Colli et al. 2011). Polymorphism analyses of these markers, in association with phylogeographic analyses based on mitochondrial DNA variation, have also provided significant insights into historical and recent evolutionary events (e.g. Heller et al. 2008, Urquhart et al. 2009). However, few studies have so far employed such makers for studying carnivores in the Neotropics, and even fewer (e.g. Pickles et al. 2012) have focused on semi-aquatic mammalian species from this region.

The Neotropical otter (*Lontra longicaudis*) is a semi-aquatic carnivore that is widely distributed in Latin America (Chehébar 1990), and despite being considered to be common across its range, its elusive behavior renders this species a difficult organism to study. As a consequence, this otter is one of the least known otter species worldwide, which resulted in an international categorization of this species as “Data Deficient” by the IUCN (Waldemarin and Alvarez 2008). Few studies have so far addressed the genetic diversity and patterns of population structure of this otter (Trinca et al. 2007, Weber et al. 2009, Trinca et al. [in press]). Recent analyses have indicated that this species harbors a high diversity of mitochondrial DNA (mtDNA) sequences across its range, which are geographically structured in at least four distinct evolutionary lineages (Trinca et al. 2007, Trinca et al. [in press]). However, no study has so far been focused on the large geographic scale analysis of nuclear patterns DNA polymorphism and population subdivision of *L. longicaudis*, which may reveal additional patterns of genetic structure, and provide valuable insights on the influence of hydrographic basins on the landscape connectivity.

Considering the importance in addressing these issues, and the usefulness of such data for the appropriated conservation of the Neotropical otter and its habitats, the present study aimed to analyze a set of microsatellite markers in order to investigate the genetic diversity, population structure, and effective population size of this otter across a large portion of its range. Observed results are interpreted and discussed in the light of the mtDNA structure patterns previously reported for this otter.

Materials and Methods

Sample collection

Forty-four Neotropical otter samples (blood, tissue, hair and faeces) were obtained from several localities across the species' range (Fig 1, Table 1). These samples were obtained from captive individuals with known geographical origin, or opportunistically collected by collaborators in the field. A salt-saturated solution (100mM Tris, 100mM EDTA, 2% SDS) was used to preserve blood samples, while tissue, faecal and hair samples were preserved in 96% ethanol. All samples were stored at -20°C for subsequent DNA extraction. In addition to newly collected samples, we also analyzed DNA available from 44 individuals previously used by Trinca et al. (in press) to investigate mtDNA markers, therefore making up a total set of 88 animals included in this study (Fig 1, Table 1).

DNA extraction and molecular analyses

Total DNA from blood and tissue samples was extracted using a standard Proteinase-K digestion and phenol-chloroform protocol (Sambrook et al. 1989). We used the ChargeSwitch® Forensic DNA Purification Kit (Invitrogen) to extract the DNA from hair and skin samples. Finally, faecal DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen) following the manufacturer's instructions with a slight modification in which the time for final elution was increased from one to 20 minutes. DNA extractions from faecal samples were carried out in a separate laboratory area dedicated to the analysis of low-quality DNA samples to avoid contamination with higher quality DNA from tissue/blood samples. Each batch of faecal DNA extraction included a negative control (reagents only) to monitor for contamination during the process. All procedures were performed in a UV-sterilized laminar flow hood and included the use of filtered tips in every step of the DNA extraction protocol.

As faecal samples usually provide DNA of low quality and amount, we initially screened the DNA extracted from these samples using the PCR-based mtDNA assay described by Trinca et al. (2007) to assess the presence of otter DNA, as a strategy to indicate which samples presented sufficient DNA amount to allow reliable amplification of the nuclear markers. Based on this first

step, only samples from which mtDNA PCR products could be confirmed on 1% agarose gels (stained with GelRed [Biotium]) were used in the subsequent analyses.

DNA extracts were typed by PCR for 11 microsatellite loci (Lut453, Lut733, Lut782, Lut701, 04OT02, 04OT04, 04OT05, 04OT17, 04OT22, RIO07, RIO11), developed for two other species of otters (*Lutra lutra* and *Lontra canadensis*) (Dallas et al. 1998, Beheler et al. 2004, 2005, Huang et al. 2005). Every forward primer was 5'-tailed with an M13 sequence (Boutin-Ganache et al. 2001), and used in combination with an M13 primer that had the same sequence but was dye-labeled (with FAM, NED or HEX) on its 5' end. PCR reactions were performed in 10ul reactions containing 1-3 ul of empirically diluted DNA, 1x PCR Buffer (Invitrogen), 1.5 - 4 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. In the case of faecal samples, we also added 10% Trehalose as an additive to empirically improve the PCR reaction yield. PCR conditions for all loci were as follows: 94^{o}C for 3min, 40 cycles of 94^{o}C for 45s, 58^{o}C - 66^{o}C (depending on the loci – Table 2) for 45s, 72^{o}C for 1.5min and final extension of 72^{o}C for 20 min. 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. PCR products were analyzed in a MegaBACE 1000 automated sequencer (GE Healthcare). Allele sizes were scored using Genetic Profiler 2.2. Negative controls were included in each batch of PCR reactions, and were also genotyped to monitor the presence of contamination.

Additionally, microsatellite DNA amplification from noninvasive samples (in this case, faeces) is known to present higher rates of allelic dropout (ADO) and false alleles (FA) due to the usually low quality of DNA recovered from this kind of material. In order to avoid such issues and to increase the reliability of the multilocus microsatellite profile from faecal DNA, we employed a multiple-tubes amplification approach (Taberlet et al. 1996) in which heterozygote and homozygote genotypes were determined after three and five independent amplifications, respectively. Additionally, heterozygote profiles were also defined when homozygote genotypes were observed at least twice for each constituent allele.

137 *Data analysis*

138 To evaluate the discriminatory power of our microsatellite data set, we estimated the
139 probability of identity (P_{ID}) index (*i.e.* the probability that two individuals in a population share
140 identical genotype profiles at random) using the software CERVUS 3.0 (Marshall et al. 1998). Since
141 it has been suggested that P_{ID} (which assumes Hardy-Weinberg equilibrium) may be biased, we
142 also estimated $P_{ID\ (sibs)}$, a more conservative index that accounts for individuals being siblings
143 (Waits et al. 2001). Additionally, as we obtained a few faecal samples from the same localities,
144 individual identification was performed by visually checking the final multilocus genotypes for allele
145 sharing. We also used the software MICRO-CHECKER 2.2.3 (Van Oosterhout et al. 2004) to
146 identify evidence of possible null alleles, ADO and scoring errors due to genotyping stutter peaks,
147 which might lead to erroneous estimates of population genetic parameters.

148 The total sample set was divided into four populations according to a geographic criterion
149 that took into account our previous results obtained with mtDNA data (Trinca et al. [in press]): i)
150 Southern South America (SSA, n = 55), ii) Northeastern Brazil (NB, n = 5), iii) Brazilian Amazon
151 (BA, n = 17), and iv) French Guiana (FG, n = 10) (Fig 1). Considering that we obtained a single
152 sample from Colombia (bLlo23), this individual was included only in global computations of the
153 Neotropical otter's genetic diversity, but not for population structure analyses due to its origin on
154 the Central Andean Cordillera, which did not allow its grouping with any other sampled population.

155 Global and population-specific levels of genetic diversity, as measured by the number of
156 alleles per locus (A), allelic richness (AR) and observed (H_O) and expected (H_E) heterozygosity for
157 each locus, were calculated using CERVUS 3.0, ARLEQUIN v. 3.5.1.2 (Excoffier and Lischer
158 2010), and FSTAT 2.9.3.2 (Goudet 2002). Significant departures from Hardy-Weinberg and linkage
159 equilibrium were estimated using CERVUS and ARLEQUIN. To correct for multiple comparisons,
160 Bonferroni adjustments (Rice 1989) with an original α of 0.05 were carried out for all tabulated
161 results. The number of private alleles was computed for each population following a rarefaction
162 method that compensates for uneven sample sizes, as implemented in the software HP-Rare
163 (Kalinowski 2004, 2005).

164 The degree of population genetic differentiation among the pre-defined populations was
165 estimated with F_{ST} indices estimated with an Analysis of Molecular Variance (AMOVA) approach,

as 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). The statistical significance of both F_{ST} and R_{ST} values was tested using 10,000 permutations.

Considering the current discussion over whether the traditional F_{ST} index may have undesirable attributes in some situations when estimated from highly polymorphic markers such as microsatellites (Jost 2008, Heller and Siegismund 2009), we also estimated a recently developed alternative measure of genetic differentiation, the *D* index (Jost 2008), as implemented in the software SMOGD 1.2.5 (Crawford 2010). The overall value of D_{EST} for each pairwise population comparison was calculated as the arithmetic mean across loci, following the recommendation outlined by Heller et al. (2010). These estimates were qualitatively compared to those obtained with F_{ST}, in order to evaluate the potential impact of heterogeneous levels of genetic diversity among populations on estimates of differentiation.

As an independent measure of the partitioning of genetic variation among groups, we employed the Bayesian clustering method implemented in the software STRUCTURE 2.3 (Pritchard et al. 2000). STRUCTURE uses a Markov Chain Monte Carlo (MCMC) approach to group microsatellite genotypes into *K* populations regardless of the geographic location of sampled 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. We estimated the number of genetic clusters by performing five independent runs for each value of *K* (ranging between 1 and 6) using 10^6 MCMC iterations after a burn-in of 10^5 steps, using no prior population information, assuming correlated allele frequencies and allowing admixture. We computed the arithmetic mean among the five runs and defined the optimal value of *K* as the one that presented the highest mean likelihood.

To evaluate the presence of genetic differentiation as a result of isolation-by-distance, we employed a Mantel test implemented in the program AIS 1.0 (Miller 2005) to assess the significance of the association between genetic and geographic distances. Statistical significance was tested using 1,000 random permutations. Finally, we estimated the Neotropical otter's effective population size (N_e) with the program LDNE (Waples and Do 2008), which is based on the linkage disequilibrium (LD) method and implements the bias correction of Waples (2006), suggested to

provide unbiased estimates under a wide range of sample sizes. We used the jackknife method, assumed a random mating model, and tested the data for critical values (P_{crit}) of 0.05, 0.02, and 0.01. Effective population size was estimated for each population separately as well as for the global set of samples to obtain a general value of Ne for the species.

Results

P_{ID} calculations and genetic diversity

Calculations of the probabilities of identity indicated that the selected microsatellite panel was powerful in distinguishing individuals, even in the case of siblings (P_{ID} = $2.94e^{-13}$; $P_{ID(sib)}$ = 0.000032). Of all the faecal samples analyzed in this study, only two presented the same multilocus genotype. Both were collected at the same locality in Rio de Janeiro state, Brazil, and were thus subsequently considered to represent the same individual (bLlo137).

General evaluation of the microsatellite data using MICRO-CHECKER with the full sample set indicated no evidence of genotyping errors due to large allelic dropout, but suggested the presence of null alleles for loci Lut782, 04OT02, 04OT04, 04OT05, 04OT17, and RIO07, while genotyping errors resulting from stuttering were also indicated for loci Lut782, 04OT02, 04OT04, and RIO07. In contrast, no evidence of genotyping errors was observed when we analyzed each of the four populations separately, except for loci 04OT02 and 04OT04, for which genotyping errors due to stuttering were still indicated for one population each. However, after double-checking the alleles at these loci, we did not find any evidence of such error, as all allelic peaks were clear and well defined when visualized with Genetic Profiler. Different loci in distinct populations were suggested to present null alleles: Lut782, 04OT02, 04OT04, 04OT05 and RIO07 in SSA, and RIO07 in both FG and BA. Additionally, significant departures from HWE and/or evidence for linkage disequilibrium were observed for the global data set as well as for SSA, NB, FG and BA populations even after Bonferroni adjustments. It is important to highlight that these populations represent large geographic areas (except for FG), so that these results may be an effect of small sample size in these regions, or perhaps a consequence of finer population structure within them. Nevertheless, based on the constant suggestion of the presence of null alleles and departure of HWE at locus RIO07, we decided to exclude it from subsequent analyses.

Of the resulting final microsatellite panel analyzed here, all 10 loci were polymorphic for global and population-specific data sets (Table 2). Moderate to high levels of genetic variability were observed for the entire sample set, with loci exhibiting between 5 and 20 alleles (except for locus 04OT04 that presented only three alleles) and mean expected and observed heterozygosities of 0.763 and 0.593, respectively (Table 2). Individual population analysis showed the expected heterozygosity ranging from 0.705 (FG) to 0.769 (BA), whereas observed heterozigosity varied between 0.6 (NB) and 0.749 (BA). Average allelic richness (AR) was also highest in the BA (AR = 4.858) and lowest in FG (AR = 3.434). Unique alleles could be observed in all populations; the largest sampled areas represented by the SSA and BA populations presenting the largest numbers of exclusive alleles (21 and eight, respectively), whereas a lower number of private alleles was observed in FG (5) and in NB (1). Nevertheless, when the rarefaction approach was applied, the BA population presented the highest estimate of private alleles (0.95), followed by FG (0.73), SSA (0.66), and NB (0.62).

Population structure

Global F_{ST} (0.041, p = 0.000) and R_{ST} (0.042, p =0.093) were low, whereas the overall value of D_{EST} was rather high (0.146). All pairwise F_{ST} values were significant, except the one between SSA and BA (F_{ST} = 0.0049, p = 0.25). The pairwise estimates of D_{EST} were also considerably high, whereas R_{ST} values were all non-significant, except for the comparison between SSA and FG (Table 3). The strongest pattern of population subdivision was observed in the comparisons involving the FG against the other three populations, while lower but still significant values of F_{ST} revealed the existence of genetic differentiation when NB was compared to the BA and SSA populations (see Table 3).

In contrast to the levels of genetic subdivision observed with the fixation indices, the Bayesian genetic clustering analyses performed with STRUCTURE using the 10 microsatellite loci indicated that the probability of the observed data was maximum when one population was assumed (K = 1; mean LnP(D) = -2786.26) indicating the absence of population subdivision. As the initial analysis indicated that loci Lut782 and 04OT04 might not be in Hardy-Weinberg equilibrium in the SSA population, we performed an additional STRUCTURE run excluding these loci, resulting in

253 a subset of eight loci and 81 individuals (we excluded those animals that presented a high
254 proportion of missing data after the exclusion of those two loci). The result of this analysis was
255 congruent with that obtained using the complete panel, also suggesting the most likely number of
256 genetic clusters as being K = 1 (mean LnP(D) = -2164.62). Finally, the Mantel test revealed no
257 evidence of correlation between genetic and geographic distances among populations (r^2 = 0.09, p
258 = 0.97) (Fig 2), thus indicating that the observed pattern of genetic differentiation suggested by the
259 pairwise population comparisons was not an effect of isolation-by-distance.
260
261 *Effective population size*
262 Overall, estimates of Ne at the population-specific level were somewhat imprecise (Table
263 4). Point estimates of Ne resulted in negative values for two of the four populations and/or
264 presented considerably variable values across the different $P_{(crit)}$. Also, in most of the cases, the
265 95% confidence interval did not reach a defined upper limit, thus ranging up to infinity. Only for the
266 FG population we could obtain a consistent Ne estimate among all values of $P_{(crit)}$ (23.6 [95% CI =
267 11.3 – 147.7], sample size = 10). To verify if this issue could be an effect of sample size, we
268 grouped all samples into a single population in order to estimate a general Ne for the species.
269 Although this resulted in consistently positive values, this analysis also yielded variable estimates
270 across the $P_{(crit)}$ values, and the upper 95% CI continued to ranged up to infinity (see Table 4),
271 suggesting an effect of sample size on an even broader scale.
272
273 **Discussion**
274 *Genetic diversity*
275 The overall levels of genetic polymorphism observed in the Neotropical otter were
276 moderate to high (H_e = 0.763 and mean of 10.9 alleles per locus). Almost all markers exhibited
277 considerably larger number of alleles in this species than reported for their original target species.
278 For example, RIO11 and 04OT05, originally developed for *Lontra canadensis* (Beheler et al. 2004)
279 and *Lutra lutra* (Hung et al. 2005), respectively, were described as presenting eight and four alleles
280 each, whereas in the present study we observed 15 and 20 alleles at these loci. The only exception
281 was observed at locus 04OT04, which we found to contain only three alleles in the Neotropical

282 otter, resulting in a lower polymorphism than that reported originally for *Lutra lutra* (Huang et al.
283 2005). However, estimates of observed heterozygosity varied between the values observed in this
284 study and those reported for the original species. Additionally, a recent genetic study of the giant
285 otter (*Pteronura brasiliensis*) encompassing a large portion of its range (where this species and *L.*
286 *longicaudis* are largely sympatric) analyzed 13 microsatellite loci (five of which were used here as
287 well), and reported low to moderate levels of observed heterozygosity (Pickles et al. 2012).
288 Therefore, the overall variability observed here for these microsatellite markers in *L. longicaudis*
289 highlights the usefulness of these loci in future population genetic and molecular ecology studies
290 involving this species. Additionally, our results suggest that these markers should be tested in the
291 other species of the genus *Lontra* that occur in South America, the marine otter, *Lontra felina*, and
292 the southern river otter, *Lontra provocax*, since they may also be valuable for investigating
293 population genetic issues of both species, as well as provide interesting comparisons of genetic
294 variability and patterns of population structure among all South American otters.

295 Considering the population-specific level of genetic polymorphism, all populations exhibited
296 high diversity, presenting moderate to high levels of observed heterozygosity even when only a
297 small sample size was assessed (NB and FG). In spite of its smaller sample size, the NB
298 population presented an allelic richness (AR = 4.1) that was not substantially lower than those of
299 the other populations, with the largest AR being observed for the BA region (AR= 4.858). Although
300 the Neotropical otter presented high genetic diversity at both global and population levels, it is
301 notable that part of the species' molecular variability seemed to be spatially subdivided. This is
302 illustrated by the evidence of most of populations presenting several private alleles, which generally
303 comprised the smallest and largest alleles in the allelic range, being usually much less frequent
304 than those of intermediate size, which were largely shared among regions. Such rare alleles may
305 be an effect of low migration rates once private alleles are inversely correlated to migration levels in
306 which the proportion of alleles that are exclusive decreases as migration rates among
307 subpopulations increases (Hartl and Clark 2007, Allendorf and Luikart 2007). Nevertheless, due to
308 the low frequencies of the observed private alleles, it is unlikely that they were included in the
309 genetic composition of the migrant individuals, so as having little effect on the genetic divergence
310 estimates such as those obtained by F_{ST} calculations (Hartl and Clark 2007) (see below).

311 *Population structure*

312 The non-spatial Bayesian genetic clustering analyses performed with STRUCTURE did not
313 suggest the presence of population subdivision among the sampled areas. Since this approach
314 searches for significant changes in allelic frequencies using simulations (Pritchard and Wen 2004),
315 it is possible that the subtle genetic differentiation observed among populations was below the level
316 detected by the program, especially if there were violations to HWE and linkage equilibrium within
317 the resulting clusters. Additional sampling at regional levels, as well as a through assessment of
318 the underlying reasons for the instances of equilibrium violations, may help clarify this result.

319 Interestingly, in contrast to the clustering analysis, allele frequency-based assessments
320 based on F_{ST} and D_{EST} revealed substantial population structure among the analyzed areas, which
321 was not observed to the same extent with R_{ST}. Pairwise F_{ST} values were significant among all
322 populations but for SSA and BA, ranging from 0.037 to 0.132 (see Table 3). D_{EST} tended to be
323 slightly higher than F_{ST} ranging from 0.039 to 0.22, whereas R_{ST} was lower, suggesting that genetic
324 drift may have been more significant than mutation in generating the observed differentiation
325 among populations. R_{ST} was significant only in the comparison between the SSA and FG
326 populations, which can be considered the most geographically distant areas among the sampled
327 groups. However, given the geographic distance among all four populations, the observed genetic
328 differentiation can be considered low, suggesting that some level of gene flow among the distinct
329 areas of the species' range is likely occurring. Although the dispersal capabilities of the Neotropical
330 otter are still virtually unknown, it is possible to hypothesize that since this species is strongly
331 associated to aquatic systems, its dispersal may be facilitated by the extensive hydrographic
332 network present in South America, which would ease some level of constant genetic
333 homogenization. Nevertheless, the highest F_{ST} and D_{EST} values were observed in the pairwise
334 comparisons involving the FG population, suggesting lower gene flow between this region and the
335 other sampled areas in South America, including the Brazilian Amazon. A somewhat similar pattern
336 of differentiation has been reported for the black caiman, *Melanosuchus niger* (de Thoisy et al.
337 2006) although in this study additional levels of genetic substructure were also observed within
338 French Guiana and Amazon regions, which were related to distinct habitats including the distinction
339 between 'black' and white' waters. At the same time, lower but significant estimates of F_{ST} and D_{EST}

values indicated a subtle differentiation between NB and the BA and SSA populations; however, NB was represented by only five individuals, which may have caused a spurious pattern of population subdivision. Additional sampling this and nearby areas may help clarify if the observed differentiation is real or was an artifact of low sample size. Still, F_{ST} and R_{ST} values were not significant between the SSA and BA populations, a finding which was corroborated by the observation of lowest value of D_{EST} for this pairwise comparison. This pattern suggests that population structure in the Neotropical otter is not strictly influenced by the limits of major hydrographic basins, as the SSA and BA regions comprise distinct ones.

In addition to the population structure analyses, the observed results of the Mantel test did not indicate population differentiation due to isolation-by-distance. This result is not surprising, since the observed pattern of genetic structure is not linear, and also because we observed low genetic differences in comparisons encompassing large geographic distances (e.g. SSA vs. BA) and vice-versa (e.g. FG vs. BA).

The observed patterns are in some ways similar to those reported in the mitochondrial DNA analyses conducted by Trinca et al. (in press). A genetic partition involving the FG region was also observed with the mtDNA, although in that marker most of the Brazilian Amazonian lineages shared a well-supported clade with those from FG. However, even the mtDNA showed some geographic swaps between clades, with one haplotype sampled in the Amazon phylogenetically placed in the clade comprising all the SSA samples that were used in that study. This finding, along with the observed absence of microsatellite partitioning between the BA and the SSA populations, supports the hypothesis of some level of recent or ongoing gene flow between these regions. Additionally, despite the low distinctiveness of the NB population observed here, the mtDNA analyses have indicated some level of differentiation of this region, as the lineage from northeastern Brazil (represented by only two samples in the mtDNA study) occupied a basal position in the clade comprising the SSA population (Trinca et al. [in press]).

Finally, in spite of these instances of congruence between the previous mtDNA analyses and the microsatellite results reported here, it is important to highlight that the levels of population structure revealed by the microsatellites are much lower than those reported for the mtDNA segments. We may hypothesize that such a difference results from contrasting male and female life

369 histories, in which females would present a more philopatric behavior whereas males would
370 disperse for longer distances, thus being the sex that would mostly mediate gene flow. Dispersal
371 would be facilitated by connectivity via the extensive hydrographic network of South America and
372 even by the sea. Another possibility could be related to a large effective population size of the
373 Neotropical otter, and particularly so in the nuclear genome, preventing strong effects of random
374 genetic drift. Along with the high variability, this could maintain some level of similarity among the
375 allelic frequencies in different regions, resulting in a scenario of lower nuclear genetic subdivision
376 than that observed in the mtDNA (whose effective size is four times smaller than nuclear single-
377 copy loci). It is noteworthy that both explanations are not mutually exclusive, and both may be
378 playing a role in the current scenario of otter population structure. Additional sampling, resulting in
379 a more complete geographic coverage, may help obtain a more comprehensive understanding of
380 both processes and even additional patterns of genetic differentiation among Neotropical otter
381 populations.
382
383 *Effective population size*
384 Ne estimates from our data set were problematic. The negative and/or variable values
385 obtained from both population-specific and global analysis seemed to be directly affected by
386 sample size. Negative results are suggested to be an effect of the empirical LD being lower than
387 the expected one due to inappropriate sampling, which would also tend to be associated with a
388 95% CI reaching infinity (Waples and Do 2008), as observed for almost all of the population-level
389 estimates. Our results thus suggest that a larger sampling size will be necessary for obtaining more
390 accurate estimates of the Ne for Neotropical otter populations and also for the species as a whole.
391 The only population that yielded a consistently positive estimate and a well-delimited 95%
392 CI was that from French Guiana. It is possible that, despite the small sample size available for FG,
393 those individuals may more closely represent a real population, since they were collected from a
394 small geographic area around the Cayenne region in French Guiana. The effective population size
395 estimate based on the 10 samples from FG indicated a small Ne of 23.6 individuals (95% CI: 11.3
396 – 147.7), which is about half of that suggested to be needed for preventing inbreeding depression
397 in the short term (Franklin 1980). Also, the recommended effective population size of 500-7000

(Franklin 1980, Lande 1995, Reed et al. 2003) is clearly several times larger than that estimated for FG population. The low effective population size, along with the considerably high level of genetic differentiation of this population, suggest that this population should be the focus of more detailed studies, and possibly the target of directed conservation strategies aiming to preserve its long-term viability along with its particular genetic composition.

Future directions

The results reported here indicated that the Neotropical otter presents high nuclear genetic diversity, and suggested some level of population structure that is somewhat congruent with that previously reported in mtDNA analyses. The less marked genetic subdivision observed with the microsatellites may be explained by male-biased gene flow, but may also be influenced by the different sensitivities of the two types of markers to deeper historical events. Moreover, additional genetic partitions are likely to exist among other populations of this species across its range, especially considering Central America and the northwestern portion of South America, whose deep differentiation was already hinted with the mtDNA (Trinca et al. [in press]). In this context, additional sampling efforts at different spatial scales should be pursued in order to provide a more detailed picture of the genetic diversity and population dynamics of this otter.

Finally, although the results presented here suggest that the Neotropical otter may be considered a genetically healthy species, this organism faces several levels of threat across its large geographic distribution, mainly caused by human activities such as habitat loss, water pollution, hydroelectric dams, reduction of prey availability and direct persecution. In a near future, all these factors may lead to increased habitat fragmentation and induce a non-natural population differentiation, likely affecting the genetic diversity of this otter and how it is distributed among its populations. In this context, the data set presented here can provide a baseline against which threatened populations may be assessed.

Acknowledgements

We would like to thank to all researchers and institutions listed on Table 1 for providing samples used in this study. Also, we are grateful to T. Haag, A. L. C. de Souza, and M. L. Fontoura-Rodrigues for their valuable help with data analyses. We also thank C. F. Jaeger, C. R. Sarturi, and P. M. Zamberlan for technical assistance. This study was supported by CNPq and FAPERGS.

References

Allendorf FW, Luikart G (2007) *Conservation and the genetics of populations*. Blackwell Publishing Ltd, Oxford.

Avise JC (2004) Molecular Markers, Natural History, and Evolution. Second Edition. Sinauer Associates, Sunderland, Massachusetts.

Balloux F, Lugon-Moulin N (2002) The estimation of population differentiation with microsatellite markers. Mol Ecol 11: 155-165.

Beebee TJC, Rowe G (2008) An introduction to molecular ecology. Second Edition. Oxford University Press, New York.

Beheler AS, Fike JA, Murfitt LM, Rhodes OE Jr, Serfass TS (2004) Development of polymorphic microsatellite loci for North American river otter (*Lontra canadensis*) and amplification in related mustelids. Mol Ecol Notes 4: 56-58.

Beheler AS, Fike JA, Dharmarajan G, Rhodes OE Jr, Serfass TL (2005) Ten new polymorphic microsatellite loci for North American river otters (*Lontra canadensis*) and their utility in related mustelids. Mol Ecol Notes 5: 602 - 604.

Biondo C, Keuroghlian A, Gongora J, Miyaki C (2011) Population genetic structure and dispersal in white-lipped peccaries (*Tayassu pecari*) from the Brazilian Pantanal. J Mammal 92: 267-274.

Boutin-Ganache I, Raposo M, Raymond M, Deschepper CF (2001) M13-tailed primers improve the readability and usability of microsatellite analyses performed with two different allele-sizing methods. Biothecniques 31: 25-28.

Chehébar CE (1990) Action Plan from Latin American Otters In: (eds. Foster-Turley P, Macdonald S, Mason C) Otters: A Plan for their Conservation. IUCN Otter Specialist Group.

456 Colli L, Cannas R, Deiana AM, Tagliavini J (2011) Microsatellite variability of Sardinian Pine
457 martens, *Martes martes*. Zool Sci 28: 580-586.
458 Crawford NG (2010) SMOGD: software for the measurement of genetic diversity. Mol Ecol Res 10:
459 556-557.
460 Dallas JF, Piertney SB (1998) Microsatellite primers for the Eurasian otter. Mol Ecol 7: 1248-1251.
461 de Thoisy B, Hrbek T, Farias IP, Vasconcellos WR, Lavergne A (2006) Genetic structure,
462 population dynamics, and conservation of Black caiman (*Melanosuchus niger*). Biol Conserv
463 133: 474-482.
464 Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: a new series of programs to perform
465 population genetics analyses under Linux and Windows. Mol Ecol Res 10: 564-567.
466 Ferrando A, Lecis R, Domingo-Roura X, Ponsà M (2008) Genetic diversity and individual
467 identification of reintroduced otters (*Lutra lutra*) in north-eastern Spain by DNA genotyping.
468 Conserv Genet 9: 129-139.
469 Frankham R, Balloux, JD, Briscoe, DA (2002) Introduction to conservation genetics. Cambridge
470 University Press, Cambridge.
471 Franklin IR (1980) Evolutionary change in small populations. In: (eds. Soulé ME, Wilcox BA)
472 Conservation Biology: An Evolutionary-Ecological Perspective. Sinauer Associates,
473 Sunderland.
474 Goudet J (2002) FSTAT: A Program to estimate and test gene diversities and fixation indices.
475 Lausanne: Institute of Ecology, Switzerland.
476 Hartl DL and Clark AG (2007) Principles of population genetics. Fourth edition. Sinauer Associates
477 Inc., Maryland.
478 Heller R, Lorenzo ED, Okello JB, Masembe C, Siegismund HR (2008) Mid-Holocene decline in
479 African buffalos inferred from Bayesian coalescent-based analyses of microsatellites and
480 mitochondrial DNA. Mol Ecol 17: 4845-4858.
481 Heller R, Siegismund HRS (2009) Relationship between three measures of genetic differentiation
482 G_{ST}, D_{EST} and G'_{ST}: how wrong have we been? Mol Ecol 18: 2080-2083.

483 Heller R, Okello JBA, Siegismund H (2010) Can small wildlife conservancies maintain genetically
484 stable populations of large mammals? Evidence for increased genetic drift in geographically
485 restricted populations of Cape buffalo in East Africa. Mol Ecol 19: 1324-1334.
486 Huang C-C, Hsu Y-C, Lee L-L, Li S-H (2005) Isolation and characterization of tetramicrosatellite
487 DNA markers in the Eurasian otter (*Lutra lutra*). Mol Ecol Notes 5: 314- 316.
488 Jost L (2008) G_{ST} and its relatives do not measure differentiation. Mol Ecol 17: 4015-4026.
489 Kalinowski ST (2004) Counting alleles with rarefaction: private alleles and hierarchical sampling
490 designs. Conserv Genet 5: 539-543.
491 Kalinowski ST (2005) HP-Rare: a computer program for performing rarefaction on measures of
492 allelic diversity. Mol Ecol Notes 5: 187:189.
493 Lande R (1995) Mutation and conservation. Conserv Biol 9: 782–791.
494 Mace GM, Smith TB, Bruford MW, Wayne RK (1996) An overview of the issues. In: (Smith TB and
495 Wayne RK eds) Molecular genetics approaches in conservation. Oxford University Press,
496 Oxford.
497 Marshall TC, Slate J, Kruuk LEB, Pemberton JM (1998) Statistical confidence for likelihood-based
498 paternity inference in natural populations. Mol Ecol 7: 639-655.
499 Miller MP (2005) ALLELES IN SPACE: Computer software for the joint analysis of inter-individual
500 spatial and genetic information. J Hered 96: 711-724.
501 Pickles RSA, Groombridge JJ, Zambrana Rojas VD, Ariani CV, Van Damme P, Jordan WC (2012)
502 Genetic diversity and population structure in the endangered giant otter, *Pteronura*
503 *brasiliensis*. Conserv Genet 13: 235-245.
504 Pritchard JK, Stephens P, Donnelly P (2000) Inference of population structure using multilocus
505 genotype data. Genetics 155: 945-959.
506 Reed DH, O'Grady JJ, Brook BW, Ballou JD, Frankham R (2003) Estimates of minimum viable
507 population sizes for vertebrates and factors influencing those estimates. Biol Conserv 113:
508 23–34.
509 Rice WR (1989) Analyzing tables of statistical tests. Evolution 43: 223-225.
510 Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning. Second Edition. Cold Spring Harbor
511 Laboratory Press, New York.

