PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL
PROGRAMA DE PÓS-GRADUAÇÃO EM ZOOLOGIA

DIVERSIDADE GENÉTICA E FILOGEOGRAFIA DE *PUMA YAGOUAROUNDI* (MAMMALIA, CARNIVORA, FELIDAE)

CARLA BETTIN PIRES

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Diversidade Genética e Filogeografia de *Puma yagouaroundi* (Mammalia, Carnivora, Felidae)

Aluna: Carla Bettin Pires

Orientador: Prof. Dr. Eduardo Eizirik

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RESUMO

Apesar da alta biodiversidade existente na região Neotropical, esta é também uma das regiões menos estudadas no mundo e onde muitas espécies ainda continuam sendo pouco conhecidas. Um exemplo é o gato selvagem jaguarundi (*Puma yagouaroundi*), que é um dos felinos Neotropicais menos conhecidos. Dos poucos estudos realizados com esta espécie, a maioria aborda aspectos ecológicos e desse modo, pouco ou quase nada se conhece em termos de sua diversidade genética, história evolutiva e estrutura populacional. Nós investigamos estes aspectos evolutivos e inferimos padrões filogeográficos do jaguarundi através da análise de 1191 pb do DNA mitocondrial e oito locos de microssatélites. Os resultados de ambos os marcadores suportaram o reconhecimento de pelo menos dois grandes grupos filogeográficos (Norte e Sul), e não corroboraram a validade das oito subespécies classicamente reconhecidas. Barreiras físicas como o rio Amazonas parecem ter influenciado a diferenciação genética destes dois grandes grupos, restringindo o fluxo gênico histórico entre essas duas regiões geográficas, em um padrão semelhante ao observado em outras espécies de felinos. Os resultados apresentados aqui contribuem para aumentar o conhecimento da história desta espécie e podem ser úteis no desenvolvimento de propostas de manejo visando a sua conservação em longo prazo.

ABSTRACT

Genetic Diversity and Phylogeography of *Puma yagouaroundi* (Mammalia, Carnivora, Felidae)

Even though the Neotropical region has a vast biodiversity, it is also one of the least studied regions in the world, and harbors many species that still remain poorly known. An example is the wild cat jaguarundi (*Puma yagouaroundi*), which is up to now one the least known Neotropical felids. Of the few studies including this species, most address ecological aspects, and therefore little is known regarding its genetic diversity, evolutionary history and population structure. We have investigated these evolutionary aspects and inferred phylogeographic patterns of the jaguarundi by analyzing 1191 bp of the mitochondrial DNA and eight microsatellite loci. The results from both markers supported the recognition of at least two major phylogeographic groups (Northern and Southern), which do not corroborate the eight subspecies classically recognized for the jaguarundi. Physical barriers such as the Amazon river appear to have influenced the genetic differentiation between these two groups by restricting the gene flow between these broad geographic areas, in a pattern reminiscent of that observed in other felids. The results presented here contribute to increase the knowledge about the evolutionary history of this felid, and may be useful in the development of management strategies fostering its conservation in the wild.

APRESENTAÇÃO

1. Introdução Geral

1.1 Biodiversidade

O Brasil é um país que possui uma alta diversidade biológica, constando na lista dos países de maior biodiversidade no mundo (LEWINSOHN; PRADO, 2004). Lewinsohn e Prado (2005) estimaram que o Brasil possua aproximadamente 13% da biodiversidade mundial, sendo esta estimativa baseada tanto em espécies conhecidas atualmente quanto em projeções daquelas ainda não descobertas ou descritas.