512 Slatkin M (1995) A measure of population subdivision based on microsatellite allele frequencies.
513 Genetics 139: 457-462.
514 Taberlet P, Griffin S, Goossens B, Questiau S, Manceau V, Escaravage N, Waits LP, Bouvet J
515 (1996) Reliable genotyping of samples with very low DNA quantities using PCR. Nucleic Acids
516 Res 24: 3189-3194.
517 Trinca CS, Waldemarin HF, Eizirik E (2007) Genetic diversity of the Neotropical otter (*Lontra*
518 *longicaudis* Olfers, 1818) in Southern and Southeastern Brazil. Braz J Biol 67: 813-818.
519 Trinca CS, de Thoisy B, Rosas FCW, Waldemarin HF, Koepfli KP, Vianna JA, Eizirik E (in press)
520 Phylogeography and demographic history of the Neotropical otter (*Lontra longicaudis*). J
521 Hered.
522 Urquhart J, Wang Y, Fu J (2009) Historical vicariance and male-mediated gene flow in the toad-
523 headed lizards *Phrinocephalus przewalskii*. Mol Ecol 18: 3714-3729.
524 Waldemarin HF, Alvarez R (2008) *Lontra longicaudis*. In: IUCN 2011. IUCN Red List of Threatened
525 Species. Version 2011.2. <www.iucnredlist.org>. Downloaded on 16 February 2012.
526 Waples RS (2006) A bias correction for estimates of effective population size based on linkage
527 disequilibrium at unlinked gene loci. Conserv Genet 7: 167-184.
528 Waples RS, Do C (2008) LDNE: a program for estimating effective population size from data on
529 linkage disequilibrium. Mol Ecol Res 8: 753-56.
530 Weber LI, Hildebrand CG, Ferreira A, Pedarassi G, Levy JA, Colares EP (2009) Microsatellite
531 genotyping from faeces of *Lontra longicaudis* from southern Brazil. Iheringia, Série
532 Zoológica 99: 5-11.
533 Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) MICRO-CHECKER: software for
534 identifying and correcting genotyping errors in microsatellite data. Mol Ecol Notes 4: 535-538.
535 Waits LP, Luikart G, Taberlet P (2001) Estimating the probability of identity among genotypes in
536 natural populations: cautions and guidelines. Mol Ecol 10: 249-256.
537
538
539
540

Table Legends

Table 1. Samples analyzed in the present study.

Table 2. Measures of genetic diversity at the 10 microsatellite loci in the four Neotropical otter populations investigated in this study.

Table 3. Pairwise F_{ST} (left number above the diagonal), D (right number above the diagonal), and R_{ST} values (below the diagonal) for the four Neotropical otter populations investigated in this study.

Table 4. Ne estimates generated with LDNE for each of the four Neotropical otter populations analyzed here, as well as for the global sample for the species.

Figure Legends

Fig 1. Map depicting the current geographic distribution (shaded area) of the Neotropical otter with approximate sample collection sites. Circles represent individuals which are colored according to the four sampled populations: i) blue: Southern South America (SSA), ii) green: Northeastern Brazil (NB), iii) yellow: Brazilian Amazon (BA), and iv) red: French Guiana (FG). The black circle represents one individual from Colombia that was only included in the genetic diversity estimates. Dotted lines are indicative of the genetic partitions observed among populations.

Fig 2. Graph depicting the estimated correlation between geographic and genetic distances of each pair of Neotropical otter samples used in this study.

Table 1. Samples analyzed in the present study.

ID	Sample	Geographic origin	Reference
bLlo01	muscle	Rio Grande do Sul State, Brazil	Trinca et al. *in press*
bLlo02	muscle	Santa Catarina State, Brazil	Trinca et al. *in press*
bLlo03	muscle	Rio Grande do Sul State, Brazil	Trinca et al. *in press*
bLlo04	muscle	Rio Grande do Sul State, Brazil	Carnívoros do RS Project
bLlo05	muscle	Gravataí, Rio Grande do Sul State, Brazil	Trinca et al. *in press*
bLlo06	muscle	Rio Grande do Sul State, Brazil	Trinca et al. *in press*
bLlo07, bLlo08	muscle	Torres, Rio Grande do Sul State, Brazil	Trinca et al. *in press*
bLlo09	muscle	Jataí, Goiânia State, Brazil	Trinca et al. *in press*
bLlo10	kidney	Mogi-Mirim, São Paulo State, Brazil	Trinca et al. *in press*
bLlo11	muscle	Rio Grande do Sul State, Brazil	Trinca et al. *in press*
bLlo12	muscle	Dois Irmãos, Rio Grande do Sul State, Brazil	Trinca et al. *in press*
bLlo14	muscle	Angra dos Reis, Rio de Janeiro State, Brazil	Trinca et al. *in press*
bLlo15	muscle	Guaratiba, Rio de Janeiro State, Brazil	Trinca et al. *in press*
bLlo16	muscle	Barra, Rio de Janeiro State, Brazil	Trinca et al. *in press*
bLlo17, bLlo18	muscle	Pantanal, Mato Grosso do Sul State, Brazil	Trinca et al. *in press*/H. F. Waldemarin
bLlo19 - bLlo22, bLlo25, bLlo41	muscle	Cayenne region, French Guiana	Trinca et al. *in press*
bLlo23	skin	Maceo, Antioquia, Colombia	Trinca et al. *in press*
bLlo24	muscle	Rio Grande do Sul State, Brazil	Trinca et al. *in press*
bLlo28	hair	Cuiabá, Mato Grosso State, Brazil	I. C. Pfeifer
bLlo29	blood	Foz do Iguaçu, Paraná State, Brazil	Trinca et al. *in press*
bLlo30	blood	Belo Horizonte, Minas Gerais State, Brazil	Trinca et al. *in press*
bLlo31	blood	Corumbá, Mato Grosso do Sul State, Brazil	Trinca et al. *in press*
bLlo34	muscle	Blumenau, Santa Catarina State, Brazil	Trinca et al. *in press*
bLlo38	muscle	P.N. Ilha Grande, Paraná State, Brazil	Trinca et al. *in press*
bLlo39	skin	Ribeirão Preto, São Paulo State, Brazil	Trinca et al. *in press*
bLlo42	muscle	Capela de Santana, Rio Grande do Sul State, Brazil	Trinca et al. *in press*
bLlo53	muscle	Novo Ayrão, Amazonas State, Brazil	Trinca et al. *in press*
bLlo54	muscle	Roraima State, Brazil	Trinca et al. *in press*
bLlo57	blood	Paranapanema River, Paraná State, Brazil	Trinca et al. *in press*
bLlo58	muscle	Osório, Rio Grande do Sul State, Brazil	Trinca et al. *in press*
bLlo60, bLlo61	blood	Recife, Pernanbuco State, Brazil	Trinca et al. *in press*
bLlo69	mucus	Manaus, Amazonas State, Brazil	CPPMA / F. C. W. Rosas
bLlo70, bLlo72	muscle	Careiro, Amazonas State, Brazil	Trinca et al. *in press*
bLlo71	muscle	Anavilhanas, Amazonas State, Brazil	Trinca et al. *in press*
bLlo73	muscle	Pantanal, Mato Grosso do Sul State, Brazil	Trinca et al. *in press*
bLlo74	muscle	Boa Vista, Roraima State, Brazil	Trinca et al. *in press*
bLlo75	muscle	Urubici, Santa Catarina State, Brazil	Trinca et al. *in press*
bLlo76	muscle	Tapes, Rio Grande do Sul State, Brazil	C. B. Kasper and M. L. Fontoura-Rodrigues
bLlo77	muscle	Campo Belo do Sul, Santa Catarina State, Brazil	Boursheid S.A Engenharia e Meio Ambiente
bLlo78	muscle	Triunfo, Rio Grande do Sul State, Brazil	Trinca et al. i*n press*
bLlo81	muscle	Itá, Santa Catarina State, Brazil	-
bLlo82	faeces	Correntoso River, Mato Grosso do Sul State, Brazil	Trinca et al. *in press*
bLlo85	muscle	Extremoz Lagoon, Rio Grande do Norte State, Brazil	UFRN / L. F. do Nascimento and F. J. L. Silva
bLlo86	muscle	Triunfo, Rio Grande do Sul State, Brazil	C. B. Kasper
bLlo87	muscle	Feliz, Rio Grande do Sul State, Brazil	C. B. Kasper
bLlo88	muscle	Canelones, Uruguay	MUNHINA / E. González
bLlo89	muscle	Tacuarembó, Uruguay	MUNHINA / E. González
bLlo90	muscle	Eldorado do Sul, Rio Grande do Sul, Brazil	L. E. Costa-Schmidt and P. Colombo
bLlo91	muscle	Uruguaiana, Rio Grande do Sul, Brazil	J. Koenemann
bLlo92	muscle	Rio Grande do Sul, Brazil	SEMA
bLlo95	blood	Ilhéus, Bahia, Brazil	IMA/ M. S. Reis and R. Veloso

Table 1. Continued.

ID	Sample	Geographic origin	Reference
bLlo96	blood	Santa Catarina, Brazil	IMA / M. S. Reis and R. Veloso
bLlo97	muscle	Pelotas, Rio Grande do Sul State, Brazil	GEMARS / R. Machado
bLlo98	muscle	Santa Fé, Argentina	F. G. Grazziotin
bLlo99	blood	Tefé, Amazonas, Brazil	IDSM / M. Marmontel
bLlo100	faeces	Itanhaém, São Paulo, Brazil	Orquidário Munic. de Santos / B. Comelli
bLlo101	faeces	Praia Grande, São Paulo, Brazil	Orquidário Munic. de Santos / B. Comelli
bLlo102	faeces	Praia Grande, São Paulo, Brazil	Orquidário Munic. de Santos / B. Comelli
bLlo106	mucus	Igarapé Preto, Amazonas, Brazil	CPPMA / F. C. W. Rosas
bLlo108	muscle	Mato Grosso State, Brazil	C. C. Cheida
bLlo111	muscle	Abreu e Lima, Pernambuco State, Brazil	P. A. C. Flores, F. Luna and A. S. Sanchez
bLlo113	muscle	Belém do Pará, Pará State, Brazil	Aquário Munic. de São Paulo / L. I. M. Moura
bLlo114	blood	Niterói, Rio de Janeiro, Brazil	Aquário Munic. de São Paulo / L. I. M. Moura
bLlo115	muscle	Guapimirim, Rio de Janeiro State, Brazil	FIOCRUZ / S. Siciliano and J. Oliveira
bLlo116	muscle	Amapá State, Brazil	UFPA / A.C. M. de Oliveira
bLlo117	faeces	São Carlos, São Paulo State, Brazil	Parque Ecológico de São Carlos / R. Miotto
bLlo118	faeces	Manaus, Amazonas State, Brazil	Parque Ecológico de São Carlos / R. Miotto
bLlo120	muscle	Rio Grande do Sul State, Brazil	GEMARS / R. Machado and P. H. Ott
bLlo121	muscle	Tramandaí, Rio Grande do Sul State, Brazil	GEMARS / R. Machado
bLlo126, bLlo127	mucus	Xingu River, Pará State, Brazil	LMA/INPA / F.C. W. Rosas
bLlo130	muscle	RSD Uatumã,Amazonas State, Brazil	S. M. Lazzarini and D. C. Ribeiro
bLlo131, bLlo134	muscle	Larivot, French Guiana	Institute Pasteur de la Guyane / B. de Thoisy
bLlo132	muscle	Mana, French Guiana	Institute Pasteur de la Guyane / B. de Thoisy
bLlo133	muscle	Matourienne, French Guiana	Institute Pasteur de la Guyane / B. de Thoisy
bLlo137	faeces	Floresta River, Rio de Janeiro State, Brazil	M. L. Rheingantz
bLlo139, bLlo140	skin	Negro River, Amazonas State, Brazil	UFMG / F. R. dos Santos

Table 2. Measures of genetic diversity at the 10 microsatellite loci in the total sample set, as well as for the four Neotropical otter populations investigated in this study.

			Global population (n = 88)					Southern South America (SSA) (n = 55)					Northeast Brazil (NB) (n = 5)					Brazilian Amazon (BA) (n = 17)					French Guiana (FG) (n = 10)				
Locus	Size range (bp)	Dye	N	A	AR	H_e	H_o	N	A	AR	H_e	H_o	N	A	AR	H_e	H_o	N	A	AR	H_e	H_o	N	A	AR	H_e	H_o
Lut453	136-146	FAM	88	6	5.829	0.698	0.671	55	6	3.904	0.761	0.726	5	2	2.000	0.533	0.800	17	4	3.126	0.629	0.647	10	4	3.553	0.616	0.600
Lut701	154-202	FAM	85	10	9.696	0.759	0.741	54	9	4.374	0.737	0.738	5	4	4.000	0.800	0.600	15	7	4.742	0.786	0.800	10	5	4.024	0.732	0.700
Lut733	150-194	NED	79	9	8.848	0.789	0.747	49	8	4.478	0.745	0.777	5	4	4.000	0.800	0.400	14	6	4.687	0.772	0.786	10	4	3.393	0.684	0.700
Lut782	162-206	NED	73	8	8.000	0.73	0.589	46	6	3.930	0.736	0.701	5	3	3.000	0.689	0.800	12	6	3.918	0.703	0.667	9	6	4.477	0.745	0.444
04OT02	142-162	HEX	73	5	5.000	0.604	0.452	46	4	3.183	0.538	0.575	5	2	2.000	0.356	0.400	11	4	3.541	0.654	0.455	10	3	2.457	0.416	0.500
04OT04	185-193	HEX	79	3	2.995	0.516	0.342	49	2	1.998	0.507	0.502	5	2	2.000	0.533	0.000	14	3	2.595	0.585	0.643	10	2	1.998	0.479	0.300
04OT05	158-250	FAM	75	20	19.917	0.896	0.720	48	18	6.790	0.839	0.891	5	5	5.000	0.844	0.800	11	10	6.604	0.900	0.818	10	8	5.889	0.868	0.600
04OT17	172-252	FAM	73	14	14.000	0.885	0.781	44	10	5.546	0.855	0.85	5	6	6.000	0.867	0.600	14	8	5.824	0.868	0.857	9	9	7.059	0.928	0.778
04OT22	143-227	FAM	84	19	18.697	0.854	0.786	52	15	5.660	0.775	0.819	5	7	7.000	0.933	1.000	16	11	6.246	0.867	0.813	10	8	5.993	0.879	0.900
RIO11	162-192	NED	85	15	14.679	0.898	0.824	53	13	6.489	0.899	0.895	5	6	6.000	0.889	0.600	16	14	7.292	0.921	1.000	10	4	3.451	0.7	0.600
Mean	-	-	-	10.9	10.766	0.763	0.593	-	9.1	4.635	0.739	0.747	-	4.1	4.100	0.724	0.600	-	7.3	4.858	0.769	0.749	-	5.3	3.434	0.705	0.612

Sample size (N), observed number of alleles (A), allelic richness (AR), observed (Ho) and expected (He) heterozigosities.

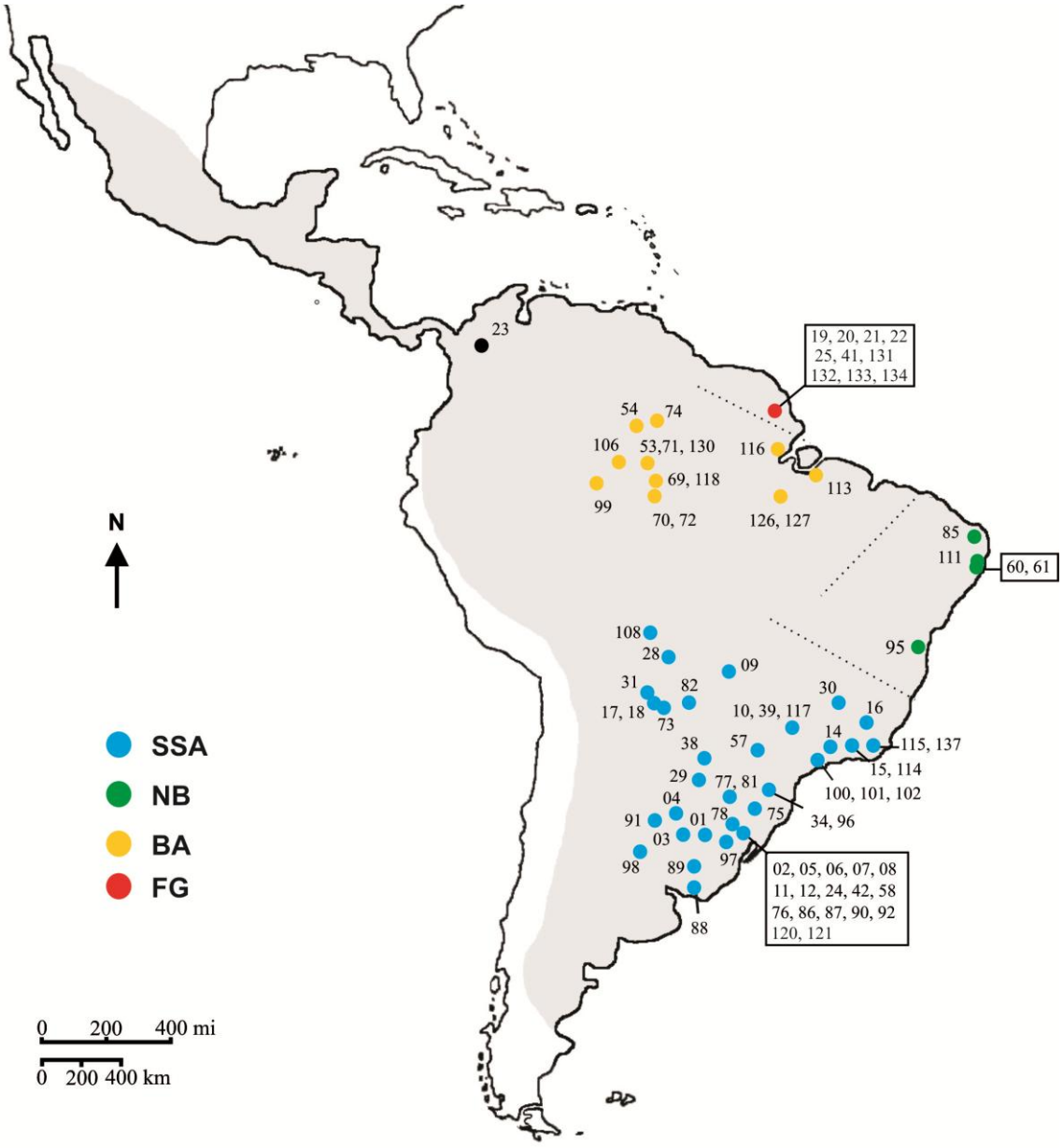
Table 3. Pairwise F_{ST} (left number above the diagonal), D (right number above the diagonal), and R_{ST} values (below the diagonal) for the four Neotropical otter populations investigated in this study.

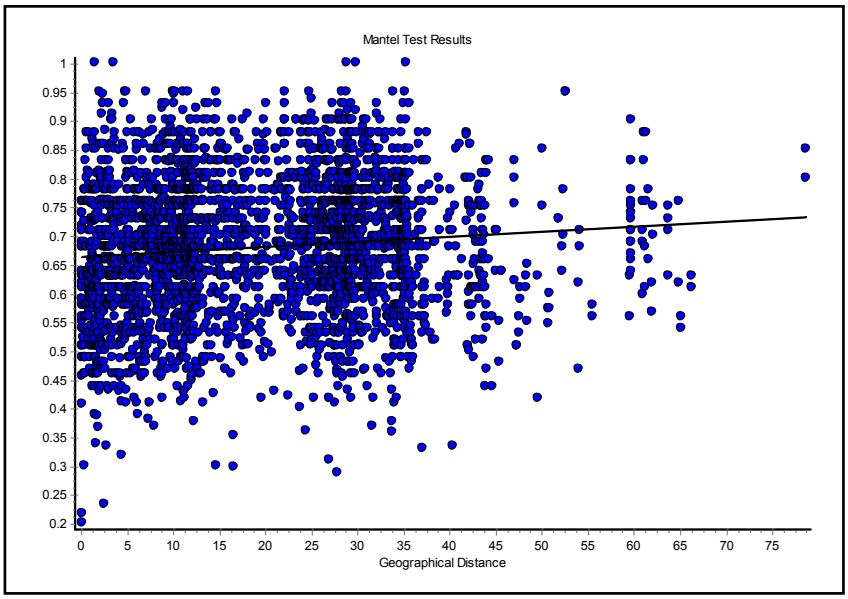
	SSA	FG	BA	NB
SSA	-	0.077**/0.13	0.005/0.011	0.037*/0.039
FG	0.086**	-	0.060**/0.14	0.132**/0.22
BA	0.028	-0.016	-	0.049*/0.062
NB	0.015	0.115	0.071	-

Significant values * $p < 0.05$, ** $p < 0.01$

Table 4. Ne estimates generated with LDNE for each of the four Neotropical otter populations analyzed here, as well as for the global sample for the species.

	LDNE Ne and 95% CI		
	$P_{(crit)}$ 0.05	$P_{(crit)}$ 0.02	$P_{(crit)}$ 0.01
SSA	543.9 (107.4 - infinity)	760.3 (157.2 - infinity)	-892.7 (170.3 - infinity)
NB	-41.6 (26.7 - infinity)	-41.6 (26.7 - infinity)	-41.6 (26.7 - infinity)
BA	-40.1 (37 - infinity)	-44.0 (263.4 - infinity)	-44.0 (263.4 - infinity)
FG	23.6 (11.3 - 147.7)	23.6 (11.3 - 147.7)	23.6 (11.3 - 147.7)
Global	864.2 (178.2 - infinity)	580.0 (207.5 - infinity)	576.5 (228.7 - infinity)


N
SSA
NB
BA
FG
0 200 400 mi
0 200 400 km
23
19, 20, 21, 22
25, 41, 131
132, 133, 134
54
74
106
53,71, 130
116
69, 118
113
99
70, 72
126, 127
85
111
60, 61
108
28
09
95
31
82
30
17, 18
73
10, 39, 117
16
14
38
57
115, 137
15, 114
29
77, 81
100, 101, 102
04
91
01
78
75
34, 96
03
98
89
97
02, 05, 06, 07, 08
11, 12, 24, 42, 58
76, 86, 87, 90, 92
120, 121
88


Mantel Test Results
1
0.95
0.9
0.85
0.8
0.75
0.7
0.65
0.6
0.55
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
Geographical Distance

Capítulo 5 – Artigo III

Molecular sexing of the Neotropical otter (*Lontra longicaudis*) noninvasive samples

Molecular sexing of Neotropical otter (*Lontra longicaudis*) noninvasive samples

Trinca CS & Eizirik E

Artigo publicado na revista *Conservation Genetics Resources*

Conservation Genet Resour
DOI 10.1007/s12686-011-9595-0

TECHNICAL NOTE

Molecular sexing of Neotropical otter (*Lontra longicaudis*) noninvasive samples

C. S. Trinca · E. Eizirik

Received: 30 September 2011 / Accepted: 21 December 2011

Abstract Noninvasive molecular assays allow the investigation of elusive, rare and/or endangered organisms, and are useful for estimates of population parameters. Sex determination of noninvasive samples provides important information of sex ratio, social structure and dispersal patterns. Still, such assays remain unavailable for most carnivore species, precluding assessments of their population biology. Here we report a standardized protocol for molecular sexing of Neotropical otter noninvasive samples collected in the field. A duplex PCR assay was employed using a multiple-tube approach, resulting in reliable gender determination for 95% of the samples and presenting a low rate (<4%) of misassignment. This assay should foster the development of molecular ecological studies of this little-known species, and likely also contribute to similar endeavors on related otters.

Keywords Sex ratio · Faeces · Mustelidae · *SRY* · Population dynamics · Conservation

C. S. Trinca (✉)
Programa de Pós-Graduação em Genética e Biologia Molecular, UFRGS, Avenida Bento Gonçalves, 9500, prédio 43323, Porto Alegre, RS 91501-970, Brazil
e-mail: cristine.trinca@gmail.com

C. S. Trinca · E. Eizirik
Laboratório de Biologia Genômica e Molecular, Faculdade de Biociências, PUCRS, Avenida Ipiranga, 6681, prédio 12C, sala 172, Porto Alegre, RS 90619-900, Brazil

E. Eizirik
Instituto Pró-Carnívoros, Av. Horácio Neto, 1030, casa 10, Atibaia, SP 12945-010, Brazil

Gender determination of wild animals is critical for understanding several aspects of population dynamics, such as mating systems, habitat use and dispersal patterns (Proctor et al. 2004; Randall et al. 2007). In elusive taxa lacking sexual dimorphism, DNA-based gender identification derived from noninvasive samples is a very useful tool for genetic, ecological and behavioral analyses (e.g. Hung et al. 2006; Zahn et al. 2007).

The Neotropical otter (*Lontra longicaudis*) is widely distributed throughout Latin America and is classified as Data Deficient by the IUCN (2011). Since ecological studies and population monitoring of this species are challenging, many aspects of its biology are still unknown. Given the difficulty in observing/capturing this species, noninvasive approaches are critical for assessment of its population dynamics. Here we report a reliable molecular sexing technique applicable to noninvasive samples (scat/anal jelly) of this otter. We also report the error rates associated with this method and suggest a multiple-tube protocol to determine gender consistently.

DNA was extracted from muscle samples from four road-killed animals (two males and two females) using a standard phenol–chloroform protocol (Sambrook et al. 1989), and from 106 scats/anal jellies using the QIAamp DNA Stool Mini Kit (QIAGEN). All batches of scat DNA extraction and PCR included a negative control (reagents only) to monitor contamination. Procedures were performed in UV-sterilized, dedicated laboratory areas free of good-quality DNA or PCR products.

Noninvasive samples were initially screened with an mtDNA assay (Trinca et al. 2007) to assess the presence of otter DNA. PCR products were checked on 1% agarose gels stained with GelRed (Biotium), and only samples that amplified for this mtDNA segment were sexed. Molecular sexing was performed in a duplex PCR using the marker

Published online: 05 January 2012

Lut-*SRY* (Dallas et al. 2000) along with the microsatellite 04OT22 (Huang et al. 2005). The latter served as a positive control which amplifies a longer segment (size range: 143–227 bp) than the *SRY* locus (70 bp). Forward primers were 5′-tailed with an M13 sequence (Boutin-Ganache et al. 2001) as described in Fontoura-Rodrigues et al. (2008).

PCRs were performed in 10 µl containing 1× Buffer, 4 mM $MgCl_2$, 200 µM dNTPs, 0.2 µM of the reverse and M13-fluorescent primer (FAM), 0.0133 µM of M13-tailed forward primers, 0.3% Trehalose, 0.1 U of *Platinum* Taq DNA Polymerase (Invitrogen) and 3 µl of DNA. PCR conditions were: 94°C for 3′, 40 cycles of 94°C for 45″, 62°C for 45″, 72°C for 1′30″ and final extension of 72°C for 20′. Genotyping was performed in a MegaBACE 1000 automated sequencer with the ET-ROX 550 (GE Healthcare). Allele sizes were called using Genetic Profiler 2.2.

We employed a multiple-tube approach (Taberlet et al. 1996) with homozygote and heterozygote profiles defined after five or three independent PCRs, respectively, to minimize genotyping errors (allelic dropout [ADO] and false alleles [FA]). This method also controlled for possible *SRY* amplification failure (false females), with a positive male identification requiring at least two independent results. In cases where three microsatellite-positive replicates presented one male and two female results, additional genotyping was performed (up to seven times), with male identification confirmed only with ≥3 corroborating genotypes.

Initial tests with the known-sex samples consistently provided the expected result regarding the amplification of the *SRY* marker. All 106 faecal samples were successfully amplified for the mtDNA segment, and then subjected to *SRY*/microsatellite amplification. Thirty-nine samples were identified as male and 62 as female, representing 36.8% and 58.5% of the total, respectively (Fig. 1). Sex of the five remaining samples (4.7%) could not be determined since amplification did not satisfy the established criterion for reliable genotyping. ADO was detected in 31.2% of the heterozygote genotypes and FA was observed in 9.74% of the amplifications. Failure in the Y fragment amplification in male DNAs occurred in 5.7% of the total PCRs. Spurious amplification of the Y fragment in female DNAs occurred in only 3.8% of the amplifications (female profile confirmed by at least three additional PCRs from the same DNA).

Despite the considerably high level of ADO observed at the microsatellite locus, the level of FA was low. Thus, the multiple-tube approach demonstrated to be critical to reduce genotyping errors. Hence, the marker set Lut-*SRY*/04OT22 proved useful for sexing of *L. longicaudis* non-invasive samples, presenting a detection rate of 95% for freshly collected samples. We suggest a minimum of three

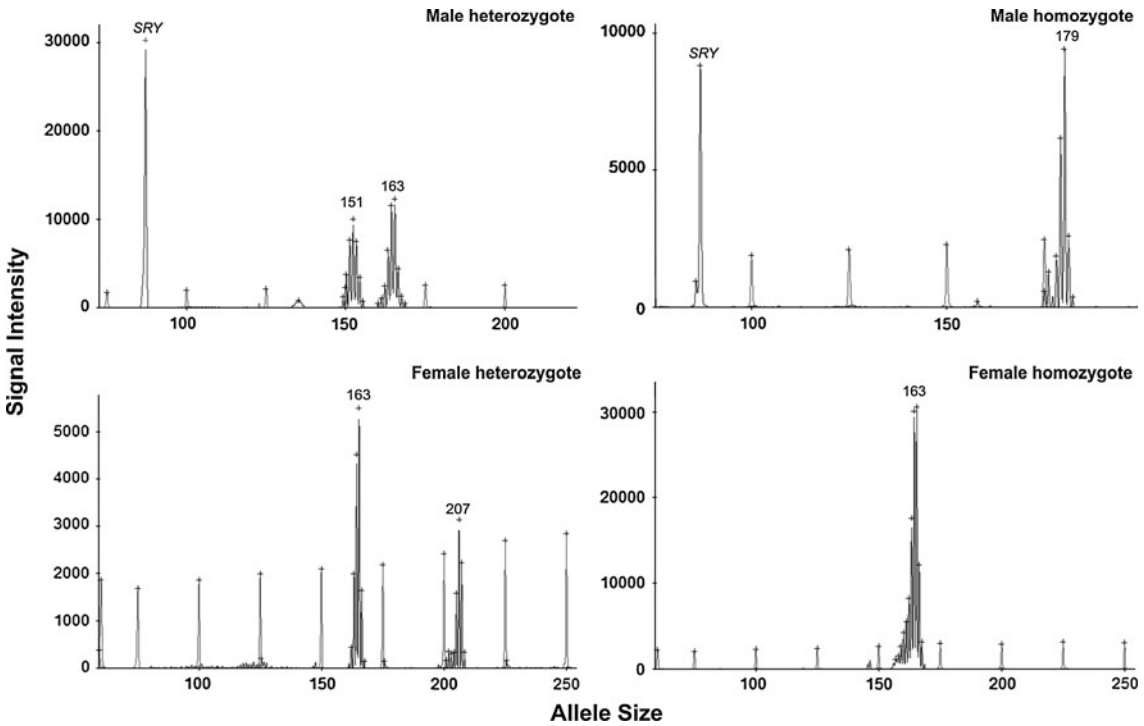


Fig. 1 Lut-*SRY*/04OT22 profiles from Neotropical otters (04OT22 alleles indicated by size [bp]). The *SRY* marker is 87 bp long (including M13 tail)

replicates, thus reducing the risk of misassignment to very low levels.

Overall, sexing of noninvasive samples is most useful when connected to individual identification. The marker set Lut-*SRY*/04OT22 can be employed along with other microsatellite loci to identify samples at individual level, and to conduct population studies. Although similar methods of sexing have already been applied for other otter species (mostly in *Lutra lutra*; Hájková et al. 2007; Arrendal et al. 2007) no previous study has focused on testing their applicability/efficacy in Latin American otters. Thus, we suggest this marker set should also be tested on the other related otter species in South America (*L. felina* and *L. provocax*) that present similar issues regarding long-term population monitoring.

Acknowledgments We thank Camila F. Jaeger for helping in DNA extractions, Cladinara R. Sarturi for laboratory assistance and Conselho Nacional de Desenvolvimento Científico e Tecnológico—CNPq (Grant number 478385/2007-2) for financial support.

References

Arrendal J, Vilà C, Björklund M (2007) Reliability of noninvasive genetic census of otters compared to field censuses. Conserv Genet 8:1097–1107

Boutin-Ganache I, Raposo M, Raymond M, Deschepper CF (2001) M13-tailed primers improve the readability and usability of microsatellite analyses performed with two different allele-sizing methods. BioTechniques 31:25–28

Dallas JF, Carss DN, Marshall F et al (2000) Sex identification of the Eurasian otter *Lutra lutra* by PCR typing of spraints. Conserv Genet 1:181–183

Fontoura-Rodrigues M, Lima-Rosa CA, Tchaicka L et al (2008) Cross-amplification and characterization of 13 tetranucleotide microsatellites in multiple species of Neotropical canids. Mol Ecol Res 8:898–900

Hájková P, Pertoldi C, Zemanová B et al (2007) Genetic structure and evidence for recent population decline in Eurasian otter populations in the Czech and Slovak Republics: implications for conservation. J Zool 272:1–9

Huang C, Hsu Y, Lee L, Li S (2005) Isolation and characterization of tetramicrosatellite DNA markers in the Eurasian otter (*Lutra lutra*). Mol Ecol Notes 5:314–316

Hung C-M, Li S-H, Lee L-L (2006) Faecal DNA typing to determine the abundance and spatial organisation of otters (*Lutra lutra*) along two stream systems in Kinmen. Anim Conserv 7:301–311

IUCN (2011) IUCN red list of threatened species. Version 2011.1. http://www.iucnredlist.org. Downloaded on 29 Sept 2011

Proctor MF, McLellan BC, Strobeck C, Barclay RMR (2004) Gender-specific dispersal distances of grizzly bears estimated by genetic analysis. Can J Zool 82:1108–1118

Randall DA, Pollinger JP, Wayne RK, Tallents LA, Johnson PJ, Macdonald DW (2007) Inbreeding is reduced by female-biased dispersal and mating behavior in Ethiopian wolves. Behav Ecol 18:579–589

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning, 2nd edn. Cold Spring Harbor Laboratory Press, New York

Taberlet P, Griffin S, Goossens B (1996) Reliable genotyping of samples with very low DNA quantities using PCR. Nucleic Acids Res 24:3189–3194

Trinca CS, Waldemarin HF, Eizirik E (2007) Genetic diversity of the Neotropical otter (*Lontra longicaudis* Olfers, 1818) in Southern and Southeastern Brazil. Braz J Biol 67:813–818

Zahn XJ, Zhang ZJ, Wu H et al (2007) Molecular analysis of dispersal in giant pandas. Mol Ecol 16:3792–3800

Capítulo 6 – Artigo IV

Molecular ecology of the Neotropical otter (*Lontra longicaudis*):
noninvasive sampling yields insights into local population dynamics

Molecular ecology of the Neotropical otter (*Lontra longicaudis*): noninvasive sampling yields insights into local population dynamics

Trinca CS, Jaeger CF, Eizirik E

Manuscrito em preparação a ser submetido à revista *Molecular Ecology*

Molecular ecology of the Neotropical otter (*Lontra longicaudis*): noninvasive sampling yields insights into local population dynamics

Cristine S. Trinca [1,2], Camila F. Jaeger [2] & Eduardo Eizirik [2,3]

[1] Departamento de Genética, Universidade Federal do Rio Grande do Sul, Avenida Bento Gonçalves, 9500, prédio 43323, Porto Alegre, RS 91501-970, Brazil.

[2] Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul, Avenida Ipiranga, 6681, prédio 12C, sala 172, Porto Alegre, RS 90619-900, Brazil.

[3] Instituto Pró-Carnívoros, Atibaia, São Paulo, Brazil.

Corresponding author:
Cristine S. Trinca
Address : Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul, Avenida Ipiranga, 6681, prédio 12C, sala 172, Porto Alegre, RS 90619-900, Brazil.
Email: cristine.trinca@gmail.com

Running title: Molecular ecology of the Neotropical otter

Keywords: Mustelidae, faecal DNA, microsatellites, population biology, otter monitoring, Neotropics

Abstract

Noninvasive genetic analysis has been successfully employed to estimate ecological and population parameters for many elusive and/or threatened species which may not be directly addressed by traditional methods. However, none of the Neotropical carnivores has so far been targeted by such studies. The Neotropical otter (*Lontra longicaudis*) is a poorly-known species for which local levels of genetic diversity and demographic parameters are virtually absent. We employed noninvasive sampling and amplification of nuclear microsatellites to characterize the genetic diversity and estimate population size, spatial organization, sex ratio, and relatedness among individuals of a wild otter population in an Atlantic forest area in southern Brazil. We identified 28 individuals which presented high levels genetic polymorphism. By using a capture-recapture method we observed larger male home ranges and several instances of spatial overlap. We were also able to reconstruct the genealogical relationships among several individuals, allowing inferences on social structure and dispersal patterns. Female close relatives presented a stronger trend for stream fidelity, suggesting a more pronounced philopatric behavior in this sex. Our results reinforced the power of noninvasive genetic sampling to investigate the population dynamics of elusive organisms, allowed the first glimpses into details of the Neotropical otter's population biology, and opened up new avenues for ecological and demographic studies of Neotropical carnivores.

Introduction

Obtaining reliable estimates of population size, temporal and spatial organization and mating systems is an important component in determining species conservation status and provides a baseline for evaluating demographic change and/or conservation success over time. However, estimating such parameters may be very difficult for rare, elusive and/or threatened species. For carnivores, population estimates such as census size and density are commonly obtained by counting tracks, active holts, and, when possible, direct observation (Gese 2001). For example, in many cases, traditional census methods using direct counting are not feasible either by the secretive behavior of the target species or lack of morphological marks that allows the distinction of individuals. Also, capture-mark-recapture techniques have been used (Gese 2001)

but may be inefficient due to low capture rates and the risk of injuries during handling (Mills et al. 2000). As a consequence, most of the world's carnivores remain virtually unknown with respect to their population biology, posing a serious obstacle to the design of adequate conservation strategies on their behalf.

In the last decade, noninvasively collected samples such as faeces and hairs have proved to be a reliable source of DNA and have become a promising alternative for conducting population studies, thus providing the opportunity to work at the individual level without the need to directly observe the animals (Taberlet et al. 1996, Taberlet & Luikart 1999). By intensively sampling a given area, it is possible to identify distinct individuals, and thus to estimate density and minimum population size (Kohn et al. 1999, Frantz et al. 2003). In addition, when associated with gender determination, individual genotypes enable the assessment of sex ratio, spatial organization, dispersal patterns and mating systems of a target species (Constable et al. 2001, Palomares et al. 2002, Dallas et al. 2003, Hung et al. 2004, Zhan et al. 2007). Finally, noninvasive genetic approaches have also been employed for monitoring a variety of wild mammal populations (Eggert et al. 2003, de Barba et al. 2010, Brøseth et al. 2010,) and have been reported to provide more precise data on population size, kinship and individual movements than conventional ecological methods (Arandjelovic et al. 2010, Arrendal et al. 2007).

The successful application of noninvasive molecular techniques to assess population-level aspects of some carnivores (e.g. Adams & Waits 2007, Lanszki et al. 2008, Koelewijn et al. 2010) suggests their potential usefulness for investigating species for which virtually no detailed information is available. This is the case of most Neotropical carnivores, none of which has so far been the target of such studies. The Neotropical otter (*Lontra longicaudis*) is a typical organism for which ecological and demographic studies are difficult to conduct since it is secretive, rarely observed, and very difficult to trap, mark and recapture. In addition, it is believed to occur at low densities and faces several threats such as habitat fragmentation/degradation, declining prey populations, human persecution and other anthropogenic impacts (Macdonald & Mason 1990) leading to a perceived decline across its range (Waldemarin & Alvarez 2008).

Currently, most of our knowledge on its ecology derives from studies of habitat use and diet, which are based on shelter and latrine monitoring and/or fecal prey content analyses that do

not need specific individual information (e.g. Chemes et al. 2010, Gallo-Reynoso et al. 2008, Kasper et al. 2008). Studies on *L. longicaudis* involving molecular approaches are few and mainly focused on the characterization of genetic diversity and analyses of population structure (Trinca et al. 2007, Weber et al. 2009, Trinca et al. [in press]). Some aspects of the Neotropical otter's behavior facilitate the application of noninvasive sampling, such as the usual defecation in distinctive and conspicuous places. This is believed to be a consequence of the role of faecal deposition in intra-specific scent communication (Kruuk 2006). Additionally, as a semi-aquatic mammal, it is strongly connected to aquatic environments, which allows the sampling strategy to be focused on linear surveys along watercourses. This is a methodological advantage when compared to organisms that range across large, two-dimensional areas (e.g. elephants, bears, and coyotes), thus requiring different strategies to allow for extensive population coverage while sampling.

However, despite the ease of collection of samples in the field, genetic analyses based on faecal DNA can be challenging, especially when working with nuclear microsatellite markers. Reported amplification success rates are highly variable and frequently low (e.g. Ernest et al. 2000, Prigioni et al. 2006a). Also, genotyping errors are common mainly due to the low quality and/or quantity of DNA that is recovered from noninvasively collected samples, potentially leading to overestimation of the number of individuals, a bias that would affect all subsequent inferences (Creel et al. 2003). There are currently several methods that aim to minimize and monitor such sources of error, including optimization of sampling and laboratory procedures and the widely employed multiple-tube approach (Taberlet et al. 1996).

In the present study, we aimed to test the applicability of noninvasive molecular techniques for studying demographic and ecological aspects of *Lontra longicaudis*. We conducted for the first time an intensive noninvasive genetic sampling of this species, which was based on the collection of faeces and anal jellies in an area of the Atlantic forest in southern Brazil. Our specific goals were to estimate: i) the minimum number of otters in the study site, ii) otter density, iii) the minimum home range for each resident individual, iv) the spatio-temporal organization, v) sex ratio, and vi) the relatedness among the identified animals.

Materials and Methods

Study area and sampling

The study site was located in the Maquiné valley, an Atlantic forest area in northeastern Rio Grande do Sul state, southernmost Brazil (Fig. 1). The Maquiné valley comprises an area of *ca.* 546 km^2 and is located in the transition between the rugged Serra Geral and the coastal plain, resulting in a landscape mosaic comprising mountains and lowlands. This area presents a rich and complex hydrographic network (strongly influenced by high pluviometric levels) composed mainly by narrow and shallow rivers and streams, with deep ponds occasionally distributed over the watercourses. Vegetation is characterized by different stages of succession, including both preserved and disturbed areas. Human influence is mainly due to agricultural use and small-scale livestock activities. Some negative impacts can be observed across the area, including water pollution by pesticides and domestic waste, silting of rivers, and removal of riparian vegetation, especially in the lower portions of the streams.

Despite these impacts, the region still sustains a considerable diversity of wildlife, and the presence of many carnivores is confirmed by the detection of vestiges and occasional sightings. However, poaching is known to occur in the region, which brings an additional threat to the persistence of these and other wildlife species in the area. Among carnivores, the Neotropical otter (*L. longicaudis*) is commonly observed by local residents, with tracks, faeces and potential dens widely distributed across the watercourses.

We performed an intensive sampling in three streams of the Maquiné region: i) Forqueta, ii) Lajeado, and iii) Encantado (Fig. 1). We regularly surveyed approximately 10 km of each stream, yielding a total linear length of *ca.* 30 km per sampling occasion. Field expeditions were conducted approximately every two months for two years (from January 2008 to December 2009; Table 1), totaling 10 sampling occasions. Each stream was divided into a set of sampling sites, with standardized starting points spaced *ca.* 1km from each other, resulting in a total of 28 collection sites (river stretches). For each site, we surveyed both banks of the stream for 30 minutes. The exact length of the surveyed stretch varied among sites and among sampling occasions, due to changes in water level and bank accessibility that altered the walking speed. The timer was

136 stopped every time an otter sign was encountered, so that the number of vestiges would not bias
137 the surveyed length traveled for a given stretch.

138 Within each sampling stretch, we collected all faeces or anal jellies that could be
139 categorized, based on field experience, as 'fresh' (0-1 day of deposition) or 'relatively fresh' (1-2
140 days after deposition). The location of each sample was recorded using Global Position System
141 (GPS). Approximately one third of each scat was collected with disposable gloves and placed in a
142 50 ml tube containing 96% ethanol. Anal jellies were collected with a sterile scoop made from cut
143 pipette tips, and also placed in 96% ethanol. All samples were kept chilled in a cooler with ice
144 during the collection, and frozen upon arrival at the field base at the end of each day. Samples
145 were then taken to the laboratory and stored at -20^{o}C for subsequent DNA extraction.

146

147 *Sample availability and temporal genotyping success*

148 Considering that defecation behavior of otters in the study area could vary among seasons
149 due to temperature, prey availability and mating periods, we categorized each sampling occasion
150 according to its respective season to test for the presence of any trend in sprainting activities
151 across the two years of the study. We also tested for differences in sample availability related to
152 the level of human-induce habitat disturbance. We divided the total sampled length of each stream
153 into three groups of sampling sites according to perceived level of habitat preservation, from the
154 least to the most disturbed: i) sampling sites 1 – 3, ii) sites 4 – 6, and iii) sites 7 – 10. Finally, we
155 assessed the proportion of collected samples that could be successfully genotyped in each season,
156 so as to test the possibility that temperature variation affects DNA integrity and microsatellite
157 amplification efficiency. Given the very small sample size obtained for the two spring season
158 occasions (numbers V and IX, respectively – Table 1) due to inclement weather (see Discussion),
159 they were excluded from these particular statistical comparisons.

160

161 *Molecular analyses*

162 Faecal DNA was extracted using the QIAamp DNA Stool Mini Kit® (Qiagen) following the
163 manufacturer's instructions with a slight modification, in which the time of elution was increased
164 from 1 to 20 minutes at room temperature. DNA extractions were performed in laboratory areas

dedicated to low-quality DNA samples, in UV-sterilized hoods and with the use of barrier tips throughout the process to avoid contamination. Every batch of DNA extraction included one negative control (reagents only) in order to monitor for contamination with exogenous DNA.

The detection of genomic DNA extracted from noninvasive samples on agarose gels does not necessarily reflect the presence of target-species DNA due to its mixing with DNA from prey items, fungi and/or bacteria. Therefore, as a quality control step for the DNA extractions, we employed a diagnostic PCR (Polymerase Chain Reaction) targeting a short mtDNA segment, as described by Trinca et al. (2007). If the presence of otter DNA was confirmed by this PCR-based assay, samples were screened for microsatellite loci to identify them at the individual level.

Eight microsatellite loci developed for two different otter species (Lut453, Lut733, Lut701, Lut782, 04OT4, 04OT17, 04OT22 [*Lutra lutra* - Dallas & Piertney 1998, Huang et al. 2005], and RIO11 [*Lontra canadensis* - Beheler et al. 2005]) were selected to conduct the individual-based analyses. We specifically targeted loci whose allelic size range in the original species was $\leq$ 250 base pairs (bp), so as to minimize the chance of amplification difficulties with low-quality DNA. We initially characterized the properties of these loci in *L. longicaudis* using a set of 28 good-quality DNA samples (obtained from blood or tissue; Supporting Information T1) collected from wild populations in southern Brazil. This assessment allowed us to determine the amplification efficiency, product size range and allelic diversity for each of these markers in this particular otter species. In addition to the microsatellite markers, we also employed a molecular sexing method for *L. longicaudis* (Trinca & Eizirik 2012), which uses a combination of microsatellite locus 4OT22 and a fragment of the *SRY* gene originally described for *Lutra lutra* (Dallas et al. 2000).

For every locus, the forward primer was 5'-tailed with an M13 sequence, and used in combination with a dye-labeled (FAM or NED) M13 primer (Boutin-Ganache et al. 2001). PCR reactions for all microsatellite loci using DNA from blood/tissue samples were performed in 10 ul containing 1x PCR buffer (Invitrogen), 2.5 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, 0.1 - 0.5 unit of Platinum Taq DNA Polymerase (Invitrogen), and 10-50 ng of genomic DNA. In the case of faeces/anal jellies samples, PCR reactions were essentially the same, except for the use of 4 mM $MgCl_2$, 3ul of DNA extract, and the inclusion of 0.3 ul of 10% Trehalose. The reaction profile for all loci was the

following: 94°C for 3', 40 cycles of 94°C for 45", 58°C - 66°C (depending on the locus; Table 2) for 45", 72°C for 1.5' and final extension of 72°C for 20'. Negative controls were included in each PCR batch to check for contamination. Faecal DNA was added to the PCR reaction in a separate laminar flow hood (distinct from that used for DNA extraction) which was UV-sterilized every time before DNA manipulation.

PCR reactions were carried out for each locus separately (except for 04OT22 and *SRY*), and products from two loci were diluted and pooled together based on fluorescent dye (one FAM and one NED), and then analyzed in a MegaBACE 1000 automated sequencer (GE Healthcare). Allele fragment sizes were scored using Genetic Profiler 2.2 software (GE Healthcare). Microsatellite genotypes are available at Dryad (XXXXX).

Data quality control

Genotyping errors associated with noninvasive analysis can lead to under- or overestimation of individuals and can result in biased population estimates. In order to minimize these problems, we employed a multiple-tube approach (Taberlet et al. 1996) where homozygote and heterozygote genotypes were defined after five and three independent and congruent PCR results, respectively. Heterozygote profiles were also identified when each of its alleles was observed at least twice. Special attention was devoted to singletons, i.e. samples with unique multilocus genotypes. In such cases, their genotypes were carefully double-checked to ensure that their uniqueness had not been caused by allelic scoring error.

The allelic dropout rate (ADO) per locus was calculated based on heterozygote genotypes. It was estimated as the number of times in which homozygote profiles were obtained from heterozygous samples (based on their ultimate consensus genotype), divided by the total number of successful PCR reactions for a given locus. The rate of false alleles (FA) was calculated as the number of times in which a spurious allele was detected in a single replicate (but could not be reproduced in subsequent attempts), divided by the total number of successful PCRs for a specific locus.

Additionally, in order to evaluate the power of the selected marker set in distinguishing samples at the individual level, we used CERVUS 3.0 (Marshall et al. 1998) to estimate the

probability of identity (P_{ID}) - defined as the probability that two distinct individuals present the same allelic combination at all loci by chance (Waits et al. 2001). We also calculated the more conservative statistic $P_{ID(sibs)}$, which is the probability of identity among siblings. These estimates were obtained for two different geographic scales: i) a regional set of samples including the same 28 individuals used to standardize and characterize the microsatellite loci, as mentioned above – Supporting Information T1); and ii) samples from the local individuals identified in this study. As a guideline, we followed the suggestion that P_{ID} estimates lower than 0.01 would be required for the data to be used for population size estimation (Mills et al. 2000, Waits et al. 2001).

Individual identification and genetic diversity

We used GENECAP (Wilberg & Dreher 2004) in Microsoft Excel and the software GIMLET (Valière 2002) to find matching genotypes among the scat samples. Genotypes from different samples were considered to represent the same individual when all alleles were identical or if there was only a single allelic mismatch (Bellemain et al. 2005). As a consequence, the resulting individual profiles were defined when they differed by at least two alleles. Matching genotypes were then given a consensus ID ("M" [male] or "F" [female] according to the sex typing, followed by a number) that was used in subsequent analyses.

We used Arlequin v. 3.5.1.2 (Excoffier & Lischer 2010) and FSTAT v. 2.9.3.2 (Goudet 2002) to test for deviations from Hardy-Weinberg equilibrium and linkage equilibrium using the larger set of samples from southern Brazil. We did not perform these tests for the local individuals identified in this study since we expected a somewhat high level of relatedness among animals. Diversity indices such as the number of alleles per locus, allelic richness, and observed/expected heterozygosities were estimated using CERVUS and FSTAT. The inbreeding coefficient (F_{is}) was computed with FSTAT.

Estimation of population size, density and sex ratio

We defined the first genotyped sample representing a given individual as a 'capture', while subsequent sampling/genotyping of that animal was considered a 'recapture'. By grouping identical multilocus genotypes, we compiled a capture-recapture history for each individual across the 10

252 sampling periods that spanned the entire two-year survey. We then estimated the local population
253 size using the software CAPWIRE (Miller et al. 2005), which has been recently developed for using
254 in DNA-based capture-mark-recapture data and performs well for small populations (n < 100). The
255 'two innate rates model' (TIRM) was chosen, since individuals could not be assumed to present
256 equal "capture" probabilities.

257 Since calculating census size using samples collected over the entire two-year study
258 period likely violate the general assumption of population closure of the model, we also used
259 CAPWIRE to estimate population size in shorter periods of intensive sampling. Thus, we run the
260 analysis for two temporally distinct scenarios: i) samples collected in each year separately; and ii)
261 dividing the entire sampling period into three relatively even subsets of sampling occasions: A)
262 comprising sampling occasions I - III (32% of the collected samples), B) occasions IV – VII (33% of
263 the samples), and C) occasions VIII – X (35% of the samples). We then compared inferences
264 made over the entire study with those from these more restricted time periods (while keeping the
265 same spatial scale).

266 In addition to estimates of population size, we calculated otter density (*D*) in our study site
267 by dividing the number of identified individuals by the total size of the sampled area. Finally, we
268 estimated the sex ratio of otters in the studied population by directly counting all distinct individuals
269 that could be reliably sexed based on the strategy outlined by Trinca & Eizirik (2012).

270

271 *Spatial organization, minimum home range and relatedness*

272 Considering the linear sampling design implemented in the study area, we defined the
273 "minimum home range" (MHR) of each individual as the total watercourse length (in km) between
274 its two furthest sampling points during the entire period. Spatial organization of the otters was
275 inferred based on the location of all samples from each individual, and an assessment of their
276 overlap with those of other animals over time.

277 We assessed the relatedness (*r*) within each pair of individuals by constructing a matrix of
278 pairwise relatedness values using the programs ML-Relate (Kalinowski et al. 2006) and Kinship
279 (Goodnight & Queller 1999). ML-Relate was also used to test the samples for the presence of null
280 alleles, as indicated by a heterozygote deficit relative to Hardy-Weinberg expectations (Rousset &

Raymond 1995). In its turn, Kinship was used to assess the significance of *r* values indicating relatedness. We investigated the relatedness among otters based on specific patterns of relationship available in ML-Relate: i) unrelated (U), ii) half-sib (HS), iii) full-sib (FS), and iv) parent–offspring (PO) and selected the most likely dyad relationship by comparing values of *r* estimated by both programs. Finally, for every inferred parent-offspring relationship we carefully checked by hand the Mendelian inheritance of all alleles.

We applied a linear correlation analysis for testing association between average spatial distance between individuals and their relatedness coefficient to investigate sex-biased patterns of dispersal. The average spatial distance between individuals was defined as the distance (in km) between the mean points of the home ranges for each of them. When a single sample was available for a given individual, we used its exact location on the watercourse to estimate pairwise distances. Correlation tests were performed for each stream separately and considered all comparisons between sexes and also each sex independently. Finally, the *r* coefficients were grouped into three categories: i) ≥ 0.5, ii) 0.49 - 0.25, and iii) < 0.24, for which the mean pairwise distance was estimated for each sex separately. We tested these values for the presence of differences among the three *r* categories in each sex, and also compared values of the same category between sexes in order to identify general patterns of spatial organization with respect to levels of relatedness.

Results

Sample collection and amplification success

Over the 10 sampling occasions, each sampling spot was surveyed, on average, for 446 m. The linear stretch covered in each sampling site was directly influenced by water level (related with season and rain intensity) and topography of each stream segment. Exceptionally, two field expeditions (V and IX) could not cover all three streams due to sudden changes in water level that submerged rocks and otter shelters, and also hampered walking along riverbanks. Both situations occurred during the spring, resulting in a small number of samples collected in this season. On sampling occasion V, only two samples were collected from the Lajeado stream, whereas on sampling occasion IX only river stretches on the Encantado stream could be covered (Table 1).

During the two-year survey we collected 253 samples of faeces/anal jellies, which were all successfully confirmed to be from *L. longicaudis* based on the PCR-based mtDNA assay. Samples were collected mainly from latrines (66.8% of samples) but were also found in small shelters, isolated on rocks or under tree roots (23.2%). From the total sample, 41.1% (corresponding to 104 samples) yielded enough quality/quantity of DNA for reliable genotyping of the selected microsatellite loci. The average rate of allelic dropout was estimated to be 40% across loci, whereas false alleles occurred at a notably lower frequency (8%) (Table 2). Amplification success was considerably higher for anal jellies (94%, 31 out of 33 samples) than for faeces (33.2%, 73 out of 220 samples) ($\chi^2 = 12.962$; $p = 0.0003$).

Sample availability and temporal genotyping success

We did not observe a significant difference in sample availability (ANOVA [$F_{0.05,\ 2}$] = 1.16; $p = 0.39$) or genotyping success (Kruskal-Wallis [$H_{0.05,\ 2}$] = 4.23; $p = 0.12$) among seasons. However, we did detect a significant discrepancy in the number of collected samples between sampling sites in the upstream (more preserved) and downstream (more disturbed) portions of the watercourses (sampling sites 1-3 *vs.* 7-10) (ANOVA [$F_{0.05,\ 2}$] = 5.26, $p < 0.05$). Neither of these groups were significantly different from the intermediate section of each watercourse (sampling sites 4-6). We also observed a significant difference in comparisons regarding the total number of collected *vs.* genotyped samples in the summer (Mann-Whitney [Wilcoxon rank-sum] $U = 2.31$; $p = 0.02$) and fall ($t_{0.05,\ 2} = 13.435$; $p = 0.006$) but not in the winter ($t_{0.05,\ 2} = 1.2162$; $p = 0.35$).

Individual identification and genetic variability

The power of the selected markers in distinguishing individuals was considerably high for both blood/tissue samples from southern Brazil ($P_{ID} = 7x10^{-9}$ and $P_{ID(sibs)} = 0.00078$) and local individuals identified in this study ($P_{ID} = 1x10^{-8}$ and $P_{ID\ (sibs)} = 0.0009$), thus revealing sufficient variability to reliably differentiate individuals in the study area. Interestingly, these observed values of P_{ID} were similar in both data sets, as a result of the high variability observed at the local level (see below). In addition to the P_{ID} analysis, the mean inbreeding coefficient (F_{is}) was estimated to be -0.131, indicating that mating between relatives is rare or absent in this population.

339 Of the 104 reliably genotyped samples, we identified 28 individuals, of which 13 were
340 determined to be males and 15 were identified as females. There was no case in which two
341 individuals presented the same genotype profile but were different in sex typing. Individuals were
342 detected 1-16 times each (mean of 3.71 detections per individual) with 18 otters (62%) being
343 detected more than once (i.e. by more than one sample in the same sampling occasion, or in
344 different occasions). There was no significant difference between the average number of multiple
345 occurrences of female (mean 4.33 ± 4.1, n = 15) and male (mean 3.0 ± 2.415, n = 13) individuals
346 (Mann-Whitney [Wilcoxon rank-sum test] U = 0.8061, p = 0.42), suggesting that both sexes
347 defecate equally in the streams.
348 Along the two years of study, the smallest number of individuals was observed in the
349 Encantado stream (n = 8), while the Forqueta and Lajeado streams presented 12 individuals each.
350 With respect to temporal population structure, we observed 18 (62%) and 19 (65.5%) of the
351 identified otters occurring in the study area in 2008 and 2009, respectively. Of these animals, nine
352 otters (32.14%; identified by codes F1, F2, F3, F7, F8, F14, M3, M7, and M8) were recorded in
353 both years (Fig 2a). These individuals, along with those found in only one year but were captured in
354 more than one sampling occasion (one in 2008 and seven in 2009) were defined as 'residents',
355 totaling 17 animals (mean number of captures of 5.22 ± 3.46). The remaining 11 individuals were
356 identified in only one sampling occasion each, and were defined as 'transients'. These individuals
357 were mostly identified by a single sample, except for the female F4 that was identified by three
358 samples on sampling occasion III (Fig 3).
359 Overall, genetic variation as measured by the number of alleles, allelic richness, and
360 observed heterozygosity was high (Table 2). All loci presented at least six alleles (except for
361 04OT04, with two alleles) and the average number of alleles per locus was 6.5. All loci were at
362 Hardy-Weinberg equilibrium, and no deviation from linkage equilibrium was observed after
363 Bonferroni corrections (Rice 1989; original α = 0.05). Finally, we found no evidence of null alleles at
364 any of the loci.
365

366 *Population size estimation, density and sex ratio*

367 Individual counts, along with the capture history of each identified otter, was used to
368 calculate population size using CAPWIRE. Based on the TIRM analysis, the estimated population
369 size was 31 individuals (95% Confidence interval [CI]: 28 – 36) when considering the two-year
370 sampling period (Fig 4). Six individuals were identified as 'easier to capture' (type A) than the
371 remaining 25 animals (classified as 'harder to capture' – type B). Such a high proportion of type B
372 individuals probably reflects the fact that *ca.* 40% of the otters were identified on the basis of a
373 single scat sample.

374 The population size analysis performed for each year of sampling yielded estimates of 28
375 (95% CI: 18 – 41) and 22 (95% CI: 19 – 27) individuals in 2008 and 2009, respectively. Finally, we
376 repeated this analysis with the samples subdivided into three shorter periods, so as to better suit
377 the assumption of population closure (see 'Materials and Methods' for details). In subset A
378 (sampling occasions I – III), 13 otters were directly identified in the area and the CAPWIRE
379 analysis indicated a population size of 23 individuals (95% CI: 13 – 35); in subset B (IV – VII) we
380 genotyped 17 animals and estimated a population of 28 otters (95% CI: 17 – 40); and in subset C
381 (VIII – X) we also identified 17 otters and estimated a population of 21 individuals (95% CI: 17 – 29)
382 (Fig 4). Several resident individuals were captured in more than one of these shorter periods given
383 their relatively constant presence in the study area. Therefore, the sum of the captures across the
384 three periods considerably exceeds our total number of identified individuals.

385 In addition to population size estimates, the linear otter density (*D*) was estimated at *ca.* 1
386 otter/km. When analyzing each year separately, *D* was estimated, on average, to be 0.66 otter/km.
387 Finally, the observed number of males (n = 13) and females (n = 15) resulted in a sex ratio (male:
388 female) of 1:1.15, which was not significantly different from 1:1 ($\chi^2_{0.05,\,1} = 0.143$, $p = 0.85$), even
389 when considering only resident animals (sex ratio of 1:1.43; $\chi^2_{0.05,\,1} = 0.529$, $p = 0.628$).

390

391 *Minimum home ranges and spatial organization*

392 Seven males (M3, M4, M7 - M11) and 10 females (F1 - F3, F7, F8, F10, F11, F13 - F15)
393 were classified as residents. Of these animals, individuals F2, F10, F14 and M11 were restricted to
394 the Forqueta stream, while F1, F3, F7, F11 and M7, M9, F8, F14, F15 occurred only on the

395 Encantado and Lajeado streams, respectively (see Fig 2 and 3). Four individuals (all males)
396 presented a broader area of occurrence as they were detected on more than one stream: M3 was
397 identified on the Forqueta and Lajeado (Fig 2a), while M4 was found on the Forqueta and
398 Encantado (Fig 2b) and M8 and M10 on the Encantado and Lajeado (Fig 2a, c). Several instances
399 of range overlap could be detected, involving all three gender combinations (male-male, female-
400 female and male-female). For example, the minimum home range (MHR) of resident male M4
401 considerably overlapped those of another male (M3) and multiple females (F1, F2, F3 and F7), all
402 of which were captured in the same temporal window (Fig 2b and Fig 3). Interestingly, one of the
403 captures of each of these two males occurred on sampling occasion II, when their vestiges were
404 collected next to each other on the same rock. Another interesting case was that of male M11 and
405 females F10 and F13, which occupied adjacent or overlapping portions of the Forqueta stream, all
406 of which were contained in the area occupied by F2 (Fig 2c).
407 Among the resident individuals, the average MHR was estimated to be 6.29 ± 5.96 km (n =
408 17). This value was significantly influenced by male movements, which almost always comprised
409 longer distances than females (Mann-Whitney [Wilcoxon rank-sum test] $U = 2.49$; $p = 0.0128$) (Fig
410 5). Average male MHR was 11.49 ± 6.45 km, while that of females was 2.97 ± 2.07 km. Male M8
411 presented the largest area of occurrence (18.3 km), followed by M3 and M4 (~17 km each) (Fig 5).
412 Among females, F8 presented the lowest MHR, occupying only 0.18 km of the Lajeado stream,
413 whereas F3 presented the largest area (~ 6 km), located on the same watercourse.
414 In addition to the resident animals, 11 individuals were categorized as transients, eight of
415 which were detected in 2008 (M1, M2, M5, M6, F4, F5, F6, F8, and F9) and three in 2009 (M12,
416 M13 and F12) (Fig 2b, c; Fig 3). Of the eight transient individuals identified in 2008, four were
417 located on the Forqueta stream and four on the Lajeado. In 2009, two transients were observed on
418 the Forqueta stream and one on the Encantado. Most of transient otters were detected within or
419 adjacent to the minimum home range of one or more resident individuals. One interesting case was
420 that of transient female F4, which was identified by three genotyped samples collected along the
421 Forqueta stream on sampling occasion III, resulting in a displacement of *ca.* 2.6 km.
422

423 *Parentage assignment*

424 The mean relatedness (*r*) among otters in the total area (*r* = 0.1011 ± 0.009) was not
425 significantly different from that observed in each stream separately (ANOVA [$F_{0.05,\ 3}$] = 2.497; *p* =
426 0.06) (Table 3). When comparing the mean relatedness coefficient of males and females, there
427 was no significant difference between the sexes (Mann-Whitney [Wilcoxon rank-sum test] *U* =
428 0.0155; *p* = 0.988). However, when each stream was analyzed separately, we observed a
429 significantly different mean relatedness between the sexes in the Encantado stream (Mann-
430 Whitney [Wilcoxon rank-sum test] *U* = 2.242; *p* = 0.025), suggesting that females tend to be more
431 related than males (Table 3). Additionally, we also compared female and male *r* coefficients among
432 streams. There was no difference in male indices of relatedness, but females from the Encantado
433 stream were significantly more related to each other than those sampled in the Lajeado, but not
434 than females from the Forqueta (ANOVA [$F_{0.05,\ 2}$] = 7.0786, *p* = 0.0036).

435 In addition, the microsatellite genotypes provided sufficient power to reconstruct a
436 genealogy for most of the sampled otters. Twenty-four individuals (86% of the total sample) could
437 be placed within a genealogical context for this population, which included 21 cases of reliable
438 assignment to a parent-offspring relationship (Fig 6). It was noteworthy that close relatives were
439 often resident individuals inhabiting the same watercourse, as observed for several inferred
440 relationships on the Forqueta and Encantado streams (see Fig 6). This general geographic trend in
441 relatedness was more marked in mother-offspring relationships, in which almost all progeny were
442 identified on the same stream as their mother, especially in the case of mother-daughter dyads (Fig
443 6).

444 Many of the inferred parent-offspring and sibling dyads exhibited geographic and temporal
445 overlap or proximity, such as individuals F2, F10, F13 and M11 on the Forqueta stream. There
446 were several cases of mother-daughter watercourse fidelity (see Figs. 2 and 6), including a
447 remarkable three-generation matriline (composed by F7, F1, F3 and F11) dominating the
448 Encantado stream. We detected only one exception to this pattern, involving a mother from the
449 Encantado stream (F3) and one of her daughters (F6), which was found on the Lajeado. Such
450 watercourse fidelity was not observed as clearly in male offspring, as there were cases in which the
451 son was sampled in a distinct river from its mother (e.g. F2 and M13; F7 and M3). With respect to

overall social structure, a particularly interesting case was that of a resident male (M4) that bred with two different females (F2 and F7), leaving resident female offspring on two different streams (see Figs. 2 and 6). More generally, it is noteworthy that we did not detect any case of mating among close relatives among otters in the study area.

Finally, we applied a linear correlation analysis to test for association between the average spatial distance and the relatedness coefficient of dyads (male-male, male-female, and female-female) identified on each stream, in order to evaluate the presence of sex-biased patterns of dispersal in this population. Overall, the observed results did not yield significant values of correlation (not shown), even when calculated only for resident individuals (when the number of pairwise comparisons was sufficient to perform the test), so that no hypothesis regarding a dispersing or philopatric sex was statistically supported. The only exception was observed on the Forqueta stream, where *r* values were significantly negative for mixed-sex comparisons (r^2 = - 0.31, p = 0.01 for all individuals on this stream; r^2 = -0.68, p = 0.005 for resident-only comparisons). However, when comparing average distances with categorized *r* coefficients (≥ 0.5, 0.49-0.25, and ≤ 0.24, with males and females treated separately) (Table 4) we did not observe significant difference between male dyads, even when considering only resident animals (ANOVA [$F_{0.05,\ 2}$] = 0.808, p = 0.55 for all male comparisons, and Kruskal-Wallis [$H_{0.05,\ 2}$] = 1.029, p = 0.59 for resident only estimates). In contrast, estimates for female dyads showed a consistent pattern of lower relatedness as the geographic distance increased (ANOVA [$F_{0.05,\ 2}$] = 9.519, p = 0.0003 for general female estimates, and $F_{0.05,\ 2}$ = 18.192, p < 0.0001 for resident-only comparisons). Finally, a statistically significant difference in mean geographic distance was observed between sexes when considering *r* coefficients ≤ 0.24 (Mann-Whitney [Wilcoxon rank-sum test] U = 2.53; p = 0.01 and U = 3.66; p = 0.0003 for estimates including all dyads in that category, and that for only residents, respectively).

Discussion

Noninvasive sampling

Scat surveys have been proved to be a relatively easy and efficient method to noninvasively sample several carnivore species that are difficult to study by employing traditional

481 methodologies (Palomares et al. 2002, Hedmark & Ellegren 2007, Mowry et al. 2011). In the case
482 of several otter species, studies based on scat collection are especially facilitated by the mostly
483 linear format of their home ranges, which are strongly associated to watercourses. Additionally,
484 otter faeces are usually easy to find due to the species behavior of conspicuous defecation, likely
485 important for intra-specific communication.

486 Although sample availability is usually not an issue in such studies, to analyze the large
487 amount of obtained material can be extremely expensive and time-consuming. To reduce these
488 issues and to design a comprehensive and standardized field strategy, we defined sampling spots
489 along the streams that aimed to allow an intensive noninvasive genetic sampling while reducing
490 excessive sampling of the same individual in a short period. By employing this sampling scheme,
491 we intensively surveyed ~30 km of three streams for two years, which enabled us to address
492 several issues on population biology of this otter which had not been investigated so far.

493 Of the total set of samples, a significantly higher number of samples was found in latrines
494 (66.8%) than isolated on rocks and shelters along the streams (23.2%). This observed bias in the
495 sprainting behavior at our study site does not necessarily represent a preference for defecating on
496 shared rocks, but may be related to the lower availability of other appropriate substrates at our
497 study site, and may differ among areas.

498

499 *Data quality control and amplification success*

500 One important issue in noninvasive genetic methods is the frequently low quality/amount of
501 the DNA recovered from the collected samples, associated with large amounts of PCR inhibitors
502 and environmental exposure to degradation. As a consequence, traditional molecular methods
503 become more prone to failure due to contamination with extrinsic DNA, as well as to genotyping
504 errors (Goossens et al. 2000, Broquet & Petit 2004, Pompanon et al. 2005). To avoid such
505 problems and to obtain accurate population estimates, it has been proved that the employment of
506 rigorous strategies for detecting and removing those sources of errors are critical (Broquet & Petit
507 2004). To address these issues, each step of our laboratory procedures was performed in isolated
508 and UV-sterilized areas. We did not observe any evidence of extrinsic DNA contamination in
509 mtDNA and microsatellite amplification, indicating that our protocols were efficient in avoiding the

510 presence of extrinsic DNA. Additionally, despite potentially time-consuming and costly, it is
511 undeniable that a multiple-tube approach is important to reliably obtain accurate genotypes, as we
512 could observe in our data.

513 The extremely high success rate (100%) in confirming the presence of otter DNA in the
514 samples by the mtDNA PCR-assay was likely a result of the freshness of the material collected in
515 the field. On the other hand, even by following the freshness criterion, the observed success of
516 microsatellite amplification considerably varied among samples and loci, resulting in the fact that
517 only 41.1% of the samples could be reliably genotyped for four or more loci. This moderate rate in
518 genotyping success may be a consequence of the environmental conditions in the region, which is
519 marked by high levels of humidity on the streamside and riparian habitats used by otters, favoring
520 fungal and bacterial proliferation and enzymatic activities (Lampa et al. 2008). Therefore, this factor
521 can decrease the amplification success, as suggested by Farrel et al. (2000), and may be more
522 pronounced when analyzing nuclear DNA (Michalski et al. 2011).

523 In spite of the moderate success in noninvasive DNA genotyping observed in this study, it
524 is within the range reported for other carnivores (e.g. Adams et al. 2007, Hedmark & Ellegren
525 2007). Among otter species, genotyping success has also proved to be greatly variable, with
526 amplification rates ranging from 14% to 73% (Jansman et al. 2001, Dallas et al. 2003, Hung et al.
527 2004, Janssens et al. 2008, Kalz et al. 2006, Prigioni et al. 2006a, b, Arrendal et al. 2007, Ferrando
528 et al. 2008, Lanszki et al. 2008). Janssens et al. (2008) reported the highest amplification success
529 rate of 73% of the total collected samples. However, their sample set included preferentially anal
530 jellies and viscous scats, which was likely related to such a high efficiency in microsatellite
531 genotyping. Their reported high efficiency in anal jelly genotyping is congruent with the success
532 rate obtained here for this type of sample (94%), as well as with reports from other studies (Lampa
533 et al. 2008, Hajková et al. 2009, Mowry et al. 2011). Such high success in amplifying anal jellies
534 may be related to the suggestions that dietary items could influence amplification results due to the
535 presence of PCR inhibitors (Reed et al. 1997, Farrel et al. 2000, Murphy et al. 2003), and that a
536 diet based mainly on fish can decrease rates of DNA extraction and PCR amplification (Murphy et
537 al. 2003). As anal jellies do not contain prey items, they would contain fewer PCR inhibitors, thus
538 yielding better DNA amplification. Also, this secretion may contain larger amounts of cells, thus

539 increasing otter DNA concentration in the final extract. However, as observed in our study, anal
540 jellies can be much less frequently found than faeces (only 33 out of the 253 samples collected
541 here), which would hamper a representative individual sampling in a given area based solely on
542 this type of material.

543 The observed average ADO rate (40%) was considerably high in our data set. However, it
544 was similar to those reported for *Lutra lutra* in China (30.7%, Hung et al. 2004) and France (54%,
545 Janssens et al. 2008). On the other hand, the low rates of FA (8%) reported for our samples
546 indicates that the probability of us having considered a spurious allele in our analysis is very low,
547 thus providing additional support for the reliability of our multilocus consensus genotypes.

548 Alternatively, quantitative PCR (qPCR) has been suggested to represent an efficient
549 method to deal with low-quality DNA by helping researchers to focus replication efforts on samples
550 that present higher DNA concentration, thus greatly optimizing success rates and reducing
551 genotyping errors (Morin et al 2001). Using qPCR, Morin et al. (2001) estimated that seven
552 replicates would be necessary to reach the correct genotype for samples with 26 – 100 pg DNA
553 concentration, a category representing a mean ADO rate of 42% and matching the
554 recommendations of Taberlet et al. (1996). Although we did not use qPCR in our samples to allow
555 a direct comparison, we obtained a similar ADO rate (40%) and our established number of
556 repetitions was in agreement with Morin et al. (2001) and Taberlet et al. (1996), although some
557 samples were exhaustively amplified (up to 12 times) to reach the necessary number of genotype
558 replicates.

559

560 *Sample availability and genotyping success*

561 Statistical analysis indicated no difference in sampling availability across seasons,
562 suggesting that sprainting behavior was similar at the study site over time. However, we could not
563 include the data from the spring (sampling occasions V and IX) in the comparisons due to the low
564 number of collected samples on those dates, derived from changes in water level and
565 inaccessibility to the sampling spots caused by intense rain immediately before and during the field
566 expeditions. Since the spring has been suggested to be the mating season of this otter in a large
567 portion of its range (Parera 1996), it is possible that during this time there could be some change

568 (likely an increase) in scent-marking behavior as a strategy for breeding. Such a possibility may be
569 tested in future studies that are not hampered by inclement weather affecting spring sampling.
570 In contrast to the observed homogeneity in sample availability over seasons, comparisons
571 between the number of collected samples and those that could be successfully genotyped in each
572 season (also excluding spring) revealed a higher efficiency of genotyping in winter. In southern
573 Brazil, climate is characterized by well-defined cold and warm seasons, which differ considerably in
574 temperature and precipitation. During our study period, the mean temperature during winter in the
575 Maquiné Valley was *ca.* 5°C during the night and early morning, whereas temperatures during the
576 summer reached up to 30°C (C. S. Trinca, personal observation). It has been suggested that
577 sampling during winter could provide higher amplification rates (Arrendal et al. 2007) due to the
578 better preservation of DNA at colder temperatures. Although southern Brazil does not present
579 extremely cold winters when compared to areas subjected to large-scale freezing, it is likely that
580 the observed low temperatures do have a similar role in preventing DNA degradation and in
581 maintaining sample freshness for a longer time.
582 Finally, the observed significantly lower presence of otter signs in the downstream portion
583 of all three sampled watercourses (see Results) may be related to less suitable substrates for otter
584 scent-marking, as rocks were too small and few riverbank shelters could be observed at these
585 sites, thus resulting in lower availability when compared to the upstream sections. In addition, these
586 areas comprised the stream segments in which habitat changes are more pronounced due to
587 higher human density and disturbance. The observed results suggest that such interference may
588 induce changes in otter habitat use and scent-marking behavior, and should be investigated by
589 further studies in the area.
590
591 *P_{ID} analysis and genetic diversity*
592 It has been suggested that the molecular markers selected for capture-recapture genetic
593 studies targeting individual identification and population size estimation should present a P_{ID} index
594 lower than 0.01 (Mills et al. 2000). The microsatellite panel used in this study (Table 2) presented
595 P_{ID} values ($1x10^{-8}$ and 0.0009 for $P_{ID(sibs)}$) that were much lower than this threshold, thus supporting
596 its usefulness in reliably differentiating individuals in the study area.

Genetic diversity of the selected loci was considerably high in the target population (Table 2), especially if one considers the spatial scale of study site (*ca.* 30 km of watercourses). Interestingly, seven alleles detected in this studied population were not observed in the blood/tissue set of samples from southern Brazil, also highlighting the great potential of these loci for population genetic studies. Levels of microsatellite polymorphism such as observed heterozigosity (H_o = 0.831) and mean number of alleles (6.5) were considerably higher in this study than those reported for two other populations from southernmost Brazil (H_o = 0.299/0.355; mean number of alleles = 4.1/4.9; [Weber et al. 2009]) based on a partially overlapping set of microsatellite loci. Interestingly, population genetic studies of the Eurasian otter (*Lutra lutra*) also reported lower levels of local molecular polymorphism at some of the same microsatellite loci, even though the markers had been developed for that species originally (Hung et al. 2004, Ferrando et al. 2008, Lanzki et al. 2008).

Population size estimates and sex ratio

In general, population size estimates were consistent among analyses and presented similar numbers for all temporally established datasets, with the actual number of genotyped otters (n = 28) included within the confidence interval observed in most of the estimates (Fig 4). For the full sampling period, population size was estimated at 31 individuals (95% CI = 28-36), while for the two partitioned datasets (by year and by sets of sampling occasions) it ranged from 21 to 28 animals (composite 95% CI = 17-41). We observed broader confidence intervals in the analyses that considered the individuals identified in 2008 and in subsets A and B (see Fig 4), suggesting that in these periods we sampled a little over half of the number of otters in the study area at those times. In contrast, the point estimates of population size for the two-year survey, for 2009 only, and for subset C (comprising 3 sampling occasions in 2009) were very close to the number of otters genotyped in each of these periods and shorter confidence intervals were obtained. It is possible that these differences between the number of genotyped individuals and the population size estimates have been caused by the larger number of transient otters that were observed in 2008, which may have inflated population size estimates involving this period. Comparing results from all datasets, we may conclude that, although our sampling design likely did not meet the assumption

626 of population closure, the value obtained for the entire period (31 otters) does not seem to seriously
627 overestimate the population size, and the effect of 2008 transients may have been diluted amidst
628 the overall number of recaptured otters.

629 Sample availability has been suggested to influence the accuracy of population size
630 estimates (Arrendal et al. 2007). We observed that efficiency in collecting otter samples did not
631 significantly vary among seasons (except for those that were affected by sudden weather
632 changes). However, capture heterogeneity likely resulted from differences in individual sprainting
633 behavior, sex, age class and resident status. Differences in individual detection due to capture
634 heterogeneity can be inferred from our data, as otters were identified by 1 – 16 samples, but this
635 difference did not seem to be related to gender variation in sprainting behavior. In spite of the
636 suggestion of spring association with mating season in several parts of the Neotropical otter's
637 range, there are indications that this species, as well as some of other otters, present a facultative
638 delayed implantation, which allows the zygote to start its development at a favorable time of the
639 year (Kruuk 2006). As a consequence, we could not infer with certainty that cubs were born in the
640 study area in the months following the putative breeding season, hampering any assessment of
641 variation in female defecation patterns (and consequent differences in capture rates) related to
642 cub-rearing.

643 The estimated Neotropical otter sex ratio (1:1.15 for the total population and 1:1.43 when
644 considering only the resident individuals) was not significantly different from 1:1 in the study area.
645 The same pattern has been reported for *Lutra lutra* by several authors (Ruiz-Olmo et al. 1998,
646 Hung et al. 2004, Arrendal et al. 2007).

647

648 *Minimum home range, spatial organization and relatedness*

649 More than a half of the identified animals in the study area were residents, while the others
650 individuals were assumed to be transients. Overall, spatial organization of the resident individuals
651 presented several instances of overlap. On average, resident males presented larger minimum
652 home ranges (MHR), of up to ~18km, and encompassed those of more than one female, which
653 were observed to be no greater than *ca.* 6 km. This general spatial pattern is congruent with the
654 typical mustelid social organization model proposed by Powell (1979).

655 It is important to note that, despite the undeniable value of noninvasive genetic methods for
656 population biology studies, we need to be cautious when interpreting the observed pattern of
657 spatial organization and MHR estimates. It is possible that noninvasive sampling may
658 underestimate home range areas since it is based solely on the sprainting behavior, and it is known
659 that otters can use areas where they do not defecate (Kruuk 2006). Also, the MHR boundaries of
660 some otters coincided with the limits of our surveyed area, such as the case for males M7, M8 and
661 M9, and females F2, F3, and F10 (see Fig 2), so that the total area comprised by their territories
662 may extend into stream sections outside our pre-established survey boundaries.
663 Overall, we observed that female MHRs usually did not present defined boundaries;
664 instead, we detected many cases of partial or total overlap of their territories along the streams.
665 Additionally, it is noteworthy that the observed female range overlaps involved exclusively closely
666 related animals which composed a somewhat female 'group range' that apparently dominated
667 almost the entire (or a large portion) of the sampled watercourses, as observed in the Forqueta and
668 the Encantado streams. Interestingly, a three-generation family composed by four females (F1, F3,
669 F7, and F11) was observed in the Encantado stream. These otters largely overlapped in their
670 MHRs, suggesting a female philopatric behavior in this species. Additionally, due to the small MHR
671 of F11 (Fig 2c and Fig 5), and its estimated relatedness to F3, it is possible to hypothesize that F11
672 may have been a juvenile following its mother in that small portion of the Encantado stream.
673 As for male spatial organization, although some individuals presented large MHR overlap,
674 these animals were often sampled on different sampling occasions or at different sampling spots
675 on the same sampling occasion, as occurred with males M4 and M8 on the Encantado stream, and
676 M3 and M10 on the Lajeado (see Fig 3; data on specific location of each sample is not shown). It is
677 likely that sprainting behavior may act as a mechanism of individual recognition (as reported for
678 other otter species); in this case, although animals inhabit and forage in the same area, they
679 recognize the presence of each other and avoid direct contact that could result in aggression for
680 territory defense (Kruuk 2006). However, we observed an interesting case in which one anal jelly
681 and one faeces were collected in the same latrine on the Forqueta stream, on the same day during
682 sampling occasion II, and were identified as belonging to the unrelated males M3 and M4,
683 respectively. Following our criterion of sample freshness, we infer that both otters defecated in that

latrine on the same 1-day interval, suggesting either tolerance of males to simultaneous spatial overlap, or territorial marking related to spatial competition between them. Both hypotheses open up interesting avenues for future research on this topic.

Additionally, we recorded other interesting cases in which at least two individuals defecated at the same place in the same period of sampling. Samples from otters M9 and F14 were collected from the same shelter on the Lajeado stream on sampling occasion VIII. This shelter were observed to be constantly used throughout the study period, and seemed to be conspicuously marked by female F14. Also, on the Forqueta stream, females F2, 10, F13 and male M11 were observed to intensively spraint at the same latrine on sampling occasion VIII. One (F13 and M11) to three (F2) samples from each of these otters were collected at this site, with all of them being equally classified as “fresh” samples. The genealogical analysis indicated that these animals were closely related (see Fig. 6), providing additional support for instances of philopatric behavior, at least during that time period. Of these individuals, the only male was M11, which occupied a short stream segment completely contained within the MHR estimated for its mother (F2). Considering that this male was only captured in the second year of sampling, we may infer that it was in fact a juvenile using the area of his mother before dispersing.

In addition to the patterns observed for resident otters, a considerable number of transient animals was identified in the study area, most of which were captured within or adjacent to the MHRs of resident individuals. Among transient females, a curious case was observed for female F4, which was identified by three samples collected at different sites on sampling occasion IV. These results indicate that this female moved *ca.* 2.6 km on a single day (based on the criterion outlined above for sample freshness - see Methods) across stream sections included in the MHRs of males M3 and M4 (Fig 2b). Thus, it is evident that otters are able to move across large distances in a short period, and there are no strong physical barriers to that. Nevertheless, our results indicate that females remain restricted to specific stream sections, likely as a consequence of territorial structure influenced by relatedness. On the other hand, it is also possible that F4 was actually a resident (since it was found in an area that seemed not to be occupied by any other female) that for some reason was not recaptured in subsequent sampling occasions. However,

712 given the observation of female ‘range groups’ on that stream, the fact that F4 was unrelated to any
713 other resident of the Forqueta stream (see Fig 6) supports the hypothesis that she was a transient.
714 Still regarding the possibility that some transient otters were, in fact, insufficiently sampled
715 residents, two main factors may be involved: the individual capture heterogeneity and water level
716 changes over time. As already mentioned, the study area is commonly affected by fast changes in
717 water level by sudden and intense rains. During the two-year survey, these events occurred at
718 least three times, two of which hampered appropriate sampling, and one (that occurred between
719 sampling occasions II and III) markedly changed the topography of the downstream section of the
720 Forqueta stream. After these episodes, several animals were no longer recaptured in the study
721 area. It is possible that these events have led otters to search for available areas on nearby
722 unaffected streams, thus reorganizing spatial distribution of the individuals, or (less likely) have
723 killed animals (especially cubs), thus influencing the number of identified transients.
724 Another interesting pattern regarding otter spatial organization in the study area was the
725 observation of fewer otter signs in the downstream portion of the sampled watercourses (see
726 above). Although this pattern may be related to the poorer habitat suitability or substrate availability
727 in these portions, these sections represent important connections among the streams, and were
728 noticed to be part of male home ranges. Therefore, we infer that, in spite of the observed scarcer
729 scent-marking in these segments, it is clear that otters are still present there, but may be using
730 them mainly as corridors to reach more suitable areas, especially in the case of males.
731 Finally, in spite of the inconsistent results from the statistical tests comparing relatedness
732 and geographic distances among individuals in each stream, our data strongly suggested that
733 otters on each of the three sampled watercourses tended to be more closely related among
734 themselves than to animals from other streams, especially when considering mother-daughter
735 relationships. Comparing each sex separately, we observed that the least related females tended
736 to have greater geographic distances between them (Table 4), reinforcing the hypothesis of female
737 philopatry. Furthermore, in spite of the rather high level of genetic relatedness among the identified
738 otters, we did not observe any evidence of close-relative mating, suggesting the presence of an
739 efficient mechanism of inbreeding avoidance in this otter, likely involving male-biased dispersal.
740

Otter density

By identifying otters at the individual level, the method employed in this study was effective in providing Neotropical otter density estimates. In general, population densities estimated here (1 otter/km for the total period of sampling and 0.66 otter/km when considering each year separately) were similar to or higher than those reported for two of the best studied otter species, the Eurasian otter (*Lutra lutra*) and the North American river otter (*Lontra canadensis*). Kruuk & Moorhouse (1991) reported a density of 1 otter/km for a *Lutra lutra* population in Shetland islands, and Hung et al. (2004) reported higher densities (1.5 – 1.8 otters per km for all otters and 0.8 – 1.1 for residents only) for the Eurasian otter in stream systems in Kinmen islands, China, whereas other studies reported much lower estimates (Kruuk et al. 1993, Prigioni et al. 2006, Lanzki et al. 2008). Considerably lower to similar densities were also reported for the river otter, with estimates varying from 0.069 to 0.51/otters/km (Melquist & Hornocker 1983, Bowyer et al. 2003, Melquist et al. 2003, Mowry et al. 2011).

The considerably high otter density observed in the study area may result from a relative good environmental quality of that system, especially in the upstream portions of the watercourses, which are less influenced by local human activities and may present abundant food resources. Ecological studies on the European otter have suggested that dense network systems and food availability are important drivers of otter density (Sidorovich & Pikulik 1998, Ruiz-Olmo et al. 2001). It is known that the stream network of the Maquiné valley is dense and complex, but information on current resource availability is absent. These issues should be addressed in future studies, aiming to investigate if that system provides habitat requirements (such as shelters and food supply) sufficient to maintain a persistent otter population, as well as to quantify the already perceived (although still not severe) threats that may also have an important effect on otter abundance and density in this region.

Final conclusions and conservation implications

Despite the reported efficiency of noninvasive genetic analyses in successfully assessing demographic characteristics of several mammals around the world (eg. Zhan et al. 2007, de Barba et al. 2010, Stenglein et al. 2011), this approach has just recently begun to be employed in

770 population genetic studies of Neotropical carnivores. These studies were mostly applied to species
771 identification and population structure (Haag et al. 2010, Miotto et al. 2011) although some new
772 approaches have also been published, such as the detection of melanism in wild jaguars from
773 faecal samples (Haag et al. 2009). Still, our study is the first to address ecological and
774 demographic aspects of a Neotropical carnivore by using genetic data from noninvasively collected
775 samples, thus confirming this method as a useful tool for studying elusive, rare and/or threatened
776 species from the Neotropics.

777 Little information on local levels of genetic diversity and population dynamics of wild
778 populations of the Neotropical otters have been published so far due to the limitations in observing
779 and monitoring the species in the field, and given the difficulty in obtaining blood and tissue
780 samples at sufficient coverage to allow population genetic studies. Our study revealed that genetic
781 analyses of otter vestiges hold great potential to increase the knowledge on this mustelid, providing
782 essential information for assessments of its conservation status and guidelines for future
783 management strategies at continental and regional scales.

784 The high level of genetic diversity, along with large number of identified animals and low
785 inbreeding coefficient observed in the studied population suggest that otters in the Maquiné valley
786 currently represent a healthy population. This may derive from still suitable resource availability,
787 and the apparent tolerance of this otter to some level of habitat disturbance. However, the
788 Neotropical otter inhabits a broad range of habitats and faces different threats, so population
789 dynamics and vulnerability to disturbance may differ considerably among areas and biomes. In this
790 context, noninvasive genetic studies may be used to investigate other populations across the
791 species' range, so as to generate a more comprehensive picture of the general patterns of
792 population dynamics of this otter. Furthermore, the results presented here raised hypotheses
793 regarding dispersal patterns, reproductive and social behavior that open new avenues on the
794 ecology and behavior of this species, which will need the conduction of long-term studies, which
795 may provide even more robust data if associated with radio-telemetry approaches. Finally, our
796 results also indicate that noninvasive sampling may represent an important tool for addressing
797 demographic parameters of the other two South American otters of the genus *Lontra* (*L. felina* and
798 *L. provocax*), which present similar difficulties to study at local population level. In the future, such

799 investigations may shed light onto the ecology and behavior of these and other elusive Neotropical
800 carnivores, improving our understanding of their life history and population dynamics.
801
802 **Aknowledgements**
803 We would like to thank F. P. Valdez, L. E. Costa-Schmidt and L. Silveira for helping in the
804 field work and M. L. Fontoura-Rodrigues for his valuable help in the parentage analyses. We also
805 thank C. R. Sarturi and P. M. Zamberlan for technical assistance. This work was supported by
806 CNPq, Brazil (postgraduate fellowship number 141882/2007-7).
807
808 **References**
809 Adams JR, Waits LP (2007) An efficient method for screening faecal DNA genotypes and detecting
810 new individuals and hybrids in the red wolf (*Canis rufus*) experimental population area.
811 *Conservation Genetics*, **8,** 123-131.
812 Arandjelovic M, Head J, Kühl H et al. (2010) Effective non-invasive genetic monitoring of multiple
813 wild western gorilla groups. *Biological Conservation*, **143**, 1780-1791.
814 Arrendal J, Vilà C, Björklund M (2007) Reliability of noninvasive genetic census of otters compared
815 to field censuses. *Conservation Genetics*, **8**, 1097-1107.
816 de Barba, M, Waits LP, Garton EO et al. (2010) The power of genetic monitoring for studying
817 demography, ecology and genetics of a reintroduced brown bear population. *Molecular*
818 *Ecology*, **19**, 3938-3951.
819 Beheler AS, Fike JA, Murfitt LM et al. (2005) Ten new polymorphic microsatellite loci for North
820 American river otters (*Lontra canadensis*) and their utility in related mustelids. *Molecular*
821 *Ecology Notes*, **5**, 602-604.
822 Bellemain E, Swenson JE, Tallmon D et al. (2005) Estimating Population Size of Elusive Animals
823 with DNA from Hunter-Collected Feces: Four Methods for Brown Bears. *Conservation Biology*,
824 **19**, 150-161.
825 Boutin-Ganache I, Raposo M, Raymond M, Deschepper CF (2001) M13-tailed primers improve the
826 readability and usability of microsatellite analyses performed with two different allele-sizing
827 methods. *Biothecniques*, **31**, 25-28.

828 Bowyer RT, Blundell GM, Ben-David M et al. (2003) Effects of the *Exxon Valdez* oil spill on river
829 otters: injury and recovery of a sentinel species. *Wildlife Monographs*, **153**.
830 Broquet T, Petit E (2004) Quantifying genotyping errors in noninvasive population genetics.
831 *Molecular Ecology*, **13**, 3601–3608.
832 Brøseth H, Flagstad O, Wärdig C et al. (2010) Large-scale noninvasive genetic monitoring of
833 wolverines using scats reveals density dependent adult survival. *Biological Conservation*, **143**,
834 113-120.
835 Chemes SB, Giraudo AR, Gil G. 2010. Dieta de *Lontra longicaudis* (Carnivora, Mustelidae) en el
836 Parque Nacional El Rey (Salta, Argentina) y su comparación con otras poblaciones de la
837 cuenca del Paraná. *Mastozoología Neotropical*, **17**, 19-29.
838 Constable JL, Ashley MV, Goodall J, Pusey AE (2001) Noninvasive paternity assignment in Gombe
839 chimpanzees. *Molecular Ecology*, **10**, 1279-1300.
840 Creel S, Spong G, Sands JL et al. (2003) Population size estimation in Yellowstone wolves with
841 error-prone noninvasive microsatellite genotypes. *Molecular Ecology*, **12**, 2003-2009.
842 Dallas JF, Piertney SB (1998) Microsatellite primers for the Eurasian otter. *Molecular Ecology*, **7**,
843 1248-1251.
844 Dallas JF, Carss DN, Marshall F et al. (2000) Sex identification of the Eurasian otter *Lutra lutra* by
845 PCR typing of spraints. *Conservation Genetics*, **1**, 181-183.
846 Dallas JF, Coxon KE, Sykes T et al. (2003) Similar estimates of population genetic composition
847 and sex ratio derived from carcasses and faeces of Eurasian otter *Lutra lutra*. *Molecular*
848 *Ecology*, **12**, 275-282.
849 Eggert LS, Eggert JA, Woodroof DS (2003) Estimating population sizes for elusive animals: the
850 forest elephants of Kakum National Park, Ghana. *Molecular Ecology*, **12**, 1389-1402.
851 Ernest HB, Penedo CT, May BP et al. (2000) Molecular tracking of mountain lions in the Yosemite
852 Valley region in California: genetic analysis using microsatelites e faecal DNA. *Molecular*
853 *Ecology*, **9**, 433-441.
854 Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: a new series of programs to perform
855 population genetics analyses under Linux and Windows. *Molecular Ecology Resources*, **10**,
856 564-567.

857 Farrel Le, Roman J, Sunquist ME (2000). Dietary separation of sympatric carnivores identified by
858 molecular analysis of scats. *Molecular Ecology*, **9**, 1583-1590.
859 Ferrando A, Lecis R, Domingo-Roura X, Ponsà M (2008) Genetic diversity and individual
860 identification of reintroduced otters (*Lutra lutra*) in north-eastern Spain by DNA genotyping of
861 spraints. *Conservation Genetics*, **9**, 129-139.
862 Frantz AC, Pope LC, Carpenter PJ et al. (2003). Reliable microsatellite genotyping of the Eurasian
863 badger (*Meles meles*) using faecal DNA. *Molecular Ecology*, **12**, 1649-1661.
864 Gallo-Reynoso JP, Ramos-Rosas NN, Rangel-Aguilar O (2008) Aquatic bird predation by
865 neotropical river otter (*Lontra longicaudis annectens)*, at Río Yaqui, Sonora, México. *Revista*
866 *Mexicana de Biodiversidad*, **79**, 275-279.
867 Gese EM (2001) Monitoring of terrestrial carnivore populations. In: (Gittleman JL, Funk SM,
868 Macdonald D, Wayne RK, eds.) *Carnivore conservation*. Cambridge University Press,
869 Cambridge, UK.
870 Goodnight KF, Queller DC (1999) Computer software for performing likelihood tests of pedigree
871 relationship using genetic markers. *Molecular Ecology*, **8**, 1231-1234.
872 Goossens B, Chikhi L, Utami SS et al. (2000) A multi-samples, multi-extracts approach for
873 microsatellite analysis of faecal samples in an arboreal ape. *Conservation Genetics*, **1**, 157-
874 162.
875 Goudet J (2002) FSTAT: A Program to estimate and test gene diversities and fixation indices.
876 Lausanne: Institute of Ecology, Switzerland.
877 Haag T, Santos AS, De Angelo C et al. (2009) Development and testing of an optimized method for
878 DNA-based identification of jaguar (*Panthera onca*) and puma (*Puma concolor*) faecal
879 samples for use in ecological and genetic studies. *Genetica*, **136**, 505-512.
880 Haag T, Santos AS, Sana DA et al. (2010) The effect of habitat fragmentation on the genetic
881 structure of a top predator: loss of diversity and high differentiation among remnant
882 populations of Atlantic Forest jaguars (*Panthera onca*). *Molecular Ecology*, **19**, 4906-4921.
883 Hájková P, Zemanová B, Bryja J et al. (2006) Factors affecting success of PCR amplification of
884 microsatellite loci from otter faeces. *Molecular Ecology Notes*, **6**, 559-562.

885 Hájková P, Zemanová B, Roche K, Hájek B (2009) An evaluation of field and noninvasive genetic
886 methods for estimating Eurasian otter population size. *Conservation Genetics*, **10**, 1667–1681.
887 Hedmark E, Ellegren H (2007) DNA-based monitoring of two newly founded Scandinavian
888 wolverine populations. *Conservation Genetics*, **8**, 843-852.
889 Huang C-C, Hsu Y-C, Lee L-L, Li S-H (2005) Isolation and characterization of tetramicrosatellite
890 DNA markers in the Eurasian otter (*Lutra lutra*). *Molecular Ecology Notes*, **5**, 314-316.
891 Hung C-M, Li S-H, Lee L-L (2004) Faecal DNA typing to determine the abundance and spatial
892 organisation of otters (*Lutra lutra*) along two stream systems in Kinmen. *Animal Conservation*,
893 **7**, 301-311.
894 Janssens X, Fontaine MC, Michaux JR et al. (2008) Genetic pattern of the recent recovery of
895 European otters in southern France. *Ecography*, **31**,176-186.
896 Jansman HAH, Chanin PRF, Dallas JF (2001) Monitoring otter populations by DNA typing of
897 spraints. *IUCN Otter Specialist Group Bulletin*, **18**, 12-19.
898 Kalz B, Jewgenow K, Fickel J (2006) Structure of an otter (*Lutra lutra*) population in Germany –
899 results of DNA and hormone analyses from faecal samples. *Mammalian Biology*, **71**, 321-335.
900 Kalinowski ST, AP Wagner, ML Taper (2006) ML-Relate: a computer program for maximum
901 likelihood estimation of relatedness and relationship. *Molecular Ecology Notes*, **6**, 576-579.
902 Kasper CB, Bastazini VAG, Salvi J, Grillo HCZ (2008) Trophic ecology and the use of shelters and
903 latrines by the Neotropical otter (*Lontra longicaudis*) in the Taquari Valley, Southern Brazil.
904 *Iheringia*, Série Zoológica, **98**, 469-474.
905 Koelewijn HP, Pérez-Haro M, Jansman HAH et al. (2010) The reintroduction of the Eurasian otter
906 (*Lutra lutra*) into the Netherlands: hidden life revealed by noninvasive genetic monitoring.
907 *Conservation Genetics*, **11**, 601-614.
908 Kohn MH, York EC, Kamradt DA et al. (1999) Estimating population size by genotyping faeces.
909 *Proceedings of the Royal Society of London B*, **266**, 657-663.
910 Kruuk H (2006) Scent marking and interactions: social behavior. In: *Otters: ecology, behavior and*
911 *conservation*. Oxford University Press, Oxford, UK.
912 Kruuk H, Moorhouse A (1991) The spatial organization of otters (*Lutra lutra* L.) in Shetland. *Journal*
913 *of Zoology*, **224**, 41-57.

914 Kruuk H, Carss DN, Conroy JWH and Durbin L (1993) Otter (*Lutra lutra* L.) numbers and fish
915 productivity in rivers in N.E. Scotland. *Symposia of the Zoological Society of London*, **65**, 171-
916 191.
917 Lampa S, Gruber B, Henle K, Hoehn M (2008) An optimisation approach to increase DNA
918 amplification success of otter faeces. *Conservation Genetics*, **9**, 201-210.
919 Lanszki J, Hidas A, Szentes K et al. (2008) Relative spraint density and genetic structure of otter
920 (*Lutra lutra*) along the Drava River in Hungary. *Mammalian Biology*, **73**, 40-47.
921 Macdonald SM, Mason CF (1990) Threats. In*: Otters: An Action Plan for their Conservation* (eds.
922 Foster-Turley P, Macdonald S, Mason C). IUCN Otter Specialist Group.
923 Marshall TC, Slate J, Kruuk LEB, Pemberton JM (1998) Statistical confidence for likelihood-based
924 paternity inference in natural populations. *Molecular Ecology*, **7**, 639-655.
925 Melquist WE, Hornocker MG (1983) Ecology of river otters in west central Idaho. *Wildlife*
926 *Monographs*, **83**.
927 Melquist WE, Polechla Jr. PJ, Toweill D (2003) River otter (*Lontra canadensis*). *In*: (Feldhamer A,
928 Thompson BC, Chapman JA, eds.) *Wild mammals of North America: biology management,*
929 *and conservation*. Second edition. Johns Hopkins University Press, Baltimore, Maryland, USA.
930 Michalski F, Valdez FP, Norris D et al. (2011) Successful carnivore identification with faecal DNA
931 across a fragmented Amazonian landscape. *Molecular Ecology Resources*, **11**, 862-871.
932 Miller CR, Joyce P, Waits LP (2005) A new method for estimating the size of small populations
933 from genetic mark-recapture data. *Molecular Ecology*, **14**, 1991-2005.
934 Mills LS, Citta JJ, Lair KP et al. (2000) Estimating animal abundance using noninvasive DNA
935 sampling: promise and pitfalls. *Ecological Applications*, **10**, 283-294.
936 Miotto RA, Begotti RA, Cervini M et al. (2011) Genetic diversity and population structure of pumas
937 (*Puma concolor*) in southeastern Brazil: implications for conservation in a human-dominated
938 landscape. *Conservation Genetics*, **12**, 1447-1455.
939 Morin PA, Chambers KE, Boesch C, Vigilant L (2001) Quantitative polymerase chain reaction
940 analysis of DNA from noninvasive samples for accurate microsatellite genotyping of wild
941 chimpanzees (*Pan troglodytes verus*). *Molecular Ecology*, **10**, 1835-1844.

942 Mowry RA, Gompper ME, Beringer J, Eggert LS (2011) River otter population size estimation using
943 noninvasive latrine surveys. *Journal of Wildlife Management*, **75**, 1625-1636.
944 Murphy MA, Waits LP, Kendall KC (2003) The influence of diet on faecal DNA amplification in
945 brown bears (*Ursus arctos*). *Molecular Ecology*, **12**, 2261-2265.
946 Palomares F, Godoy JA, Piriz A et al (2002) Faecal genetic analysis to determine the presence and
947 distribution of elusive carnivores: design and feasibility for the Iberian lynx. *Molecular Ecology*,
948 **11**, 2171-2182.
949 Parera A (1996) Las nutrias verdaderas de La Argentina. *Boletín Técnico de Fundación Vida*
950 *Silvestre Argentina*. Buenos Aires, Argentina.
951 Pompanon F, Bonin A, Bellemain E, Taberlet P (2005) Genotyping errors: causes, consequences
952 and solutions. *Nature Reviews Genetics*, **6**, 847-856.
953 Powell RA (1979) Mustelid spacing patterns: variations on a theme by *Mustela*. *Zeitschrift für*
954 *Tierpsychologie*, **51**, 153-165.
955 Prigioni C, Remonti L, Balestrieri A (2006a) Otter *Lutra lutra* movements assessed by genotyped
956 spraints in Southern Italy. *Hystrix Italian Journal of Mammalogy*, **17**, 91–96.
957 Prigioni C, Remonti L, Balestrieri A et al. (2006b) Estimation of European otter (*Lutra lutra*)
958 population size by fecal DNA typing in Southern Italy. J Mammal, 87: 855–858.
959 Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus
960 genotype data. *Genetics*, **155**, 945-959.
961 Reed JZ, Tollit DJ, Thompson PM, Amos W (1997) Molecular scatology: the use of molecular
962 genetic analysis to assign species, sex and individual identity to seal faeces. *Molecular*
963 *Ecology*, **6**, 225-324.
964 Rice WR (1989) Analyzing tables of statistical tests. *Evolution*, **43**, 223-225.
965 Rousset F, Raymond M (1995) Testing heterozygote excess and deficiency. *Genetics*, **140**, 1413-
966 1419.
967 Ruiz-Olmo J, Delibes M, Zapata SC (1998) External morphometry, demography and mortality of
968 the otter *Lutra lutra* (Linnaeus, 1758) in the Iberian peninsula. *Galemys*, **10**, 239-251.
969 Ruiz-Olmo J, Lopez-Martin JM, Palazon S (2001) The influence of fish abundance on the otter
970 (*Lutra lutra*) populations in Iberian Mediterranean habitats. *Journal of Zoology*, **254**, 325-336.

971 Sandell M (1989) The mating tatics and spacing patterns of solitary carnivores. In: (Gittleman JL
972 ed.) *Carnivore behavior, ecology, and evolution*. Cornell University Press, Ithaca, NY.
973 Sidorovich VE, Pikulik MM (2002) Factors allowing high density of otters in Eastern Europe. *IUCN*
974 *Otter Specialist Group Bulletin*, **19A**, 326-333.
975 Stenglein JL, Waits LP, Ausband DE et al. (2011) Estimating grey wolf pack size and family
976 relationships using noninvasive genetic sampling at rendezvous sites. *Journal of Mammalogy*,
977 **92**, 784-795.
978 Taberlet P, Griffin S, Goossens B et al. (1996) Reliable genotyping of samples with very low DNA
979 quantities using PCR. Nucleic Acids Researches, **24**, 3189-3194.
980 Taberlet P, Luikart G (1999) Noninvasive genetic sampling and individual identification. *Biological*
981 *Journal of the Linnean Society of London*, **68**, 41 – 55.
982 Trinca CS, Waldemarin HF, Eizirik E (2007) Genetic diversity of the Neotropical otter (*Lontra*
983 *longicaudis* Olfers, 1818) in Southern and Southeastern Brazil. *Brazilian Journal of Biology*,
984 **64**, 813–818.
985 Trinca CS, Eizirik E (2012) Molecular sexing of the Neotropical otter (*Lontra longicaudis*)
986 noninvasive samples. *Conservation Genetics Resources*, DOI 10.1007/s12686-011-9595-0.
987 Trinca CS, de Thoisy B, Rosas FCW, Waldemarin HF, Koepfli KP, Vianna JA, Eizirik E (in press)
988 Phylogeography and demographic history of the Neotropical otter (*Lontra longicaudis*). J
989 Hered.
990 Valière N (2002) GIMLET: a computer program for analysing genetic individual identification data.
991 *Molecular Ecology Notes*, **2**, 377–379.
992 Waits LP, Luikart G, Taberlet P (2001) Estimating the probability of identity among genotypes in
993 natural populations: cautions and guidelines. *Molecular Ecology*, **10**, 249-256.
994 Waldemarin, H.F. & Alvarez, R. (2008) *Lontra longicaudis*. In: *IUCN 2011*. IUCN Red List of
995 Threatened Species. Version 2011.1. <www.iucnredlist.org>. Downloaded on 02 February
996 2012.
997 Weber LI, Hildebrand CG, Ferreira A et al. (2009) Microsatellite genotyping from faeces of
998 *Lontra longicaudis* from southern Brazil. *Iheringia, Série Zoológica*, **99**, 5 – 11.

999 Wilberg MJ, Dreher BP (2004) GENECAP: a program for analysis of multilocus genotype data for
1000 non-invasive sampling and capture-recapture population estimation. *Molecular Ecology Notes*,
1001 **4**, 783 - 785.
1002 Zhan XJ, Zhang ZJ, Wu H et al. (2007) Molecular analysis of dispersal in giant pandas. *Molecular*
1003 *Ecology*, **16**, 3792-3800.
1004
1005 **Table Legends**
1006 Table 1. Sampling information for the Neotropical otter noninvasively collected samples along the
1007 two-year survey. Each sampling occasion is indicated along with their respective periods of
1008 sampling and the correspondent season. Number of collected and successfully genotyped samples
1009 are also indicated along with the number of individuals identified in each period.
1010
1011 Table 2. Measures of microsatellite diversity in the noninvasively identified otters from the Maquiné
1012 valley investigated in this study.
1013
1014 Table 3. Values of relatedness (*r*) among individuals in the study area.
1015
1016 Table 4. Mean distances of related dyads of both genders estimated for different classes of
1017 relatedness.
1018
1019 **Figure Legends**
1020 Fig 1. Study area in the Maquiné river basin, southern Brazil, showing the location of the three
1021 sampled streams: Lajeado (L), Forqueta (F), and Encantado (E).
1022
1023 Fig 2. Spatial distribution of the identified individuals in the study area during the two-year survey:
1024 A) resident individuals that were present in the area on both years of sampling; B) resident and
1025 transient otters identified in 2008; and C) resident and transient animals observed in 2009.
1026 Individuals are represented by their identifier codes (ex. F1) and the approximate location of each

capture is indicated by colored circles. Multiple captures at the same location are represented by a single circle.

Fig 3. Minimum home range (MHR) of the identified otters represented as straight lines, along the Forqueta (F), Lajeado (L), and Encantado (E) streams. Transient individuals are indicated by a dot at the approximate location of the sampling spot where they were captured. Sampling occasions and the number of samples representing each individual are also indicated. Individuals that were observed on both years of sampling are represented by two lines, representing their recorded movements on each year. Male otters that were identified on two watercourses are represented in both respective graphs.

Fig 4. Population size and 95% CI estimates for each set of temporally grouped sampling occasions.

Fig 5. Minimum home range (MHR) of resident otters observed during the total sampling period. Dark grey bars represent the stream area occupied by males, and light grey bars indicate female area of occurrence.

Fig 6. Heredogram depicting the reconstructed genealogical relationships among otters identified in this study. Colors are representative of each watercourse: orange: Forqueta stream, blue: Encantado, and green: Lajeado. Two-colored squares indicate those males captured on more than one stream. Question marks indicate presumed individuals that were not captured in the study. Thicker edges indicate resident individuals, while thin edges represent transient otters. Dotted lines represent cases in which the exact relationship was uncertain.

Supporting Information

T1. List of the blood/tissue samples of the Neotropical otter used for genetic characterization of the microsatellite marker panel employed in this study.

Table 1. Sampling information for the Neotropical otter noninvasively collected samples along the two-year survey. Each sampling occasion is indicated along with their respective periods of sampling and the corresponding season. Number of collected and successfully genotyped samples are also indicated along with the number of individuals identified in each period.

Year of collection	Sampling occasion	Period of sampling	N° of collected samples	N° of genotyped samples	N° of individuals identified	Season
2008	I	23 - 26/January	21	6	5	Summer
	II	15 -17/March	33	10	6	Summer
	III	07- 08, 14 - 15/June	27	10	7	Fall
	IV	06 - 08/August	30	13	9	Winter
	V*	22 - 23/November	2	2	2	Spring
2009	VI	04 - 06/February	23	9	7	Summer
	VII	10 - 12/April	29	8	7	Fall
	VIII	11 - 12, 18 - 19/July	43	32	14	Winter
	IX*	26 - 28/September	11	6	4	Spring
	X	27 - 30/December	35	8	6	Summer

* sampling occasions affected by water level changes.

Table 2. Measures of microsatellite diversity in the noninvasively identified otters from the Maquiné valley investigated in this study.

Locus	Original Species	Dye	Annealing (C°)	N	A[a]	Size range (bp)[b]	AR	H_o	H_e	Freq (Null)	F_{IS}	ADO	FA
Lut453[1]	*Lutra lutra*	FAM	58	26	6 (7)	136-148	5.540	0.808	0.682	-0.0995	-0.189	0.37	0.15
Lut733[1]	*Lutra lutra*	NED	58	25	6 (8)	154-194	5.597	0.840	0.735	-0.0764	-0.147	0.31	0.08
Lut701[1]	*Lutra lutra*	FAM	64	28	7 (9)	154-202	5.710	0.679	0.691	0.0002	0.018	0.66	0.11
Lut782[1]	*Lutra lutra*	NED	58	16	7 (9)	162-194	6.000	0.938	0.786	-0.1059	-0.200	0.38	0.12
04OT04[2]	*Lutra lutra*	FAM	62	28	2 (2)	185-189	2.000	0.571	0.486	-0.0900	-0.180	0.48	0.03
04OT17[2]	*Lutra lutra*	FAM	64	26	6 (9)	172-204	5.603	0.923	0.777	-0.1063	-0.193	0.33	0.09
04OT22[2]	*Lutra lutra*	FAM	64	28	11 (14)	143-207	9.446	0.929	0.879	-0.0365	-0.056	0.36	0.10
RIO11[3]	*Lontra canadensis*	NED	66	24	8 (12)	166-190	7.775	0.958	0.858	-0.0694	-0.120	0.28	0.09
Mean	-	-	-	-	6.5 (8.75)	-	5.959	0.831	0.737	-0.0730	-0.131	0.40	0.08

[1] Reference: Dallas & Piertney 1998.

[2] Refenrece: Huang et al. 2005.

[3] Reference: Beheler et al. 2005.

[a] Values in parentheses indicate the number of alleles detected when a broader sample set is assessed, which comprises good-quality DNA from a regional set of individuals (n = 28), in addition to those identified noninvasively here (see text for details).

[b] Estimated from the full set of individuals, including non-invasive samples (n = 28) and good-quality DNA (n = 28).

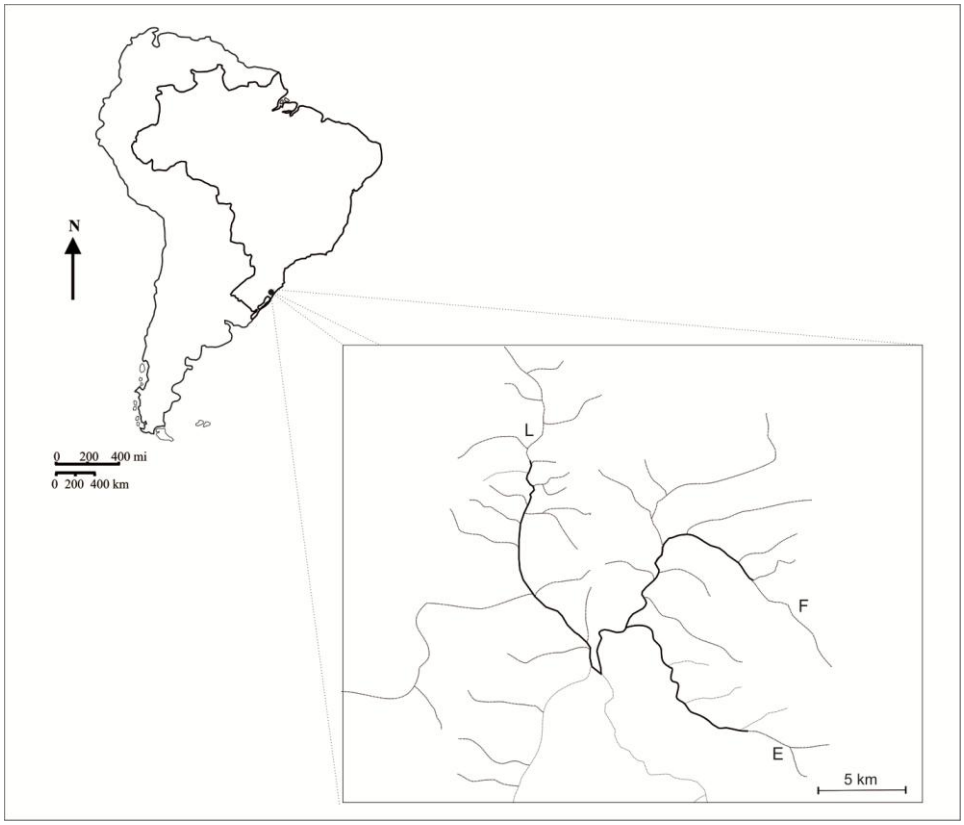
Table 3. Values of relatedness (*r*) among individuals in the study area.

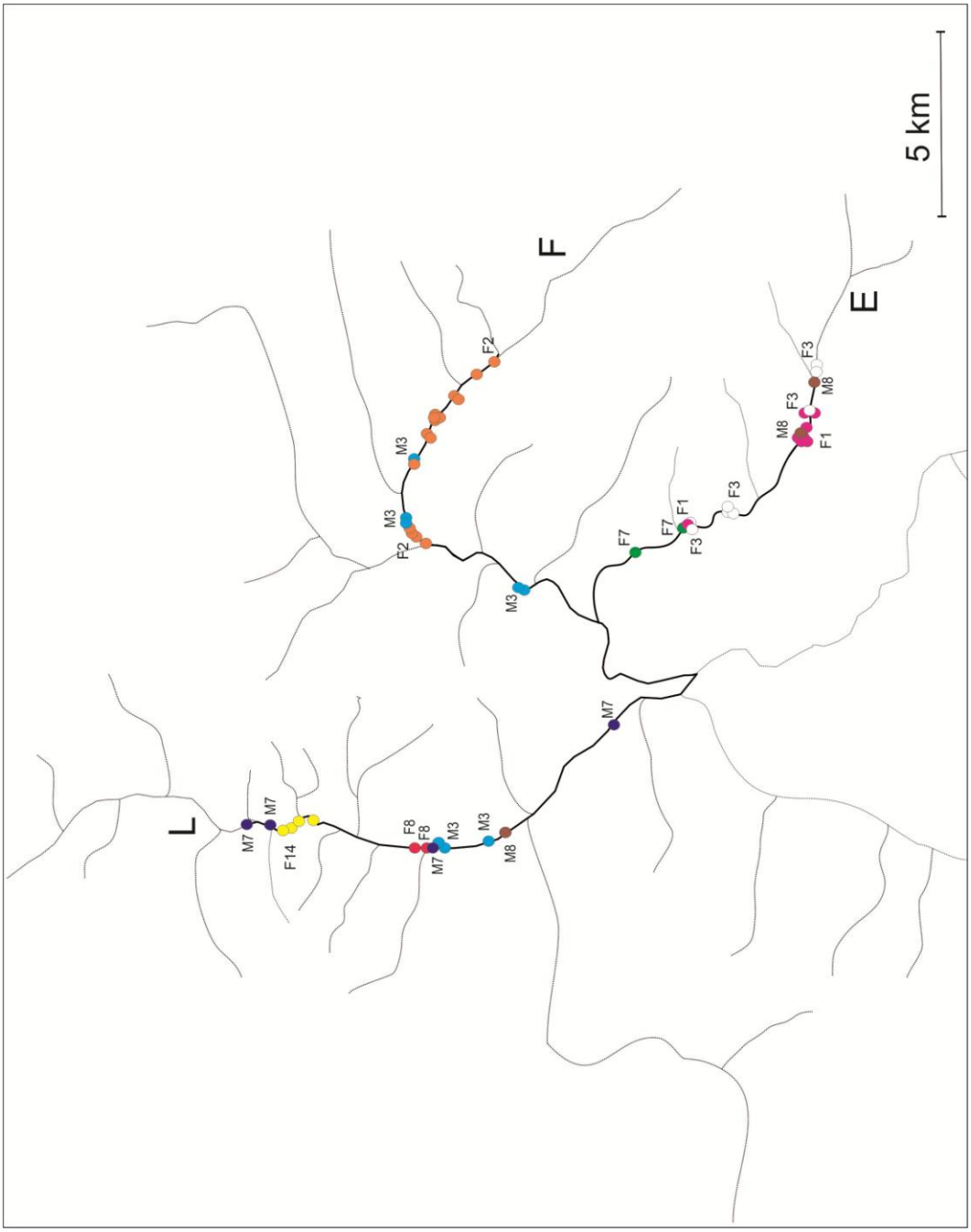
	All streams	Lajeado	Encantado	Forqueta
Female	0.1119 (SE: 0.187)	0.062 (SE: 0.059)	0.490 (SE: 0.069)	0.237 (SE: 0.065)
Male	0.1012 (SE: 0.020)	0.136 (SE: 0.039)	0.128 (SE: 0.090)	0.081 (SE: 0.052)
Total	0.1011 (SE: 0.009)	0.115 (SE: 0.018)	0.196 (SE: 0.045)	0.122 (SE: 0.024)

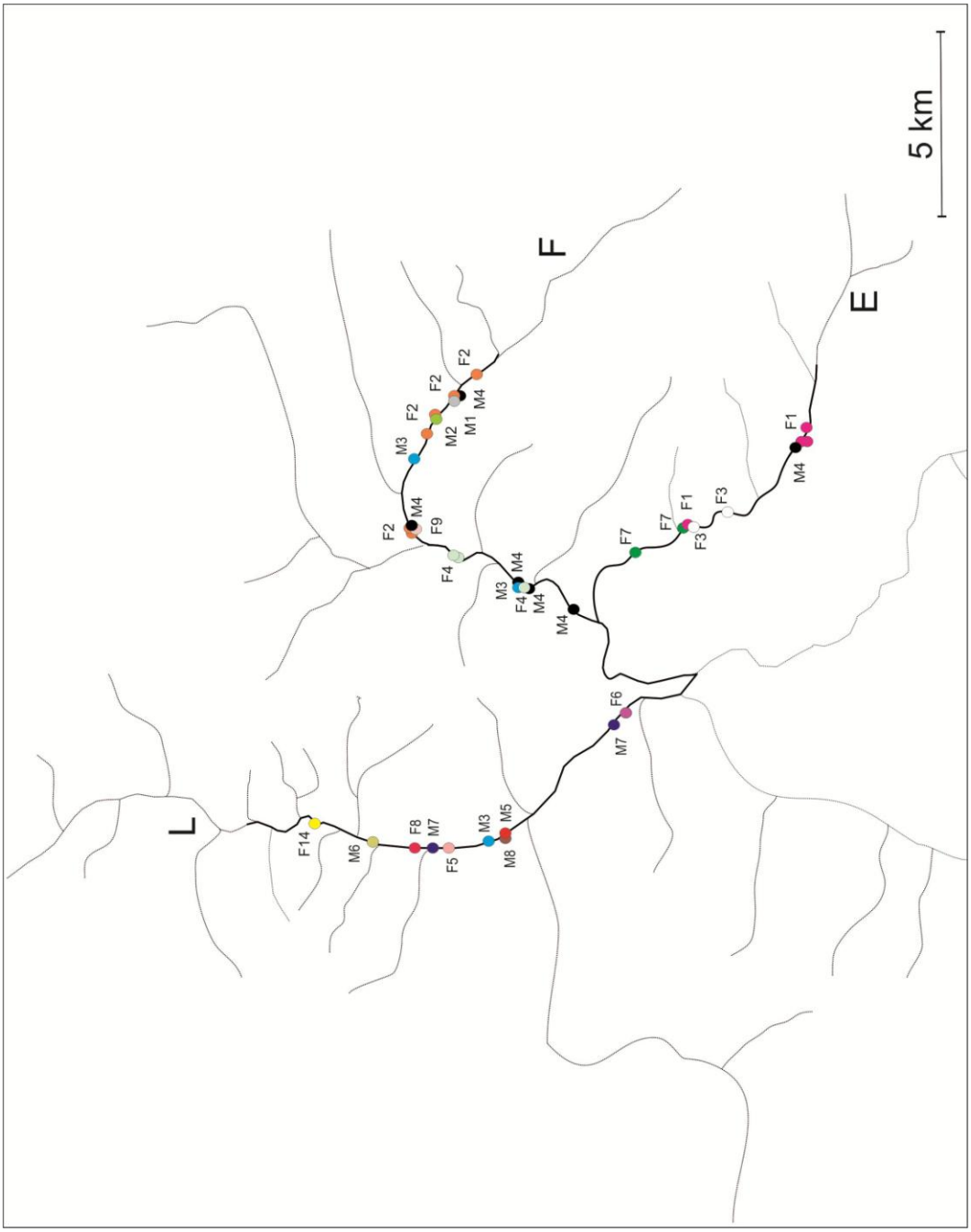
Table 4. Mean distances of related dyads of both genders estimated for different classes of relatedness.

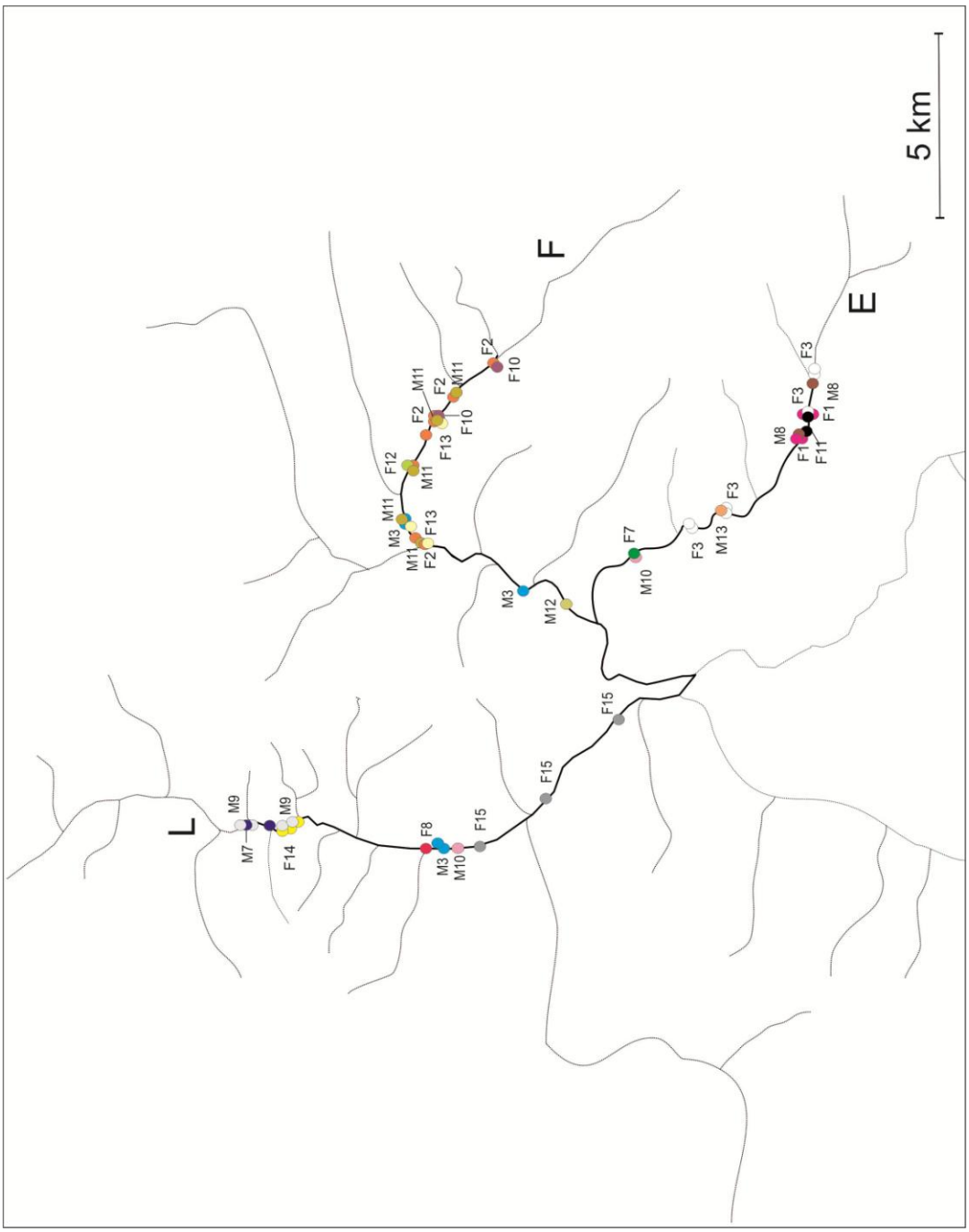
r coefficient category	Male dyad distance (km)[a, b]	Female dyad distance (km)[a, b]
≥ 0.5	6.87 ± 3.62 (7)/9.56 ± 0.02 (2)	4.76 ± 4.6 (13)/4.26 ± 4.26 (7)
0.25 - 0.49	10.88 ± 4.58 (4)/9.66 ± 6.72 (2)	8.59 ± 6.27(8)/1.65 ± 1.65 (2)
≤ 0.24	9.23 ± 5.66 (67)/7.6 ± 5.54 (21)	11.43 ± 5.28 (84)/13.74 ± 4.61 (36)*

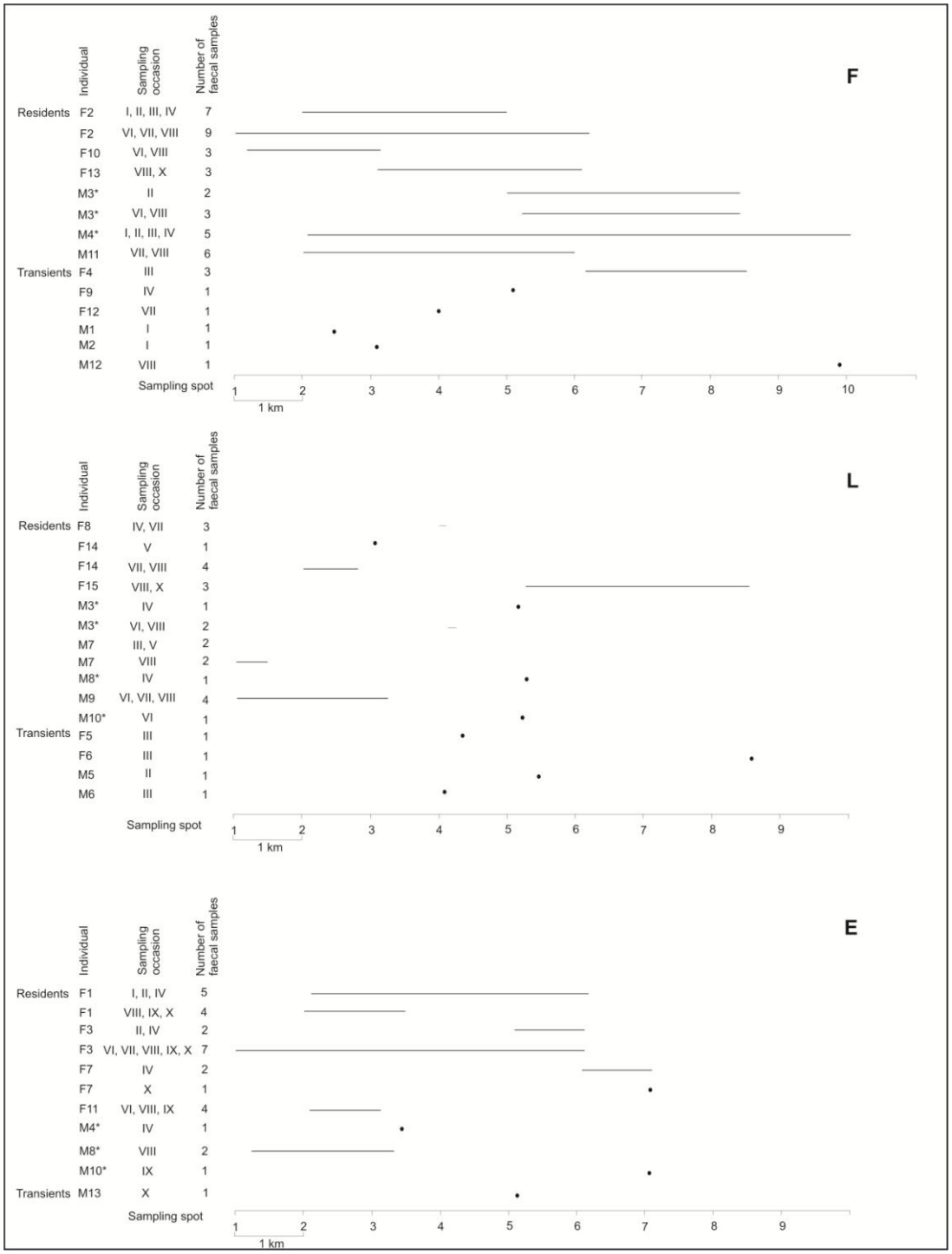
[a] Values are given for all animals included in each category/considering residents only.
[b] Numbers in parentheses indicate the number of comparisons included in a particular estimate.
* Significant for $\alpha = 0.05$.

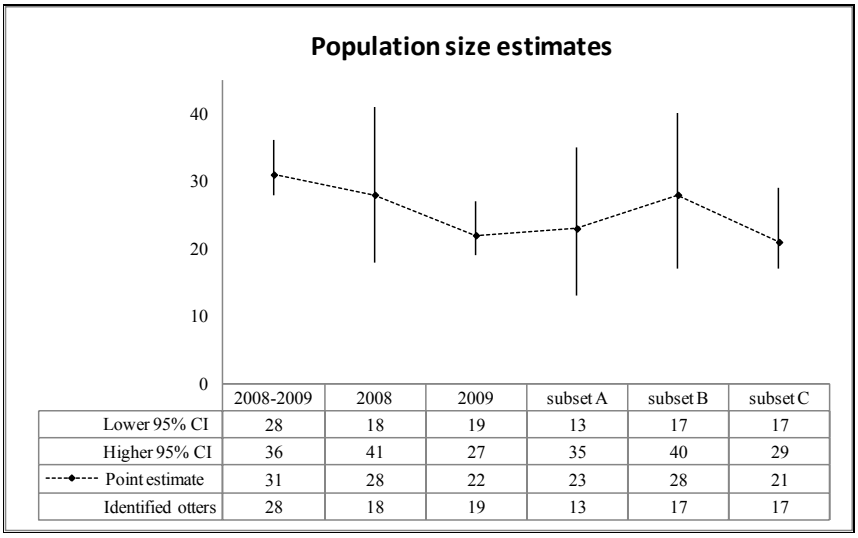

N
0 200 400 mi
0 200 400 km
L
F
E
5 km


5 km
L
F
E
M7
M7
F14
F8
F8
M7
M3
M3
M8
M7
M3
M3
F2
F2
M3
F7
F7
F3
F1
F3
M8
F3
F1
M8
F3

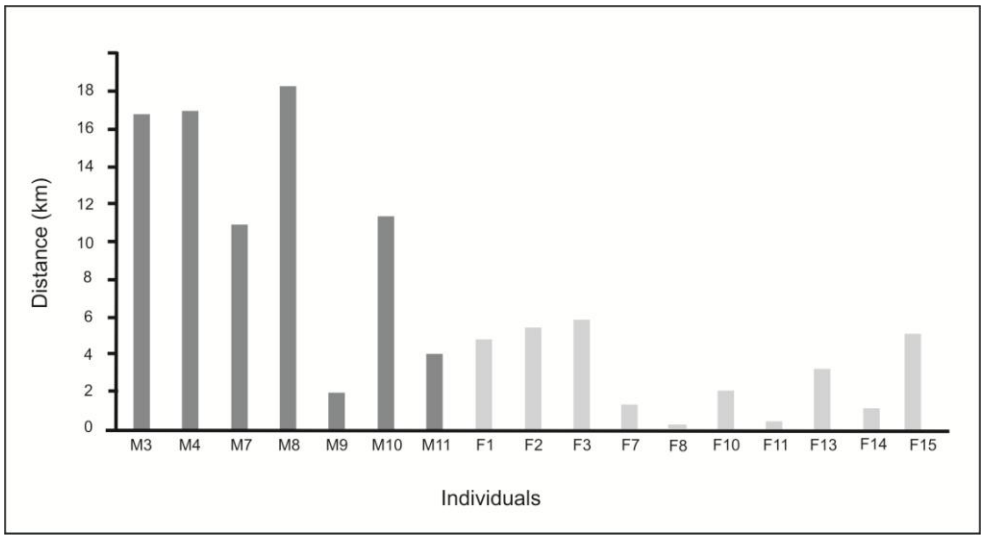

5 km
L
F
E
F14
M6
F8
M7
F5
M3
M5
M8
F6
M7
M4
F4
M4
M3
F4
M4
F2
M4
F9
F4
F2
M3
F2
M2
F2
M1
M4
F7
F7
F1
F3
F3
F1
M4

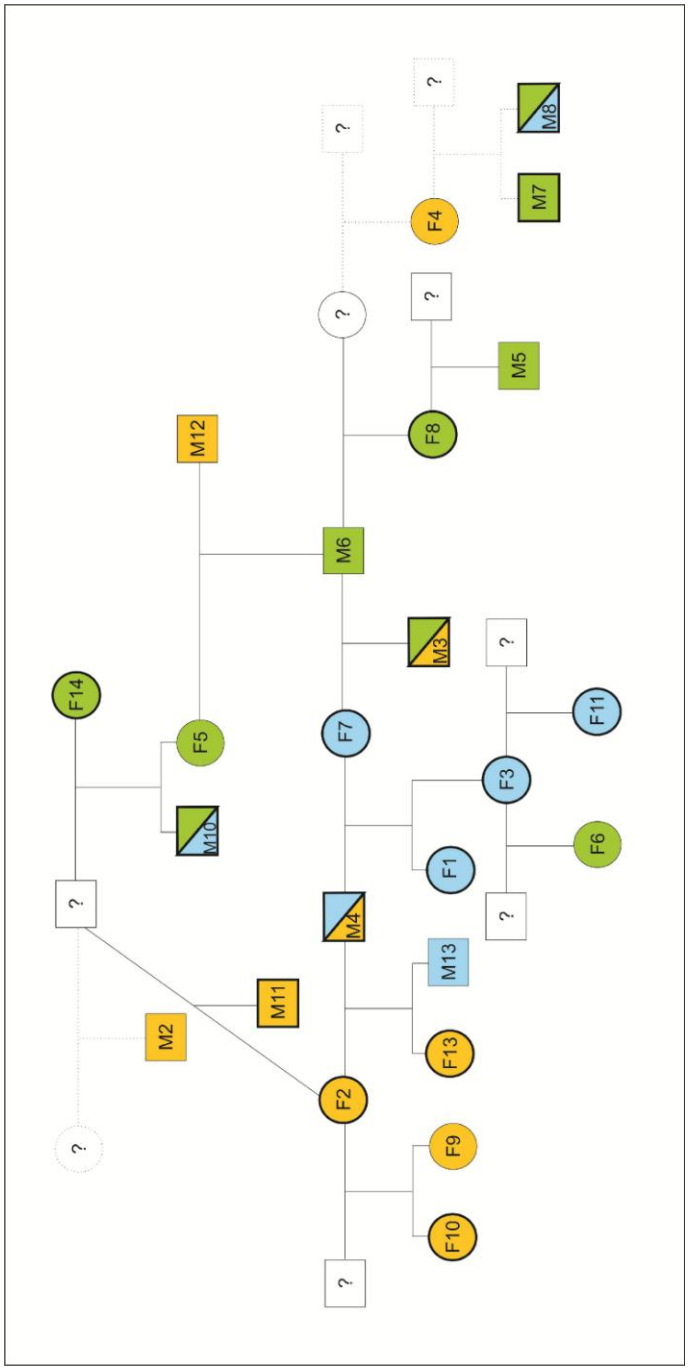

5 km
L
F
E
M9
M7
F14
M9
F8
M3
M10
F15
F15
F15
M3
M12
F7
M10
F3
M13
F3
F3
M8
F1
F11
F3
F1
M8
F2
F10
M11
F2
M11
F10
F2
F13
F12
M11
M11
M3
F13
M11
F2


F
Individual
Sampling occasion
Number of faecal samples
Residents F2 I, II, III, IV 7
F2 VI, VII, VIII 9
F10 VI, VIII 3
F13 VIII, X 3
M3* II 2
M3* VI, VIII 3
M4* I, II, III, IV 5
M11 VII, VIII 6
Transients F4 III 3
F9 IV 1
F12 VII 1
M1 I 1
M2 I 1
M12 VIII 1
Sampling spot
1 2 3 4 5 6 7 8 9 10
1 km
L
Individual
Sampling occasion
Number of faecal samples
Residents F8 IV, VII 3
F14 V 1
F14 VII, VIII 4
F15 VIII, X 3
M3* IV 1
M3* VI, VIII 2
M7 III, V 2
M7 VIII 2
M8* IV 1
M9 VI, VII, VIII 4
M10* VI 1
Transients F5 III 1
F6 III 1
M5 II 1
M6 III 1
Sampling spot
1 2 3 4 5 6 7 8 9
1 km
E
Individual
Sampling occasion
Number of faecal samples
Residents F1 I, II, IV 5
F1 VIII, IX, X 4
F3 II, IV 2
F3 VI, VII, VIII, IX, X 7
F7 IV 2
F7 X 1
F11 VI, VIII, IX 4
M4* IV 1
M8* VIII 2
M10* IX 1
Transients M13 X 1
Sampling spot
1 2 3 4 5 6 7 8 9
1 km



	2008-2009	2008	2009	subset A	subset B	subset C
Lower 95% CI	28	18	19	13	17	17
Higher 95% CI	36	41	27	35	40	29
Point estimate	31	28	22	23	28	21
Identified otters	28	18	19	13	17	17


Distance (km)
0
2
4
6
8
10
12
14
16
18
M3
M4
M7
M8
M9
M10
M11
F1
F2
F3
F7
F8
F10
F11
F13
F14
F15
Individuals


?
?
?
M8
M7
F4
?
?
M5
F8
M12
M6
M3
?
F14
F5
F7
F11
F3
M10
F1
F6
?
M4
M13
M11
M2
F13
F2
F9
?
F10
?

T1. List of the blood/tissue samples of the Neotropical otter used for genetic characterization of the microsatellite marker panel employed in this study.

ID	Sample	Geographic origin	Reference
bLlo01	muscle	Rio Grande do Sul, Brazil	G. Bencke and J. R. Marinho
bLlo02	muscle	Santa Catarina, Brazil	Sapucaia do Sul Zoo
bLlo03	muscle	Rio Grande do Sul, Brazil	Carnívoros do RS Project
bLlo04	muscle	Rio Grande do Sul, Brazil	Carnívoros do RS Project
bLlo05	muscle	Gravataí, Rio Grande do Sul, Brazil	G. L. Gonçalves
bLlo06	muscle	Rio Grande do Sul, Brazil	P. H. Ott
bLlo07, bLlo08	muscle	Torres, Rio Grande do Sul, Brazil	P. Colombo
bLlo11	muscle	Rio Grande do Sul, Brazil	A. P. Brandt
bLlo12	muscle	Dois Irmãos, Rio Grande do Sul, Brazil	Prefeitura de Dois Irmãos / I. Fick
bLlo24	muscle	Rio Grande do Sul, Brazil	T. R. O. de Freitas
bLlo34	muscle	Blumenau, Santa Catarina, Brazil	FURB / S. Althoff
bLlo42	muscle	Capela de Santana, Rio Grande do Sul, Brazil	Canoas Mini Zoo / M. Martins
bLlo58	muscle	Osório, Rio Grande do Sul, Brazil	P. Colombo, C. Zank and G. Volkmer
bLlo75	muscle	Urubici, Santa Catarina, Brazil	C. Castilho and L.G. M. de Sá
bLlo76	muscle	Tapes, Rio Grande do Sul, Brazil	C. B. Kasper and M.L. Fontoura-Rodrigues
bLlo77	muscle	Campo Belo do Sul, Santa Catarina, Brazil	Boursheid S.A Engenharia e Meio Ambiente
bLlo78	muscle	Triunfo, Rio Grande do Sul, Brazil	FZB-RS / M. Jardim
bLlo81	muscle	Itá, Santa Catarina, Brazil	-
bLlo86	muscle	Triunfo, Rio Grande do Sul, Brazil	C. B. Kasper
bLlo87	muscle	Feliz, Rio Grande do Sul, Brazil	C. B. Kasper
bLlo90	muscle	Eldorado do Sul, Rio Grande do Sul, Brazil	L. E. Costa-Schmidt and P. Colombo
bLlo91	muscle	Uruguaiana, Rio Grande do Sul, Brazil	J. Konemann
bLlo92	muscle	Rio Grande do Sul, Brazil	SEMA
bLlo96	blood	Santa Catarina, Brazil	IMA / M. S. Reis and R. Veloso
bLlo97	muscle	Pelotas, Rio Grande do Sul, Brazil	GEMARS / R. Machado
bLlo120	muscle	Rio Grande do Sul, Brazil	GEMARS / R. Machado and P. H. Ott
bLlo121	muscle	Tramandaí, Rio Grande do Sul, Brazil	GEMARS / R. Machado

Capítulo 7 – Discussão Geral

O trabalho aqui apresentado teve como principais objetivos avaliar os níveis de diversidade genética e os padrões de estruturação populacional da lontra neotropical (*Lontra longicaudis*) através da análise de segmentos do DNA mitocondrial e de locos de microssatélite, padronizar metodologias para embasar o uso de amostras não-invasivas em estudos moleculares, bem como investigar diversos parâmetros demográficos desta espécie através da análise de DNA extraído de amostras não-invasivas coletadas em campo.

Além das conclusões e interpretações aqui apresentadas, as informações obtidas abrem caminho para inúmeras outras perguntas, apresentando assim novas perspectivas para o estudo de diversos aspectos da biologia e história evolutiva desta lontra. Desta forma, em última análise, este trabalho tem como objetivo final servir como base para estudos moleculares futuros, que visem tanto à continuidade das análises filogeográficas aqui iniciadas, quanto à investigação aspectos ecológicos e populacionais em escala local e/ou regional. Ambos os direcionamentos são fundamentais para aumentar o atual escasso conhecimento sobre esta espécie e, consequentemente, tem implicações diretas para a sua conservação.

O conjunto de dados apresentados nos capítulos 3 e 4 revelaram que a lontra neotropical apresenta moderada a alta variabilidade genética em ambos os marcadores moleculares analisados (DNA mitocondrial [DNAmt] e microssatélites). Um grande número de haplótipos foi observado nos segmentos de DNAmt empregados, bem como uma grande diversidade de alelos nos locos de microssatélite, resultando em uma variabilidade que pode ser considerada, de forma geral, maior do que a reportada para outros carnívoros neotropicais com distribuição semelhante, bem como para outras espécies de lontra.

As análises do DNAmt sugeriram que a variabilidade genética da lontra neotropical encontra-se subdividida em pelo menos quatro linhagens distintas. Dois clados foram observados, um representado prioritariamente por haplótipos encontrados no Brasil (grupo geográfico denominado ‘ESA’ no capítulo 3) e outro incluindo quase todas as amostras da região amazônica e Guiana Francesa (chamado de ‘Amazon’). Além destes dois clados, duas outras linhagens distintas foram identificadas, uma na Bolívia e outra, apresentando uma divergência mais profunda, localizada na região da Cordilheira Central dos Andes, na Colômbia. Infelizmente, estas

duas últimas foram representadas por uma apenas amostra cada, o que impediu inferências mais aprofundadas dos padrões filogeográficos envolvendo estas regiões.

A análise específica dos dois principais clados indicou a ocorrência de haplótipos intimamente relacionados, conectados a um haplótipo central mais frequente, sugerindo ainda para o clado 1 (predominantemente brasileiro) a ocorrência de expansão populacional deste grupo. Além disso, estes dados indicaram a separação recente dos grupos de populações de lontra na América do Sul, enquanto que a maior divergência genética observada em relação ao haplótipo da Colômbia, evidenciada pelo grande número de passos mutacionais, sugeriu uma história evolutiva mais antiga nesta região. A localização geográfica deste haplótipo na Cordilheira Central dos Andes sugere que esta cadeia de montanhas possa estar relacionada com o processo de diferenciação das linhagens de *L. longicaudis* e destaca a importância de se estudar esta região em mais detalhe no futuro.

O padrão geral observado de estruturação genética mostrou ampla associação com a distribuição geográfica, sugerindo a presença de barreiras históricas ao fluxo gênico da espécie, as quais requerem análises biogeográficas aprofundadas. A inferência de uma partição genética marcada entre as regiões Noroeste e Leste da América do Sul, bem como a estrutura filogeográfica observada no grupo ESA, sugerem que o processo de colonização da América do Sul pela lontra neotropical possa ter ocorrido de acordo em um sentido Norte-Sul, o qual teria iniciado após eventos de especiação ocorridos já neste continente que seguiram a uma invasão inicial viabilizada pelo fechamento do Istmo do Panamá há cerca de 3 milhões de anos. Todavia, os fatores que levaram à diferenciação das linhagens da região amazônica daquelas observadas no restante do Brasil, ainda não são claros, dada a ausência de barreiras físicas claras entre as regiões. É ainda importante salientar que, considerando a amostragem parcial da distribuição da espécie, a obtenção de amostras provenientes de regiões intermediárias entre os dois grupos geográficos encontrados contribuirá para o refinamento do padrão filogeográfico observado, podendo até mesmo vir a indicar uma maior conectividade entre as duas regiões.

As análises dos marcadores nucleares microssatélite (capítulo 4) também revelaram uma partição genética significativa em *L. longicaudis*, embora em uma magnitude consideravelmente menor. Como reportado para o DNAmt, foi observada uma diferenciação genética moderada

envolvendo a região da Guiana Francesa (FG) e as outras áreas amostradas na América do Sul (Nordeste brasileiro [NB] e Sul da América do Sul [SSA]). Todavia, em contraste aos resultados observados para os segmentos mitocondriais, esta região apresentou valores significativos de F_{ST} e D_{EST} também em relação à região amazônica (Amazônia brasileira [BA]), apesar de a diferenciação genética observada ter sido pequena (F_{ST} = 0.06, p < 0.01; D_{EST} = 0.14). Embora baixos, valores significativos de F_{ST} também foram encontrados para as comparações entre SSA e BA x NB (representada por 5 amostras, enquanto que na análise do DNAmt foram analisadas apenas dois indivíduos), o que pode ser devido à presença de alelos privados em todas as populações associada a diferenças na frequência de ocorrência dos alelos mais comuns.

Em comparação com os dados mitocondriais, a moderada a baixa subdivisão populacional observada nos locos nucleares poderia resultar de um padrão diferencial de dispersão entre os sexos, resultando em um fluxo gênico mediado por machos de *L. longicaudis*. Um resultado que corrobora esta hipótese é a observação de ausência de diferenciação genética entre as populações da Amazônia brasileira (BA) e do sul da América do Sul (SSA) (F_{ST} = 0.005, D_{EST} = 0.01, R_{ST} = 0.028). Outra hipótese pode estar relacionada ao maior tamanho efetivo e as altas taxas de mutação apresentadas pelos microssatélites. Estes fatores poderiam retardar o aparecimento de padrões de diferenciação populacional (Zhang e Hewitt 2003), enquanto que o DNAmt, devido a sua herança materna e tamanho efetivo menor, seria mais suscetível à deriva genética entre grupos com algum grau de isolamento (Hare 2001, Zhang e Hewitt 2003). O padrão de subdivisão populacional indicado pelo DNAmt, na ausência de barreiras geográficas evidentes como observado para os clados 1 e 2, pode ser resultante de um comportamento filopátrico de fêmeas. Todas estas hipóteses poderão ser testadas mediante uma maior amostragem de *L. longicaudis* ao longo da sua área de ocorrência. Nesse sentido, embora com uma amostragem consideravelmente maior do que aquela analisada para o DNAmt, a ainda esparsa amostragem da região nordeste e de algumas áreas da região norte do Brasil, bem como a carência de amostras que representem a América Central, limita as inferências sobre os fatores envolvidos na distribuição da diversidade genética desta lontra e ressalta a importância destas áreas para a compreensão da história evolutiva desta espécie na totalidade de sua distribuição geográfica.

Ainda, considerando o hábito semi-aquático da lontra neotropical, e consequentemente sua forte associação a corpos d'água continentais, poderia ser esperado que os padrões filogeográficos da espécie estivessem de alguma forma relacionados com a configuração de bacias hidrográficas. Contudo, os dados apresentados mostram que, ao menos no que diz respeito ao território brasileiro, as bacias hidrográficas não parecem ter um papel importante na diferenciação genética desta espécie. Estudos futuros poderão testar esta hipótese em mais detalhe, e investigar se este padrão pode ser observado também em outras áreas com sistemas hidrográficos complexos, como por exemplo, o extremo norte da América do Sul, o qual tem sido apontado como importante na diferenciação populacional de outras espécies de vertebrados (p. ex. Garcia-Rodriguez et al. 1998, Banguera-Hinestrosa et al. 2001).

Por fim, no que diz respeito à atualmente questionada delimitação taxonômica da espécie, a partição genética observada especialmente nas linhagens do DNA mitocondrial revelou uma parcial concordância com a classificação de subespécies previamente proposta, a qual foi baseada em apenas uma característica da morfologia externa da espécie. Segundo esta classificação, *L. longicaudis* estaria subdividida em três subespécies geograficamente distintas, onde *L. longicaudis annectens* seria distribuída pelo México, Nicarágua, Costa Rica, Panamá, Colômbia, Venezuela e Equador, *L. longicaudis enudris* ocorreria nas regiões da Guiana Francesa, Suriname, Trinidad e Peru, enquanto que *L. longicaudis longicaudis* estaria distribuída pela maior parte da América do Sul, incluindo as regiões do Brasil e Uruguai (Larivière 1999, van Zyll de Jong 1972). Com base nos resultados apresentados aqui, a continuidade das análises filogeográficas torna-se ainda mais necessária, pois não envolve apenas o aumento do conhecimento dos padrões de estruturação populacional desta lontra, mas tem relação direta com a definição de unidades taxonômicas empregadas em estratégias de conservação. Ainda, esses estudos devem vir acompanhados de uma investigação detalhada da ocorrência de variações morfológicas intraespecíficas, através de análises morfométricas de material osteológico que possam ser relacionadas com a distribuição geográfica da espécie.

Tendo em vista a evidente necessidade de análise de amostras provenientes de outras regiões da distribuição de *L. longicaudis*, é importante destacar que um dos maiores impedimentos à condução de estudos moleculares com esta lontra é justamente a dificuldade de obtenção de

material biológico desta espécie. Uma vez que a visualização e captura de indivíduos de vida livre é bastante difícil devido principalmente ao seu comportamento arredio, a aquisição de material para análises genéticas torna-se dependente principalmente da coleta de amostras de indivíduos encontrados atropelados em estradas e de animais de cativeiro com procedência geográfica conhecida.

Entretanto, nos últimos anos, métodos de amostragem não-invasiva, baseados na coleta de vestígios tais como pelos e fezes, têm sido considerados uma fonte alternativa de DNA, uma vez que mesmo espécies difíceis de localizar em campo costumam deixar vestígios que podem ser identificados com certa facilidade (BeeBee e Rowe 2008). Este é o caso da lontra neotropical, em que a defecação, além da função básica de eliminação de resíduos, tem um papel importante na comunicação entre os indivíduos (Kruuk 2006). O uso de material fecal desta espécie já tem sido amplamente utilizado para análises de dieta, indicando a facilidade de coleta deste tipo de amostra. Além disso, amostras fecais já têm sido utilizadas para estudos genéticos de algumas outras espécies de lontra (p. ex. Hung et al. 2004, Mowry et al. 2011), sugerindo sua utilidade para abordagens moleculares com a lontra neotropical. Desta forma, a utilização de amostras não-invasivas representa uma grande oportunidade para expandir significativamente a amostragem desta espécie, contribuindo para o preenchimento das lacunas geográficas atualmente existentes e, assim, permitindo uma melhor compreensão dos padrões filogeográficos observados neste estudo, bem como a possível descoberta de partições genéticas adicionais. Algumas amostras de fezes e muco de *L. longicaudis* já foram empregadas nas análises descritas nos capítulos 3 e 4, as quais atestam a utilidade deste tipo de material nos estudos moleculares desta lontra.

Além da aplicabilidade nas análises filogeográficas, a coleta de amostras não-invasivas desta lontra realizada em escalas local e regional pode possibilitar a condução de estudos sobre níveis de conectividade e fluxo gênico entre populações, bem como a investigação de parâmetros demográficos e ecológicos até agora difíceis de obter por métodos tradicionais de ecologia. Neste contexto, os capítulos 5 e 6 apresentam a padronização e otimização de metodologias para o emprego deste tipo de amostra em abordagens ecológicas, bem como as primeiras estimativas de alguns parâmetros populacionais e demográficos de uma população natural de *L. longicaudis*.

Uma vez que a informação de gênero é importante para várias inferências ecológicas, e que na maioria das vezes esta informação é ausente para *L. longicaudis* devido à dificuldade de visualização destes animais em campo, no capítulo 5 foi desenvolvido um método de sexagem a partir da utilização de DNA extraído das fezes. Métodos similares já haviam sido descritos para a determinação sexual de outros mustelídeos, mas nenhum havia sido testado na lontra neotropical. O método apresentado é simples e baseia-se na amplificação conjunta de um loco de microssatélite e um segmento do gene *SRY*. Os resultados demonstraram que o método é altamente eficiente para a sexagem de amostras frescas de fezes e muco de *L. longicaudis* e, quando associado a marcadores hipervariáveis que identifiquem indivíduos, pode ser útil na realização de inferências sobre sistemas de acasalamento, organização espacial e padrões de dispersão da espécie.

Nesse contexto, o manuscrito do capítulo 6 apresenta o primeiro estudo de ecologia molecular de uma população natural de *L. longicaudis*, o qual foi baseado exclusivamente na análise de DNA extraído de amostras não-invasivas coletadas em campo. O estudo foi realizado na região do Vale do rio Maquiné, em um fragmento de Mata Atlântica remanescente do sul do Brasil.

O alto nível de polimorfismo observado nos locos de microssatélite viabilizou a identificação confiável de 28 indivíduos na área de estudo, um número que pode ser considerado bastante alto, considerando-se o tamanho da área amostrada (~30 km). A grande variação nas taxas de recaptura ente os indivíduos sugere que este é um fator que deve ser levado em consideração na estimativa de tamanho populacional, e que a amostragem periódica de uma mesma área é uma abordagem interessante que aumenta as chances de captura dos animais. Vários indivíduos foram amostrados em períodos de coleta distintos, sendo considerados como residentes. Estes indivíduos mostraram um padrão de estrutura social bastante interessante, indicando uma tendência de organização espacial relacionada com estruturas familiares, especialmente no que diz respeito às fêmeas, indicando uma tendência à filopatria deste gênero à respectiva área de nascimento.

Além disso, as estimativas de área de vida indicam que a lontra neotropical apresenta um padrão de organização espacial que se encaixa naquele reportado para mustelídeos de uma

forma geral, onde machos ocupam territórios maiores e que estes abrangem a área de mais de uma fêmea. Embora a sobreposição de territórios também tenha sido observada para pares de indivíduos machos, em geral estes animais foram identificados em períodos de coleta distintos ou em pontos de amostragem separados, o que sugere que o comportamento de defecação possa atuar de forma a evitar a presença simultânea de machos em uma mesma porção de rio.

Além disso, as análises de parentesco indicaram que a espécie apresenta um sistema de comportamento poligâmico e que, apesar do alto grau de parentesco estimado para a maioria dos indivíduos, nenhuma evidência de endocruzamento foi observada. Estes dados sugerem a atuação de mecanismos que evitem a ocorrência de cruzamentos consanguíneos na população. Considerando que a área de estudo representa apenas uma porção de um sistema hidrográfico mais complexo e com conexões a áreas adjacentes, a entrada periódica de indivíduos vindos de outros rios pode contribuir para evitar o endocruzamento. Alguns resultados sugerem ainda que a dispersão de machos também pode estar relacionada com o baixo índice de endocruzamento observado; todavia, esse é um campo que merece investigações adicionais.

Ainda, os resultados sugerem uma maior atividade das lontras e, consequentemente, uma maior disponibilidade de amostras nas porções dos rios mais preservadas e com menor presença humana. Isto pode estar diretamente associado à disponibilidade de recursos (ex. alimento e abrigo), que poderia ser maior nas áreas a montante dos rios. Contudo, essa hipótese precisaria ser testada através da análise de cobertura vegetal, de disponibilidade de presas e também dos níveis de contaminação da água nas diferentes porções dos rios.

De uma forma geral, os resultados apresentados neste capítulo confirmam o alto potencial da utilização de amostras não-invasivas para o estudo de diversas questões sobre a ecologia e comportamento de *L. longicaudis*. Todavia, é de grande importância salientar que padronização inicial dos métodos de amplificação dos locos de microssatélite em amostras de DNA de boa qualidade foi essencial para a melhor eficiência em DNA fecal. Além disso, a utilização do método de múltiplos tubos proposto por Taberlet et al. (1996) foi fundamental para assegurar a confiabilidade dos genótipos obtidos, dada a baixa qualidade já bastante conhecida do DNA neste tipo de amostra.

É importante destacar que este trabalho apenas inicia os estudos moleculares voltados para a investigação dos parâmetros populacionais de *L. longicaudis*, e que muitas outras questões permanecem em aberto, as quais devem ser investigadas através de estudos de longo prazo e, quando possível, em associação com métodos de rádio-telemetria. Pesquisas adicionais devem ser conduzidas com outras populações, em diferentes áreas e biomas, incluindo regiões onde se estima haver outras populações saudáveis da espécie (p.ex. Pantanal e Amazônia), bem como áreas em que diferentes níveis de ameaça já foram identificados e onde a persistência da espécie em longo prazo pode estar comprometida. Na medida em que novos dados forem gerados utilizando-se uma abordagem multidisciplinar, será possível traçar um padrão populacional para a lontra neotropical como um todo, e identificar áreas prioritárias de preservação, fornecendo assim dados sólidos para embasar futuros planos para sua conservação e manejo em âmbitos internacional, nacional e regional.

Por fim, a análise genética de amostras não-invasivas representa um campo promissor para o estudo não apenas da lontra neotropical, mas também para muitas outras espécies de carnívoros e outros mamíferos da região Neotropical, para os quais a disponibilidade de amostras biológicas convencionais também é um fator limitante. A realização de estudos na área da ecologia molecular utilizando-se este tipo de método pode contribuir significativamente para uma melhor compreensão dos impactos da fragmentação e perda de habitat na história de vida e dinâmica populacional de diversas espécies neotropicais consideradas raras e/ou ameaçadas, de forma a embasar a elaboração de estratégias que viabilizem sua persistência nestes ambientes em longo prazo.

.

Referências Bibliográficas

Allendorf FW and Luikart G (2007) *Conservation and the genetics of populations*. Blackwell Publishing Ltd, Oxford, 642 pp.

Arrendal J, Vilà C and Björklund M (2007) Reliability of noninvasive genetic census of otters compared to field censuses. Conserv Genet 8: 1097–1107.

Avise JC, Arnold J, Ball RM, Bermingham E, Lamb T, Neigel JE, Reeb CA and Saunders NC (1987) Intraspecific Phylogeography: the mithocondrial DNA bridge between populations genetics and systematics. Annu Rev Ecol Syst 18: 489:522.

Avise JC (1994) Molecular markers, natural history e evolution. Chapman & Hall, New York, 511 pp.

Avise JC (2000) Phylogeography – The history and formation the species. Harvard University Press, Cambridge, 447 pp.

de Barba, M, Waits LP, Garton EO, Genovesi P, Randi E, Mustoni A, Groff C (2010) The power of genetic monitoring for studying demography, ecology and genetics of a reintroduced brown bear population. Mol Ecol 19: 3938–3951.

Baker CS, Pery A, Bannister JL, Weinrich MT, Abernethy RB, Calambokidis K, Lien J, Lambertsen RH, Urban-Ramirez J, Vasquez O et al. (1993) Abundant mitochondrial DNA variation and world-wide population structure in humpback whales. Proc Natl Acad Sci 90: 8239-8243.

Ballard JWO and Whitlock MC (2004) The incomplete natural history of mitochondria. Mol Ecol 13: 729-744.

Banguera-Hinestroza E, Cárdenas H, Ruíz-Garcia M, Marmontel M, Gaitán E, Vásquez R, García-Vallejo F (2002) Molecular Identification of Evolutionary Significant Units in the Amazon River Dolphin Inia sp. (Cetacea: Iniidae). J Hered 93: 312-322.

Beebee TJC and Rowe G (2008) An introduction to molecular ecology. Second Edition. Oxford University Press, New York, 400 pp.

Bellemain E, Swenson JE, Tallmon D, Brunberg S and Taberlet P (2005). Estimating population size of elusive animals with DNA from hunter-collected feces: four methods for Brown bears. Conserv Biol 19: 150–161.

Bermingham E and Moritz C (1998) Comparative phylogeography: concepts and applications. Mol Ecol 7: 367-369.

Bertonatti C and Parera A (1994) Lobito de rio. Revista Vida Silvestre, nuestro Libro Rojo. Fundación Vida Silvestre Argentina, Ficha n°34.

Blacher C (1987) Ocorrência e preservação de Lutra longicaudis (Mammalia, Mustelidae) no litoral de Santa Catarina. Boletim da Fundação Brasileira para Conservação da Natureza 22: 105-117.

Blundell GM, Ben-David M, Groves P, Bowyer RT and Geffen E (2004) Kinship and sociality in coastal river otters: are they related? Behav Ecol 15: 705–714.

Broquet T and Petit E (2004) Quantifying genotyping errors in noninvasive population genetics. Mol Ecol 13: 3601–3608.

Brøseth H, Flagstad O, Wärdig C, Johansson M and Ellegren H (2010) Large-scale noninvasive genetic monitoring of wolverines using scats reveals density dependent adult survival. Biol Conserv 143: 113–120.

Brown GG, Gadalata G, Pepe G, Saccone C and Sbisa E (1986) Structural conservation and variation in D-loop containing region of vertebrate mitochondrial DNA. J Mol Biol 192: 503-511.

Bruford MW and Wayne RK (1993) Microsatellite and their application to population genetic studies. Curr Opin Genetics Dev 3: 939-943.

Cabrera A (1957) Catálogo de los mamíferos de América del Sur. I (Metatheria-Unguiculata-Carnivora). Revista del Museo Argentino de Ciências Naturales “Bernardino Rivadavia” e Instituto Nacional de Investigación de las Ciências Naturales, Ciências Zoológicas 4: 1-307.

Cegelski CC, Waits LP and Anderson NJ (2003) Assessing population structure e gene flow in Montana wolverines (Gulo gulo) using assignment-based approaches. Mol Ecol 12: 2907-2918.

Chebez JC (1999) Los que se van – Espécies argentinas em peligro. Editorial Albatroz Saci, Buenos Aires, 606 pp.

Chehébar CE (1990) Action Plan from Latin American Otters In: Foster-Turley P, Macdonald S and Mason C (eds) Otters: A Plan for their Conservation. IUCN Otter Specialist Group Bulletin, pp 64-73.

Cimardi AV (1996) Mamíferos de Santa Catarina. Primeira edição. Fundação de Amparo à Tecnologia e Meio Ambiente, Florianópolis, 302 pp.

Colares EP and Silva MNF (1987) Efeito da redução da alimentação na digestibilidade em lontras Lutra longicaudis (Mammalia: Mustelidae). In: Anais da 2ª Reunião de trabalhos de especialistas em mamíferos aquáticos da América do Sul.

Colares EP and Best R (1991) Blood parameters of amazon otters (Lutra longicaudis, Pteronura brasiliensis) (Carnivora, Mustelidae). Comp Biochem Physiol 99: 513-515.

Colares EP and Waldemarin HF (2000) Feeding of the Neotropical river otter (Lontra longicaudis) in the coastal region of the Rio Grande do Sul state, Southern Brazil. IUCN/SSC Otter Specialist Group Bulletin 17: 6-13.

Crandall KA, Bininda-Emond ORP, Mace GM and Wayne RK (2000) Considering evolutionary process in conservation biology. Trends Ecol Evolut 15: 290-295.

Curtis C, Stewart BS and Karl SA (2011) Genetically effective population sizes of Antarctic seals estimated from nuclear genes. Conserv Genet 12: 1435–1446.

Dalerum F, Loxterman J, Shults B, Kunkel K, Cook JA (2007) Sex-specific dispersal patterns of wolverines: Insights from microsatellite markers. J Mammal 88: 793–800.

Dallas JF, Carss DN, Marshall F, Koepfli KP, Kruuk H, Piertney SB and Bacon PJ (2000) Sex identification of the Eurasian otter Lutra lutra by PCR typing of spraints. Conserv Genet 1: 181–183.

Dallas JF, Coxon KE, Sykes T, Chanin PRF, Marshall F, Carss DN, Bacon PJ, Piertney SB and Racey PA (2003) Similar estimates of population genetic composition and sex ratio derived from carcasses and faeces of Eurasian otter Lutra lutra. Mol Ecol 12: 275–282.

DeSalle R and Amato G (2004) The expansion of conservation genetics. Nature Rev Genet 5: 702-712.

DeYoung RW and Honeycutt RL (2005) The molecular toolbox: genetic techniques in wildlife ecology and management. J Wildl Manage 69: 1362–1384.

Dugdale HL, Macdonald DW, Pope LC, Johnson PJ and Burke T (2008) Reproductive skew and relatedness in social groups of European badgers, Meles meles. Mol Ecol 16: 5294–5306.

Eguiarte LE and Souza V (2007) Introducción a ecología molecular. In: Eguiarte LE, Souza V, Aguirre X (Orgs) Ecología Molecular. Universidad Nacional Autónoma de México, Comisión Nacional para el Conocimiento y Uso de la Biodiversidad, pp 1-6.

Eisenberg JF and Redford KH (1999) Mammals of the Neotropics, Vol. 3. The Central Tropics: Ecuador, Peru, Bolivia, Brazil. The University of Chicago Press, Chicago, 609 pp.

Eizirik E (1996) Ecologia molecular, genética da conservação, e o conceito de Unidades Evolutivamente Significativas. Rev Bras Genet 19: 23-29.

Eizirik E, Kim J, Menotti-Raymond M, Crawshaw Jr PG, O'Brien SJ and Johnson WE (2001) Phylogeography, population history e conservation of jaguars (Panthera onca, Mammalia, Felidae). Mol Ecol 10: 65-79.

Emmons L H (1990) Neotropical Rainforest Mammals: A field guide. The University of Chicago Press, Chicago, 281 pp.

Ernest HB, Penedo CT, May BP, Syvanen M and Boyce WM (2000) Molecular tracking of mountain lions in the Yosemite Valley region in California: genetic analysis using microsatellites e faecal DNA. Mol Ecol 9: 433-441.

Ferreira ME (2001) Técnicas e estratégias para a caracterização molecular e uso de recursos genéticos. In: Garay I, Dias B (eds) Conservação da biodiversidade em ecossistemas tropicais. Editora Vozes, Rio de Janeiro, pp 223-267.

Farrel LE, Roman J and Sunquist ME (2000) Dietary of simpatric carnivores identified by molecular analysis of scats. Mol Ecol 9: 1583-1590.

Fernando P, Vidya TNC, Rajapakse C, Dangolla A and Melnick DJ (2003) Reliable Noninvasive Genotyping: Fantasy or Reality? J Hered 94: 115-123.

Ferrando A, Lecis R, Domingo-Roura X and Ponsà M (2008) Genetic diversity and individual identification of reintroduced otters (Lutra lutra) in north-eastern Spain by DNA genotyping of spraints. Conserv Genet 9: 129-139.

Fleming MA and Cook JA (2002) Phylogeography of endemic ermine (Mustela erminea) in southeast Alaska. Mol Ecol 11: 795 –807.

Foster-Turley P, Macdonald SM and Mason CF (1990) Otters, an action plan for their conservation. International Union for the Conservation of Nature, Gland, Switzerland, 126 pp.

Frankham R, Ballou JD and Briscoe DA (2002) Introduction to Conservation Genetics. Cambridge University Press, Cambridge, 617 pp.

Frantz AC, Pope LC, Carpenter PJ, Roper TJ, Wilson GJ, Delahay RJand, Burke T (2003) Reliable microssatélite genotyping of the Eurasian badger (Meles meles) using faecal DNA. Mol Ecol 12: 1649-1661.

Fraser DJ and Bernatchez L (2001) Adaptive evolutionary conservation: towards a unified concept for defining conservation units. Mol Ecol 10: 2741–2752.

Garcia DM, Marmontel M, Rosas FCW and Santos FR (2007) Conservation genetics of the giant otter (Pteronura brasiliensis (Zimmerman, 1780)) (Carnivora, Mustelidae). Braz J Biol 67: 819-827.

Garcia-Rodriguez AI, Bowen BW, Domning D, Mignucci-Giannoni AA, Marmontel M, Montoya-Spina RA, Morales-Vela B, Rudin M, Bonde RK and McGuirre PM (1998) Phylogeography of the West Indian manatee (Trichechus manatus): how many populations and how many taxa? Mol Ecol 7: 1137-1149.

Goossens B, Waits LP and Taberlet P (1998) Plucked hair samples as a source of DNA: reliability of dinucleotide microsatellite genotyping. Mol Ecol 7: 1237-1241.

Gómez-Moliner BJ, Cabria MT, Rubines J, Garin I, Madeira MJ, Elejalde A, Aihartza J, Fournier P and Palazón S (2004) PCR-RFLP identification of mustelid species: European mink (Mustela lutreola), American mink (M. vison) and polecat (M. putorius) by analysis of excremental DNA. J Zool 262: 311–316.

Graur D and Li W (2000) Fundamentals of molecular evolution. Sinauer Associates, Inc., Massachusetts, 480 pp.

Haag T, Santos AS, Sana DA, Morato RG, Cullen Jr L, Crawshaw Jr PG, de Angelo C, di Bitetti MS, Salzano FM, Eizirk E (2010) The effect of habitat fragmentation on the genetic structure of a top predator: loss of diversity and high differentiation among remnant populations of Atlantic Forest jaguars (Panthera onca). Mol Ecol 19: 4906-4921.

Hájková P, Pertoldi C, Zemanová B, Roche K, Hajék B, Bryja J and Zima J (2007) Genetic structure and evidence for recent population decline in Eurasian otter populations in the Czech and Slovak Republics: implications for conservation. J Zool 272: 1-9.

Hare MP (2001) Prospects for nuclear phylogeography. Trends Ecol Evolut 16: 700-706.

Harris CJ (1968) Otters: A study of the recent Lutrinae. Weinfield and Nicholson, London, 397 pp.

Hartl DL and Clark AG (2007) Principles of population genetics. Fourth edition. Sinauer Associates Inc., Maryland, 565 pp.

Hedmark E, Flagstad O, Segerström P, Persson J, Landa A and Ellegren H (2004) DNA-based individual and sex identification from wolverine (Gulo gulo) faeces and urine. Conserv Genet 5: 405–410.

Hedmark E and Ellegren H (2006) A test of the multiplex pre-amplification approach in microsatellite genotyping of wolverine faecal DNA. Conserv Genet 7: 289-293.

Helder J and de Andrade HK (1997) Food and feeding habitats of the Neotropical river otter Lontra longicaudis (Carnivora, Mustelidae). Mammalia 61: 193-203.

Hoffman JI and Amos W (2005) Microsatellite genotyping errors: detection approaches, common sources e consequences for paternal exclusion. Mol Ecol 14: 599-612.

Höss M, Kohn M, Pääbo S, Knauer F and Schröder W (1992) Excrement analysis by PCR. Nature 359: 199.

Hung C-M, Li S-H and Lee L-L (2004). Faecal DNA typing to determine the abundance and spatial organisation of otters (Lutra lutra) along two stream systems in Kinmen. Anim Conserv 7: 301–311.

Indrusiak CB and Eizirik E (2003) Carnívoros. In: Livro vermelho da fauna ameaçada de extinção no Rio Grande do Sul. Edipucrs, Porto Alegre, pp 507-534.

Janecka JE, Jackson R, Yuquang Z, Diqiang L, Munkhtsog B, Buckley-Beason V and Murphy WJ (2008) Population monitoring of snow leopards using noninvasive collection of scat samples: a pilot study. Anim Conserv 11: 401–411.

Johnson WE, Pecon-Slattery J, Eizirik E, Kim JH, Menotti-Raymond M, Bonacic C, Cambre R, Crawshaw P, Nunes A, Seuánez HN, Seymour KL, Simon F, Swanson W and O'Brien SJ (1999) Disparate phylogeography patterns of molecular genetic variation in four closely related South American small cat species. Mol Ecol 8: 79-92.

Johnson WE, Eizirik E, Roelke-Parker M and O'Brien SJ (2001) Applications of genetic concepts and molecular methods to carnivore conservation. In: Gittleman JL, Funk SM, MacDonald

D and Wayne RK (eds) Carnivore Conservation. Cambridge University Press, Cambridge, pp 335-358.

Johnson WE, Eizirik E, Waits L and O'Brien SJ (2006) Molecular ecology and carnivore conservation: the application of molecular techniques for inferring identity, kinship, and social structure in the Neotropics. In: Morato RG, Rodrigues FHG, Eizirik E, Mangini PR and, Azevedo FCC (orgs) Manejo e conservação de carnivoros neotropicais. Instituto Brasileiro do Meio Ambiente, Brasília, pp 67-84.

Kalinowski ST, Taper ML and Creel S (2006) Using DNA from non-invasive samples to identify individuals and census populations: an evidential approach tolerant of genotyping errors. Conserv Genet 7: 319-329.

Kalz B, Jewgenow K and Fickel J (2006) Structure of an otter (Lutra lutra) population in Germany - results of DNA and hormone analyses from faecal samples. Mamm Biol 71: 321-335.

Kays RW, Gittleman JL and Wayne RK (2000) Microsatellite analysis of kinkajou social organization. Mol Ecol 9: 743 – 751.

Ketmaier V and Bernardini C (2005) Structure of the Mitochondrial Control Region of the Eurasian Otter (Lutra lutra; Carnivora, Mustelidae): Patterns of Genetic Heterogeneity and Implications for Conservation of the Species in Italy. J Hered, 96: 318–328.

Koepfli KP and Wayne RK (1998) Phylogenetic relationships of otters (Carnivora: Mustelidae) based on mitochondrial cytochrome b sequences. J Zool 246: 401-406.

Koepfli KP and Wayne RK (2003) Type ITS Markers Are More Informatives than Cytocrome b in Phylogenetic Reconstruction of the Mustelidae (Mammalia: Carnivora). Syst Biol 52: 571-593.

Koepfli KP, Deere KA, Slater GJ, Begg C, Begg K, Grassman L, Lucherini M, Veron G and Wayne R (2008) Multigene phylogeny of the Mustelidae: Resolving relationships, tempo and biogeographic history of a mammalian adaptive radiation. BMC Biol 6: 10.

Kohn MH, York EC, Kamradt DA, Haught G, Sauvajot RM and Wayne RK (1999) Estimating population size by genotyping faeces. Proc R Soc Lond [Biol] 266: 657-63.

Kohn MH, Murphy WJ, Ostrander EA and Wayne RK (2006) Genomics and conservation genetics. Trends Ecol Evolut 21: 629-637.

Kruuk R (2006) Otters. Ecology, behavior and conservation. Oxford University Press, Oxford, 280 pp.

Larivière S (1999) Lontra longicaudis. Mamm Species 609: 1-5.

Latch EK, Scognamillo DG, Fike JA, Chamberlain MJ and Rhodes Jr OE (2008) Deciphering Ecological Barriers to North American River Otter (Lontra canadensis) Gene Flow in the Louisiana Landscape. J Hered 99: 265–274.

Lanszki J, Hidas A, Szentes K, Révay T, Lehoczky S and Weiss S (2008) Relative spraint density and genetic structure of otter (Lutra lutra) along the Drava River in Hungary. Mamm Biol 73: 40–47.

Larson S, Jameson R, Bodkin J, Staedler M and Bentzen P (2002) Microsatellite DNA and mitochondrial DNA variation in remnant and translocated sea otter (Enhydra Lutris) populations. J Mammal 83: 893–906.

Larson S, Monson D, Ballachey B, Jameson R and Wasser SK (2009) Stress-related hormones and genetic diversity in sea otters (Enhydra lutris). Mar Mam Sci 25: 351-372.

Leakey R and Lewin R (1995) The Sixth Extinction. Doubleday Publishing/Weidenfeld and Nicolson, London.

Lynch AB and Brown MJF (2006) Molecular sexing of pine marten (Martes martes): how many replicates? Mol Ecol Notes 6: 631-633.

Macdonald SM (1990) Surveys. In: Foster-Turley P, Macdonald S and Mason C (eds) Otters, an Action Plan for their Conservation. IUCN Otter Specialist Group, pp 8-10.

Macdonald SM and Mason CF (1985) Otters, their habitat and conservation in northeast Greece. Biol Conserv 31: 191-210.

Macdonald SM and Mason CF (1990) Threats. In: Foster-Turley P, Macdonald S and Mason C (eds) Otters, an Action Plan for their Conservation. IUCN/SSC Otter Specialist Group Bulletin, pp 11-14.

Machado ABM, Fonseca GAB, Machado RB, Aguiar LMS and Lins LV (1998). Livro vermelho das espécies ameaçadas de extinção da fauna de Minas Gerais. Fundação Biodiversitas, Belo Horizonte, 605 pp.

Marmi J, López-Giraldéz F and Domingo-Moura X (2004) Phylogeny, evolutionary history and taxonomy of the Mustelidae based on sequences of the cytochome b gene and a complex repetitive flanking region. Zool Scripta 33: 481-499.

Marmi, J, López-Giráldez F, Macdonald DW, Calafell F, Zholnerovskaya E and Domingo-Roura X (2006) Mitochondrial DNA reveals a strong phylogeographic structure in the badger across Eurasia. Mol Ecol 15: 1007-1020.

Martin A and Palumbi SR (1993) Body size, metabolic rate, generation time, and the molecular clock. Proc Natl Acad Sci 90: 4087-4091.

Mason C (1990) An Introduction to the Otters In: Foster-Turley P, Macdonald S and Mason C (eds). Otters: An Action Plan for their Conservation. IUCN/SSc Otter Specialist Group Bulletin, pp 4-7.

Michaux JR, Hardy OJ, Justy F, Fournier P, Kranz A, Cabria M, Davison A, Rosoux R and Libois R (2005) Conservation genetics and population history of the threatened European mink Mustela lutreola, with an emphasis on the west European population. Mol Ecol 14: 2373-2388.

Miotto RA, Begotti RA, Cervini M, Figueiredo MG and Galetti Jr PM (2011) Genetic diversity and population structure of pumas (Puma concolor) in southeastern Brazil: implications for conservation in a human-dominated landscape. Conserv Genet 12: 1447–1455.

Ministério do Meio Ambiente (2008) Livro vermelho da fauna brasileira ameaçada de extinção. Machado ABM, Drummond GM, Paglia AP (eds), Fundação Biodiversitas, Brasília, 1420 pp.

Moritz C (1994) Defining “Evolutionary Significant Units” for conservation. Trends Ecol Evolut 9: 373-375.

Moritz C and Faith D (1998) Comparative phylogeography and the identification of genetically divergent areas for conservation. Mol Ecol 7: 419-429.

Mowry RA, Gompper ME, Beringer J and Eggert LS (2011) River otter population size estimation using noninvasive latrine surveys. J Wildl Manage 75: 1625-1636.

Mucci N, Delibes M, Arrendal J, Ferrando A, Ansorge H, Fournier P, Bailey M, Fournier C, Bodner M and Godoy JA et al. (2010) Genetic diversity and landscape genetic structure of otter (Lutra lutra) populations in Europe. Conserv Genet 11: 583–599.

Mullins J, Statham MJ, Roche T, Turner PD and O'Reilly C (2010) Remotely plucked hair genotyping: a reliable and non-invasive method for censusing pine marten (Martes martes, L. 1758) populations. Eur J Wildl Res 56: 443–453.

Murray BW (1996) The estimation of genetic distance and population substructure from microsatellite allele frequency data. http: //helix.biology.mcmaster.ca//brent.

Neigel JE and Avise JC (1986) Phylogenetic relationships of mitochondrial DNA under various demographic models of speciation. In: Nevo E, Karlin S (eds) Evolutionary processes and theory. Academy Press, New York, pp. 515-534.

Nyström V, Angerbjörn A and Dalén L (2006) Genetic consequences of a demographic bottleneck in the Scandinavian arctic Fox. Oikos 114: 84-94.

Ottaviani D, Panzacchi M, Lasininio GJ, Genovesi P and Boitani L (2009) Modelling semi-aquatic vertebrates' distribution at the drainage basin scale: The case of the otter Lutra lutra in Italy. Ecol Model 220: 111–121.

Palomares F, Godoy JA, Piriz A, O'Brien SJ and Johnson WE (2002) Faecal genetic analysis to determine the presence and distribution of elusive carnivores: design and feasibility for the Iberian lynx. Mol Ecol 11: 2171-2182.

Paraná (Secretaria do Estado do Meio Ambiente) (1995) Lista vermelha de animais ameaçados de extinção no Estado do Paraná. SEMA/GTZ, Curitiba.

Pardini R (1998) Feeding ecology of the Neotropical river otter Lontra longicaudis in an Atlantic Forest Stream, southeastern Brazil. J Zool 245: 385-391.

Pardini R and Trajano E (1999) Use of shelters by the Neotropical river otter (Lontra longicaudis) in an Atlantic forest stream, southeastern Brazil. J Mammal 80: 600-610.

Parera A (1993) The Neotropical River Otter Lutra longicaudis in Iberá Lagoon, Argentina. IUCN/SSC Otter Specialist Group Bulletin 8: 13-16.

Pease K, Freedman AH, Pollinger JP, McCormack JE, Buermann W, Rodzen J, Banks J, Meredith E, Bleich VC, Schaefer RJ et al. (2009) Landscape genetics of California mule deer

(Odocoileus hemionus): the roles of ecological and historical factors in generating differentiation. Mol Ecol 18: 1848–1862.

Pertoldi C, Breyne P, Cabria MT, Halfmaerten D, Jansman HAH, Berge KVD, Madsen AB and Loeschcke V (2006) Genetic structure of the European polecat (Mustela putorius) and its implication for conservation strategies. J Zool 270: 102–115.

Pickles RSA, Groombridge JJ, Zambrana Rojas VD, Van Damme P, Gottelli D, Kundu S, Bodner R, Ariani CV, Iyengar A and Jordan WC (2011a) Phylogeography and identification of evolutionary significant units in the giant otter. Mol Phylogenet Evol doi:10.1016/j.ympev.2011.08.017

Pickles RSA, Groombridge JJ, Zambrana Rojas VD, Ariani CV, Van Damme P and Jordan WC (2012) Genetic diversity and population structure in the endangered giant otter, Pteronura brasiliensis. Conserv Genet 13: 235–245.

Pilot M, Gralak B, Goszczynski J and Posluszny M (2007) A method of genetic identification of pine marten (Martes martes) and stone marten (Martes foina) and its application to faecal samples. J Zool 271: 140–147.

Pohle H (1920) Die Unterfamilie der Lutrinae. Eine systematisch-tiergeographische Studie an dem Material der Berliner Messen. Archiv für Naturgeschichte 85: 1-247.

Prugh LR, Ritland CE, Arthur SM and Krebs CJ (2005) Monitoring coyote population dynamics by genotyping faeces. Mol Ecol 14: 1585-1596.

Quadros J and Monteiro-Filho ELA (2000) Fruit occurrence in the diet of the Neotropical otter, Lontra longicaudis, in Southern Brazilian Atlantic Forest and its implication for seed dispersion. Mastozoología Neotropical 7: 33-36.

Ramírez-Bravo OE (2010) Neotropical Otter (Lontra longicaudis) Records in Puebla, Central Mexico. IUCN/SSC Otter Specialist Group Bulletin 27: 134-136.

Rheingantz ML, Waldemarin HF, Rodrigues L and Moulton TP (2011) Seasonal and spatial differences in feeding habits of the Neotropical otter Lontra longicaudis (Carnivora: Mustelidae) in a coastal catchment of southeastern Brazil. Zoologia 28: 37–44.

Riddle AE, Pilgrim KL, Mills LS, McKelvey KS and Ruggiero LF (2003) Identification of mustelids using mitochondrial DNA and non-invasive sampling. Conserv Genet 4: 241–243.

Schlötterer C (1998) Microssatellites. In: Hoelzel AR (ed) Molecular genetic analysis of populations: a practical Approach. Second edition. Oxford University Press, New York, pp 237-261.

Schlötterer C (2004) The evolution of molecular markers – just a matter of fashion? Nature Rev Genet 5: 63-69.

Silva F (1994) Mamíferos silvestres do Rio Grande do Sul. Fundação Zoobotânica do Rio Grande do Sul, Porto Alegre, 246 pp.

Slatkin M (1994) Gene flow and population structure. In: Real LA (ed) Ecological Genetics. Princeton University Press, New Jersey, pp 3-34.

Small MP, Stone KD and Cook JA (2003) American marten (Martes americana) in the Pacific Northwest: population differentiation across a landscape fragmented in time and space. Mol Ecol 12: 89-103.

Smith DA, Ralls K, Hurt A, Adams B, Parker M and Maldonado JE (2006) Assessing reliability of microsatellite genotypes from kit fox faecal samples using genetic and GIS analyses. Mol Ecol 15: 387-406.

Soulé M (1985) What is Conservation Biology? Bioscience 35: 727-734.

Stanton DWG, Hobbs GI, Chadwick EA, Slater FM and Bruford MW (2009) Mitochondrial genetic diversity and structure of the European otter (Lutra lutra) in Britain. Conserv Genet 10: 733-737.

Stenglein JL, Waits LP, Ausband DE, Zager P and Mack CM (2011) Estimating gray wolf pack size and family relationships using noninvasive genetic sampling at rendezvous sites. J Mammal 92: 784–795.

Strausberger BM and Ashley MV (2001) Eggs yield nuclear DNA from egg-laying female cowbirds, their embryos and offspring. Conserv Genet 2: 385-390.

Taberlet P and Bouvet J (1991) A single plucked feather as a source of DNA for bird genetic studies. Auk 108: 959-960.

Taberlet P and Bouvet J (1992) Bear conservation genetics. Nature 358: 197.

Taberlet P, Griffin S, Goossens B, Questiau S, Manceau V, Escaravage N, Waits LP and Bouvet J (1996) Reliable genotyping of samples with very low DNA quantities using PCR. Nucleic Acids Res 24: 3189-3194.

Taberlet P, Waits LP and Luikart G (1999) Noninvasive genetic sampling : Look before you leap. Trends Ecol Evolut 8: 323-327.

Trinca CS, Waldemarin HF and Eizirik E (2007) Genetic diversity of the Neotropical otter (Lontra longicaudis Olfers, 1818) in Southern and Southeastern Brazil. Braz J Biol 67: 813-818.

Valière N, Bonenfant C, Toïgo C, Luikart G, Gaillard J and Klein F (2007) Importance of a pilot study for non-invasive genetic sampling: genotyping errors and population size estimation in red deer. Conserv Genet 8: 69–78.

Valière N, Fumagalli L, Gielly L, Miquel C, Lequette B, Poulle M-L, Weber J-M, Arlettaz R and Taberlet P (2003) Long-distance wolf recolonization of France and Switzerland inferred from non-invasive genetic sampling over a period of 10 years. Anim Conserv 6: 83-92.

Valière N and Taberlet P (2000) Urine collected in the field as a source of DNA for the species and individual identification. Mol Ecol 9: 2150-2154.

Valqui J, Günther BH and Zachos FE (2010) Non-invasive genetic analysis reveals high levels of mtDNA variability in the endangered South-American marine otter (Lontra felina). Conserv Genet 11: 2067–2072.

van Zyll de Jong CG (1972) A systematic review of the Nearctic and Neotropical river. otters (Genus Lontra, Mustelidae, Carnivora). Life Sci Cont Royal Ontario Museum 80: 1-104.

van Zyll de Jong CG (1987) A phylogenetic study of the Lutrinae (Carnivora; Mustelidae) using morphological data. Can J Zool 65: 2536-2544.

Vianna JA, Ayerdi P, Medina-Vogel G, Mangel GC, Zeballos H, Apaza M and Faugeron S (2010) Phylogeography of the Marine Otter (Lontra felina): Historical and Contemporary Factors Determining Its Distribution. J Hered 101: 676-689.

Vianna JA, Medina-Vogel G, Chehébar C, Sienfield W and Faugeron S (2011) Phylogeography of the Patagonian otter Lontra provocax: adaptive divergence to marine habitat or signature of southern glacial refugia? BMC Evol Biol 11: 53.

Waldemarin HF and Colares EP (2000) Utilization of resting sites and dens by the Neotropical river otter (Lontra longicaudis) in the south of Rio Grande do Sul State, Southern Brazil. IUCN/SSC Otter Specialist Group Bulletin 17: 14-19.

Waldemarin HF and Alvarez R (2008) Lontra longicaudis. In: IUCN 2011. IUCN Red List of Threatened Species. Version 2011.2. <www.iucnredlist.org>. Downloaded on 04 December 2011.

Waits LP (2004) Using Noninvasive Genetic Sampling to Detect and Estimate Abundance of Rare Wildlife Species. In: Thompson WL (ed) Sampling Rare and Elusive Species - Concepts, Designs, and Techniques for Estimating Population Parameters. Island Press, Washington D. C., pp 211-228.

Weber LI, Hildebrand CG, Ferreira A, Pedarassi G, Levy JA and Colares EP (2009) Microsatellite genotyping from faeces of Lontra longicaudis from southern Brazil. Iheringia, Série Zoológica 99: 5-11.

Wisely SM, Buskirk SW, McDonald DB and Ostrander EA (2002) Genetic diversity and fitness in black-footed ferrets before e during a bottleneck. J Hered 93: 231-237.

Wozencraft WC (2005) Order Carnivora. In: Wilson DE and Reeder DM (eds) Mammal species of the world: a taxonomic and geographic reference. Third Edition. The Johns Hopkins University Press, Baltimore, pp 532-628.

Wright S (1978) Evolution and the Genetics of Populations. The University of Chicago Press, Chicago, 590 pp.

Zagrebel'nyi SV, Fomin VV and Burdin AM (2008) Dynamics of abundance and population structure of sea otters, Enhydra lutris L., on the Commander Archipelago and activity of their migrations between islands. Russ J Ecol 39: 41-47.

Zhang DX and Hewitt GM (2003) Nuclear DNA analyses in genetic studies of populations: practices, problems and prospects. Mol Ecol 12: 563-584.

Anexo

Genetic diversity of the Neotropical otter (*Lontra longicaudis* Olfers, 1818) in Southern and Southeastern Brazil

Cristine S. Trinca, Helen F. Waldemarin & Eduardo Eizirik

Artigo publicado na revista *Brazilian Journal of Biology*

Genetic diversity of the Neotropical otter (*Lontra longicaudis* Olfers, 1818) in Southern and Southeastern Brazil

Trinca, CS.[a,c]*, Waldemarin, HF.*[b] *and Eizirik, E.*[c,d]*

[a]Departamento de Genética, Universidade Federal do Rio Grande do Sul – UFRGS, CEP 91501-970, Porto Alegre, RS, Brazil

[b]Associação Ecológica Ecomarapendi, Projeto Ecolontras, CEP 22210-080, Rio de Janeiro, RJ, Brazil

[c]Laboratório de Biologia Genômica e Molecular, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul – PUCRS, Av. Ipiranga 6681, prédio 12 C, sala 172, Bairro Partenon, CEP 90619-900, Porto Alegre, RS, Brazil

[d]Instituto Pró-Carnívoros, CEP 12945-010, Atibaia, SP, Brazil

Received May 25, 2007 – Accepted August 30, 2007 – Distributed December 1, 2007

*e-mail: eduardo.eizirik@pucrs.br

(With 1 figure)

Abstract

The Neotropical otter is one of the least known otter species, and it is considered to be threatened to various degrees throughout its geographic range. Little information exists on the ecological characteristics of this species, and no genetic study has been published about it until now, hampering the design of adequate conservation strategies for its populations. To contribute with genetic information to comprehensive conservation efforts on behalf of *L. longicaudis*, we characterized the molecular diversity of the 5' portion of the mtDNA control region in samples from this species collected in Southern and Southeastern Brazil. The sequence analysis revealed a high level of haplotype diversity (h = 0.819; SE = 0.0052) and nucleotide variability ranging from 0.0039 to 0.0067. One of the sampled haplotypes was the most common in both regions and, from this sequence, several other (locally occurring) haplotypes could be derived by single point mutations. No significant genetic differentiation was observed between the Southern and Southeastern regions.

Keywords: Lontra longicaudis, mitochondrial DNA, control region, genetic diversity.

Diversidade genética da lontra Neotropical (*Lontra longicaudis* Olfers, 1818) no Sul e Sudeste do Brasil

Resumo

A lontra Neotropical é uma das espécies de lontras menos conhecidas e apresenta diferentes graus de ameaça ao longo de sua distribuição geográfica. Pouca informação existe a respeito de aspectos ecológicos desta espécie e nenhum estudo genético foi publicado até o momento, dificultando a delimitação de estratégias adequadas de conservação para suas populações. Para contribuir com informação genética aos esforços de conservação de *L. longicaudis*, a diversidade molecular da porção 5' da região controladora do DNA mitocondrial foi caracterizada em amostras desta espécie coletadas nas regiões Sul e Sudeste do Brasil. A análise das seqüências revelou um alto nível de diversidade haplotípica (h = 0,819; SE = 0,0052) e variabilidade nucleotídica entre 0,0039 a 0,0067. Um dos haplótipos encontrados foi o mais comum em ambas as regiões e, desta seqüência, diversos outros haplótipos (de ocorrência restrita) podem ter se derivado através de mutações pontuais. Nenhuma diferenciação genética significante foi observada entre as regiões Sul e Sudeste.

Palavras-chave: Lontra longicaudis, DNA mitocondrial, região controladora, diversidade genética.

1. Introduction

Accurate estimates of genetic diversity and its patterns of geographic structuring are extremely important for the ultimate adequacy and success of conservation efforts. However, due to the elusive behavior of most carnivore species, this kind of information is often very difficult to obtain, as is the case of *Lontra longicaudis* (Mamalia, Carnivora, Mustelidae). This medium-sized, semi-aquatic carnivore, is widely distributed in the Neotropical region, and currently faces threats such as habitat destruction and pollution in several parts of its distribution (Chehébar, 1990). Information on the genetic diversity of this species, and its connection with ecological data, are required steps towards the effective conservation and management of this organism.

The mitochondrial DNA (mtDNA) control region has been extensively used in conservation studies of many species of vertebrates (Eizirik et al., 1998; Möller et al., 2001; Montoya-Burgos, 2003; Cantanhede et al., 2005; Márquez et al., 2006; Barnett et al., 2006; Tchaicka et al., 2007). Due to the high polymorphism on its 5' end in vertebrates (Avise, 1994; Taberlet, 1996), this segment has been widely used in populational studies of mustelids such as *Gulo gulo* (Wilson et al., 2000; Chappell et al., 2004), *Mustela putorius* (Davison et al., 2000), *Martes foina* (Davison et al., 2001), *Enhydra lutris* (Larson et al., 2002), and *Lutra lutra* (Mucci et al., 1999; Cassens et al., 2000; Pérez-Haro et al., 2005).

Although the Neotropical otter is one of the least known otter species, several ecological studies focusing on *L. longicaudis* have been conducted in different localities in Brazil (Pardini, 1999; Colares and Waldemarin, 2000; Quadros and Monteiro-Filho, 2001). In contrast, no study has been published so far addressing the genetic variability of this species. For this reason, in this paper we aim to describe and analyze the diversity of the 5' portion of the mtDNA control region in this species, based on samples collected in Southern and Southeastern Brazil.

2. Material and Methods

Tissue (footpad, muscle and kidney) and blood samples were obtained from 20 individuals of *L. longicaudis* from several localities in the South and Southeast regions of Brazil. The former includes the states of Rio Grande do Sul, Santa Catarina, and Paraná; the latter includes samples from the states of São Paulo, Rio de Janeiro, and Minas Gerais. In addition, four fecal samples were obtained by collaborators working in the field or with captive individuals (Table 1). Both tissue and scat samples were preserved

Table 1. Samples of *Lontra longicaudis* and *Pteronura brasiliensis* used in this study.

Sample ID	Material	Geographic Origin[a]	Geographic region	Source Institution / Contact
bLlo01	muscle	RS	Southern	G. Bencke and JR. Marinho
bLlo03	muscle	RS	Southern	Carnívoros do RS Project
bLlo04	footpad	RS	Southern	Carnívoros do RS Project
bLlo05	muscle	Gravataí, RS	Southern	GL.Gonçalves
bLlo06	muscle	RS	Southern	PH. Ott
bLlo07	muscle	Curumim, RS	Southern	P. Colombo
bLlo12	muscle	Dois Irmãos, RS	Southern	Prefeitura de Dois Irmãos / I. Fick
bLlo26	faeces	Eldorado do Sul, RS	Southern	Sapucaia do Sul Zoo / R. von Hohendorff
bLlo37	faeces	Nova Santa Rita, RS	Southern	Canoas Minizoo / M. Martins
bLlo42	muscle	Capela de Santana, RS	Southern	Canoas Minizoo / M. Martins
bLlo58	muscle	Osório, RS	Southern	P. Colombo, C. Zank and L. Volkmer
bLlo02	muscle	SC	Southern	Sapucaia do Sul Zoo
bLlo34	muscle	Blumenau, SC	Southern	FURB / S. Althoff
bLlo29	blood	Foz do Iguaçu, PR	Southern	São Paulo Zoo / K. Kassaro
bLlo38	muscle	P. N. de Ilha Grande, PR	Southern	L. Kroposki
bLlo57	blood	Paranapanema River, PR	Southeastern	L. Koproski
bLlo10	kidney	Mogi-Mirim, SP	Southeastern	CENAP/IBAMA
bLlo36	faeces	Sumaré, SP	Southeastern	Campinas Zoo / E. Ferraz
bLlo39	muscle	Ribeirão Preto, SP	Southeastern	Ribeirão Preto Zoo / M. dos Santos
bLlo51	faeces	P.E. da Ilha do Cardoso, SP	Southeastern	E. Nakano
bLlo14	muscle	Angra dos Reis, RJ	Southeastern	HF. Waldemarin
bLlo15	muscle	Guaratiba, RJ	Southeastern	HF. Waldemarin
bLlo16	muscle	Barra, RJ	Southeastern	HF. Waldemarin
bLlo30	blood	Belo Horizonte, MG	Southeastern	São Paulo Zoo / K. Kassaro
bPbr01	muscle	Negro River, Pantanal, MS	Center-West	HF. Waldemarin
bPbr02	muscle	Negro River, Pantanal, MS	Center-West	HF. Waldemarin

[a]RS: Rio Grande do Sul State; SC: Santa Catarina State; PR: Paraná State; SP: São Paulo State; RJ: Rio de Janeiro State; MG: Minas Gerais State; MS: Mato Grosso do Sul State. bLlo: *Lontra longicaudis*; bPbr: *Pteronura brasiliensis*

in ethanol 96% and blood samples were preserved in a salt saturated solution (100mM Tris, 100mM EDTA, 2% SDS). All samples were stored at –20 °C prior to DNA extraction. Total DNA was extracted from tissue and blood samples following a standard phenol-chloroform protocol (Sambrook et al., 1989). DNA from scats was recovered using the QIAamp DNA Stool Mini Kit (Qiagen). The scat DNA extractions were carried out in a separate room to avoid contamination with other DNA sources.

The 5' portion of the mtDNA control region (CR), containing the first hypervariable segment, was amplified by Polymerase Chain Reaction (PCR) using the primer pair MTLPRO2 and CCR-DR1 (Tchaicka et al., 2007). Since the DNA present in scat samples is often degraded and at low concentrations, the sequencing of long fragments from this type of material can be difficult. To address this issue, we developed internal primers for the otter CR, dividing it into three shorter fragments of approximately 250 base pairs (bp) each. We did this by initially sequencing multiple *L. longicaudis* individuals for the complete CR segment, along with two samples of the distantly related giant otter (*Pteronura brasiliensis* Gmelin, 1788), to identify internal segments for primer design that will likely be conserved across the subfamily Lutrinae. The resulting primers are: LonCR-R1 (reverse to MTLPRO2) (5'-ATGGTTTCTCGAGGCATGGT-3'), LonCR-F2 (forward to CCR-DR1) (5'-AACTATACCTGGCATCTGGTTCTT-3'), and the internal pair LonCR-F1 (5'- GGTTTGCCCCATGCATATAA-3') + LonCR-R2 (5'-TGTGTGATCATGGGCTGATT-3').

Each 20 µL PCR reaction contained 1-2 µL of empirically diluted DNA, 1x PCR Buffer (Invitrogen), 1.5-2.0 mM $MgCl_2$, 200 µM dNTPs, 0.2 µM of each primer, and 0.5 unit of Taq DNA Polymerase (Invitrogen). The PCR conditions were the following: 10 cycles (*Touchdown*) of 94 °C for 45 seconds, 60-51 °C for 45 seconds, and 72 °C for 1.5 minute; followed by 30 cycles of 94 °C for 45 seconds, 50 °C for 45 seconds, and 72 °C for 1.5 minute, and final extension of 72 °C for 3 minutes. Products were checked on a 1% agarose gel stained with ethidium bromide, purified with PEG8000, sequenced using the DYEnamic ET Dye Terminator Sequencing Kit (GE Healthcare), and analyzed in a MegaBACE 1000 automated sequencer (GE Healthcare). Sequences were deposited in GenBank under accession numbers EU251949-EU251960.

Sequences were visually checked and manually corrected using CHROMAS 2.0 (http://www.technelysium.com.au/chromas.html), and subsequently aligned with the CLUSTALW algorithm implemented in MEGA 3.1 (Kumar et al., 2004). MEGA was also used to perform sequence comparisons and computations of variability. The program Network 4.1.1.2 (www.fluxus-engineering.com) was used to construct a haplotype network depicting the evolutionary relationships among the sequences. To test whether there is genetic differentiation between the Southern and Southeastern Brazilian regions, we employed an Analysis of Molecular Variance (AMOVA) approach (Excoffier et al., 1992) implemented in ARLEQUIN 2.0 (Schneider et al., 2000). Additional population genetic analyses were performed with DnaSP (Rozas et al., 2003).

3. Results

A fragment of 516 bp was obtained from the 5' end of the mtDNA control region of 24 Neotropical otters. Removal of a 25 bp portion whose alignment was ambiguous resulted in a final data set of 491 bp, which was used in all subsequent analyses. Timines and adenines were quite prevalent in the otter CR (T: 30.12%; A: 26.50%; C: 24.44%; G: 18.94%). Fifteen polymorphic positions were identified (seven of which were parsimony-informative), allowing the detection of 12 different mitochondrial haplotypes (Table 2). The overall observed

Table 2. List of individuals that bear each mtDNA control region haplotype. The localities of occurrence of each haplotype are also indicated.

Haplotype[a]	Individuals	Haplotype occurrence
L-1	bLlo01, bLlo03,bLlo24	Rio Grande do Sul
L-2A	bLlo02, bLlo06, bLlo12, bLlo14, bLlo15, bLlo26, bLlo34, bLlo38, bLlo58	Rio Grande do Sul, Santa Catarina, Paraná and Rio de Janeiro
L-2B	bLlo30	Minas Gerais
L-3	bLlo05, bLlo39	Rio Grande do Sul and São Paulo
L-4	bLlo07	Rio Grande do Sul
L-5	bLlo10	Sao Paulo
L-6	bLlo16	Rio de Janeiro
L-7	bLlo29, bLlo37	Paraná and Rio Grande do Sul
L-8	bLlo36	São Paulo
L-9	bLlo42	Rio Grande do Sul
L-10	bLlo51	São Paulo
L-11	bLlo57	Paraná

[a]Haplotypes with the same number and different letters (e.g. L-2A, 2B) are collapsed into a single haplotype when all sites with missing information or indels are excluded (e.g. Figure 1). bLlo: *Lontra longicaudis*; bPbr: *Pteronura brasiliensis*

haplotype diversity was high (0.8188), but the nucleotide diversity was low, even considering both sampled regions (0.0049; SE: 0.0015) and within each geographic region (Table 3). Haplotype L-2A was clearly the most common and, along with haplotype L-3, it was found in both analyzed regions (see Tables 1 and 2), while the other 10 haplotypes were restricted to either the South or the Southeast. The haplotype network (Figure 1) shows some reticulation, indicating the occurrence of homoplasy likely due to saturation at variable sites. The haplotypes were divergent mostly by a single difference, indicating a recent origin and no major divergence among these sequences. The analysis of genetic differentiation based on the AMOVA approach showed no evidence for significant divergence between the Southern and Southeastern populations ($F_{ST} = 0.0511$; $p = 0.1113$). To assess whether alternative subdivision scenarios could indicate any genetic partitioning, we also tested two other structuring schemes allowed by our sampling: (i) placing the haplotypes sampled in Paraná State (the Northernmost of the Southern States) into the Southeastern population; and (ii) considering Rio Grande do Sul State (the Southernmost of all sampled states) as a separate population from the other areas. Both scenarios yielded non-significant F_{ST} values (scheme (i): $F_{ST} = 0.0048$; $p = 0.3467$; scheme (ii): $F_{ST} = -0.0097$; $p = 0.5009$), supporting the inference of no genetic subdivision between these areas.

4. Discussion

The Neotropical otter populations analyzed here exhibit low levels of nucleotide variation on the 5' end of the mitochondrial DNA control region, which is known to be a highly polymorphic marker in other vertebrates (Avise, 1994). However, they present high haplotype diversity, indicating that they are not genetically depauperate, but rather may result from a recent process of population diversification in this region.

Observed levels of genetic variability in Neotropical otters were similar or lower ($\pi = 0.0049 \pm 0.0015$; $h = 0.8188 \pm 0.0052$) than values observed for other vertebrates. For example, Eizirik et al. (2001) found that the nucleotide diversity of jaguars (*Panthera onca* Linnaeus, 1758) throughout their range is 0.00771 (SE: 0.00010); Cantanhede et al. (2005) obtained nucleotide and haplotype diversity values for the Amazonian manatee (*Trichechus inunguis* Natterer, 1883) of 0.624 (SE: 0.384) and 0.887 (SE: 0.026), respectively. Moreover, the same control region segment was used by Tchaicka et al. (2007) in phylogeographic analyses of the crab-eating fox (*Cerdocyon thous* Linnaeus, 1766), a genetically diverse Neotropical canid. Indeed, the comparison between *L. longicaudis* and *C. thous* indicates that the nucleotide diversity is substantially higher in the latter ($\pi = 0.019 \pm 0.002$), while the haplotype diversity is similar in both species ($h = 0.83 \pm 0.032$ in *C. thous*).

Comparing the results obtained in this study with analyses performed with other mustelids, we observed that the levels of nucleotide variability estimated for the sampled *L. longicaudis* populations are among the lowest recorded so far in this carnivore family (e.g. *Mustela lutreola* ($\pi = 0.0012 \pm 0.0003$ in Southeastern Europe; and 0.012 ± 0.0014 in Northeastern Europe populations; Michaux et al., 2005), *Gulo gulo* ($\pi = 0.0055 \pm 0.0040$ and 0.0153 ± 0.0091 in North and Prairie Canadian populations, respectively; Chappell et al., 2004), *Enhydra lutris* ($\pi = 0.098 \pm 0.029$; Larson et al., 2002). Nevertheless, the nucleotide diversity of *L. longicaudis* is still much

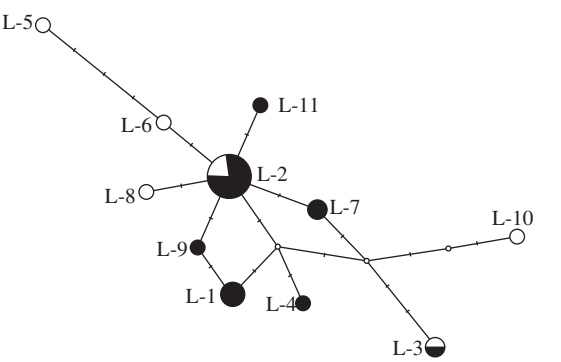


Figure 1. Median-joining network of *L. longicaudis* mtDNA control region haplotypes (using 491 bp; all sites containing indels or missing information were excluded). Each haplotype is represented by a circle, whose area is proportional to its population frequency. White circles (or areas within circles) represent samples from Southeastern Brazil, whereas black coloration indicates samples from Southern Brazil. Bars on branches indicate nucleotide substitutions inferred to have occurred in each lineage.

Table 3. mtDNA control region diversity estimates for the Neotropical otter.

Geographic Region	N	N. of haplotypes	S[a]	PI[b]	π[c]	h[d]
Southern	16	7	9	3	0.0039 ± 0.0014	0.7917 ± 0.0078
Southeastern	8	7	10	4	0.0067 ± 0.0021	0.9167 ± 0.0085
Total	24	12	15	7	0.0049 ± 0.0015	0.8188 ± 0.0052

[a]S = Polymorphic sites.
[b]PI = Parsimony-informative sites.
[c]Nucleotide diversity per site.
[d]Haplotype diversity.

higher than the levels observed so far for *Lutra lutra* in Europe ($\pi = 0.0006$; Ferrando et al., 2004).

In contrast to the low nucleotide diversity, the haplotype diversity found in the Neotropical otter is much higher than levels observed for other otter species, such as *Enhydra lutris* (h = 0.412; Larson et al., 2002) and *Lutra lutra* (h = 0.16 ± 0.06; Ferrando et al., 2004). In contrast to *L. longicaudis*, large-scale studies on European populations of *Lutra lutra* based on 300 bp of the 5' end mtDNA control region have so far described only six haplotypes, all of which differing from each other by a single nucleotide, demonstrating very low levels of genetic variability for that species (Effenberger and Suchentrunk, 1999; Mucci et al., 1999; Cassens et al., 2000; Pérez-Haro et al., 2005). Future assessments using standardized DNA segments and analytical methods should allow more detailed comparisons of the genetic diversity and underline the demographic history of the world's otter species.

This study provides a first description of the genetic variability of the Neotropical otter, based on the analysis of some populations originated from only a small part of the species' geographic distribution. The observed patterns suggest a recent origin and possibly a high demographic connectivity between the sampled areas, with no major genetic structuring. However, the samples outside Rio Grande do Sul State were very reduced (N = 9 over 3,000 km), which could also be responsible for the lack of significance in the F_{ST} comparisons. Further studies must be conducted to provide a broader perspective on the genetic diversity of this species in its entire geographic range. The expansion of these genetic analyses, both in terms of geographic coverage and use of multiple marker systems, should contribute to increase the current knowledge on the history and population biology of the Neotropical otter, aiding in the development and implementation of conservation strategies targeting this species and its habitats.

Acknowledgments — We would like to thank the people and institutions that helped us to collect the samples used in this study, and to an anonymous reviewer who provided useful comments on a previous version of this paper. The authors are also grateful to Centro Nacional de Pesquisas para a Conservação de Predadores Naturais – CENAP/IBAMA, Instituto Pró-Carnívoros, and CNPq for the financial support of this project.

References

AVISE, JC., 1994. *Molecular markers, natural history e evolution*. New York, Chapman & Hall, 511p.

BARNETT, R., YAMAGUCHI, N., BARNES, I. and COOPER, A., 2006. Lost populations and preserving genetic diversity in the lion *Panthera leo*: implications for its ex situ conservation. *Conserv. Genet*., vol. 7, no. 4, p. 507-514.

CANTANHEDE, AM., DA SILVA, VMF., FARIAS, IP., HRBEK, T., LAZZARINI, SM. and ALVES-GOMES, J., 2005. Phylogeography and population genetics of the endangered Amazonian manatee, *Trichechus inunguis* Natterer, 1883 (Mammalia, Sirenia). *Mol. Ecol*., vol. 14, no. 2, p. 401-413.

CASSENS, I., TIEDEMANN, R., SUCHENTRUNK, F. and HARTL, GB., 2000. Mitochondrial DNA variation in the European otter (*Lutra lutra*) and the use of spatial autocorrelation analysis in conservation. *J. Hered*., vol. 91, no. 1, p. 31-41.

CHAPPELL, DE., VAN DEN BUSSCHE, RA., KRIZAN, J. and PATTERSON, B., 2004. Contrasting levels of genetic differentiation among populations of wolverines (*Gulo gulo*) from northern Canada revealed by nuclear and mitochondrial loci. *Conserv. Genet*., vol. 5, no. 6, p. 759-767.

CHEHÉBAR, CE., 1990. Action Plan from Latin American Otters. In FOSTER-TURLEY, P., MACDONALD, S. and MASON, C. (eds.). *Otters: An Action Plan for their Conservation*. IUCN Otter Specialist Group, p. 64-73.

COLARES, EP. and WALDEMARIN, HF., 2000. Feeding of the neotropical river otter (*Lontra longicaudis*) in the coastal region of the Rio Grande do Sul State, Southern Brazil. *IUCN Otter Specialist Group Bulletin*, vol.17, no. 1, p. 6-13.

DAVISON, A., GRIFFITHS, HI., BROOKES, RC., MORAN, T., MACDONALD, D., SIDOROVICH, V., KITCHENER, AC., IRIZIAR, I., VILLATE, I., GONZÁLES-ESTEBAN, J., CEÑA, A., MOYA, I. and PALAZÓN, S., 2000. Mitochondrial DNA and paleontological evidence for the origins of endangered European mink, *Mustela lutreola*. *Anim. Conserv*., vol. 3, no. 4, p. 345-355.

DAVISON, A., BIRKS, JDS., BROOKES, RC., MESSENGER, JE. and GRIFFITHS, HI., 2001. Mitochondrial phylogeography and population history of pine martens *Martes martes* compared with polecats *Mustela putorius*. *Mol. Ecol*., vol. 10, no. 10, p. 2479-2488.

EFFENBERGER, S. and SUCHENTRUNK, F., 1999. RFLP analyses of the mitochondrial DNA of otters (*Lutra lutra*) from Europe - implications for conservation of a flagship species. *Biol. Conserv*., vol. 90, no. 3, p. 229-234.

EIZIRIK, E., BONATTO, SL., JOHNSON, WE., CRAWSHAW, Jr. PG., VIÉ, JC., BROUSSET, DM., O'BRIEN, SJ. and SALZANO, FM., 1998. Phylogeographic patterns and mitochondrial DNA control region evolution in two Neotropical cats (Mammalia, Felidae). *J. Mol. Evol*., vol. 47, no. 5, p. 613-624.

EIZIRIK, E., KIM, J., MENOTTI-RAYMOND, M., CRAWSHAW, Jr. PG., O'BRIEN, SJ. and JOHNSON, WE., 2001. Phylogeography, population history e conservation of jaguars (*Panthera onca*, Mammalia, Felidae). *Mol. Ecol*., vol. 10, no. 1, p. 65-79.

EXCOFFIER, L., SMOUSE, P. and QUATTRO, J., 1992. Analysis of Molecular Variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*, vol. 131, no. 2, p. 479-491.

FERRANDO, A., PONSÀ, M., MARMI, J. and DOMINGO-ROURA, X., 2004. Eurasian otters, *Lutra lutra*, have a dominant mtDNA haplotype from the Iberian Peninsula to Scandinavia. *J. Hered*., vol. 95, no. 5, p. 430-435.

KUMAR, S., TAMURA, K. and NEI, M., 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinf*., vol. 5, no. 2, p. 150-163.

LARSON, SE., JAMESON, RJ., BODKIN, JL., STAEDLER, M. and BENTZEN, P., 2002. Microsatellite DNA and mtDNA variation within and among remnant and translocated sea otter, *Enhydra lutris*, populations. *J. Mamm*., vol. 83, no. 3, p. 893-906.

MÁRQUEZ, A., MALDONADO, JE., GONZÁLEZ, S., BECCACECI, MD., GARCIA, JE. and DUARTE, JMB., 2006. Phylogeography and Pleistocene demographic history of the endangered marsh deer (*Blastocerus dichotomus*) from the Río de La Plata Basin. *Conserv. Genet*., vol. 7, no. 4, p. 563-575.

MICHAUX, JR., HARDY, OJ., JUSTY, F., FOURNIER, P., KRANZ, A., CABRIA, M., DAVISON, A., ROSOUX, R. and LIBOIS, R., 2005. Conservation genetics and population history of the threatened European mink *Mustela lutreola*, with an emphasis on the West European populations. *Mol. Ecol*., vol. 14, no. 8, p. 2373-2388.

MÖLLER, LM., BEHEREGARAY, LB., HARCOURT, RG. and KRÜTZEN, M., 2001. Alliance membership and kinship in wild male bottlenose dolphins (*Tursiops aduncus*) of southeastern Australia. *Proc. R. Soc. Lon., B*, vol. 268, no. 1479, p. 1941-1947.

MONTOYA-BURGOS, JI., 2003. Historical biogeography of the catfish genus *Hypostomus* (Siluriformes: Loricariidae), with implications on the diversification of Neotropical ichthyofauna. *Mol. Ecol*., vol. 12, no. 7, p. 1855-1867.

MUCCI, N., PERTOLDI, C., MADSEN, AB., LOESCHCKE, V. and RANDI, E., 1999. Extremely low mitochondrial DNA control-region sequence variation in the otter *Lutra lutra* population of Denmark. *Hereditas*, vol. 130, no. 3, p. 331-336.

PARDINI, R., 1999. Use of shelters by the neotropical river otter (*Lontra longicaudis*) in an Atlantic Forest stream, southeastern Brazil. *J. Mamm*., vol. 80, no. 2, p. 600-610.

PÉREZ-HARO, M., VIÑAS, J., MAÑAS, F., BATET, A., RUIZ-OLMO, J. and PLA, C., 2005. Genetic variability in the complete mitochondrial control region of the Eurasian Otter (*Lutra lutra*) in the Iberian Peninsula. *Biol. J. Linn. Soc*., vol. 86, no. 4, p. 397-403.

QUADROS, J. and MONTEIRO-FILHO, ELA., 2001. Diet of the Neotropical otter, *Lontra longicaudis*, in Atlantic Forest area, Santa Catarina State, southern Brazil. *Stud. Neotrop. Fauna Environ*., vol. 36, no. 1, p. 15-21.

ROZAS, J., SÁNCHEZ-DEL BARRIO, JC., MESSEGUER, X. and ROZAS, R., 2003. DNASP, DNA polymorphism analyses by the coalescent and others methods. *Bioinformatics*, vol. 19, no. 18, p. 2496-2497.

SAMBROOK, J., FRITSCH, EF. and MANIATIS, T., 1989. *Molecular Cloning*. 2nd. edition. New York, Cold Spring Harbor Laboratory Press.

SCHNEIDER, S., ROESSLI, D. and EXCOFFIER, L., 2000. ARLEQUIN: *A Software for Population Genetic Data Analysis*, Version 2.0. Geneva, Switzerland, Genetics and Biometry Laboratory, Department of Anthorpology, University of Geneva. 140p.

TABERLET, P., 1996. The use of mitochondrial DNA control region sequencing in conservation genetics. In SMITH, TB. and WAYNE, RK. (eds.). *Molecular approaches in conservation*. New York, Oxford University Press, p. 125-142.

TCHAICKA, L., EIZIRIK, E., OLIVEIRA, TG., CÂNDIDO, Jr JF., FREITAS, TRO., 2007. Phylogeography and population history of the crab-eating fox (*Cerdocyon thous*). *Mol. Ecol*., vol. 16, no. 4, p. 819-838.

WILSON, GM., VAN DEN BUSSCHE, RA., KENEDY, PK., GUNN, A. and POOLE, K., 2000. Genetic variability of wolverines (*Gulo gulo*) from the Northwest territories, Canada: conservation implications. *J. Mamm*., vol. 81, no. 1, p. 186-196.