Neste contexto, a fauna de mamíferos no Brasil é extremamente diversa, com mais de 530 espécies descritas, e muitas a serem descobertas e catalogadas (BRANDON *et al.*, 2005; COSTA *et al.*, 2005). Costa e colaboradores (2005) ressaltam que apesar de os mamíferos serem um grupo de animais bem conhecido, quando comparado a outros grupos, poucas regiões do Brasil foram adequadamente inventariadas, as listas locais de espécies são geralmente incompletas e a falta de conhecimento sobre muitas espécies dificulta iniciativas conservacionistas e de manejo. No que tange especificamente aos carnívoros neotropicais, grupo focal deste estudo, Oliveira (2006) ressaltou que, apesar do recente aumento na quantidade e abrangência dos estudos conduzidos sobre este grupo, pouco ainda se conhece sobre aspectos básicos da ecologia e história natural da maioria das espécies contidas neste táxon.

De um modo geral, os vertebrados, e os mamíferos em particular, têm um papel muito importante em uma série de processos na maior parte dos ecossistemas. Estes processos acabam influenciando a diversidade e estruturação da comunidade de plantas e seus consumidores primários (HOWE; MIRITI, 2004; PARDINI *et al.*, 2003). Nesse aspecto, Stoner *et al.* (2007) relatam a importância dos mamíferos na determinação e manutenção da estrutura de florestas Neotropicais, e destacam ainda que alterações nas populações de mamíferos (devido a ações como fragmentação e perda de

habitat, entre outras) podem, portanto, ter conseqüências na estrutura vegetal encontrada em uma floresta (PARDINI *et al.*, 2003).

Estendendo esta avaliação para os carnívoros em particular, Miller et al. (2001) destacam como estas espécies são importantes para a "saúde" de um ecossistema, participando da cadeia trófica, da transferência de energia e também influenciando a diversidade de espécies presentes naquele ambiente. Esta conclusão é baseada no fato de que os herbívoros controlam a biomassa das plantas, e os carnívoros por sua vez, controlam a biomassa dos herbívoros (CROOKS; SOULÉ, 1999; MILLER *et al.*, 2001; TERBORGH *et al.*, 2001). Assim sendo, a atividade predatória dos carnívoros influencia toda a cadeia trófica de um ecossistema, e por este e outros motivos, as ações de manejo realizadas em uma região devem levar em conta o papel dos carnívoros naquele ambiente (CROOKS; SOULÉ, 1999; MILLER *et al.*, 2001; TERBORGH *et al.*, 2001). Complementando esta idéia, Chiarello *et al.* (2008) ressaltam que, como os carnívoros necessitam de grandes áreas para manter populações viáveis, os esforços para conservar áreas suficientes à manutenção/conservação deste grupo acabam por preservar também outras espécies da comunidade. No entanto, o ambiente em que esses animais vivem vem sendo constantemente degradado por ações causadas por humanos.

1.2 Genética da Conservação

A destruição, alteração e fragmentação dos biomas terrestres têm aumentado consideravelmente nas últimas décadas, sendo decorrentes de um desenvolvimento econômico desordenado, que acarreta inúmeros danos às espécies de mamíferos em geral (COSTA *et al.*, 2005). Dentre as principais ações antropogênicas que afetam direta ou indiretamente as espécies estão: a destruição de habitats naturais, poluição atmosférica e aquática, a sobre-exploração dos recursos, o crescimento de áreas cultivadas e urbanas (COSTA *et al.*, 2005), e a introdução de espécies em regiões nas quais não são nativas (WILCOVE *et al.*, 1998; FRANKHAM; BALLOU; BRISCOE, 2008). Devido a seus efeitos negativos, atualmente, a perda ou destruição de habitats é considerada a maior causa promotora da perda de biodiversidade mundial

(WILCOVE *et al.*, 1998; FAHRIG, 2003; PIRES; FERNANDEZ; BARROS, 2006), sendo que esta é a principal ameaça as populações de mamíferos brasileiros (COSTA *et al.*, 2005; HAYWARD, 2009).

Em carnívoros, em especial no caso das espécies da família Felidae, fatores adicionais de interferência humana são ameaças importantes, como por exemplo, a perseguição direta (EIZIRIK; JOHNSON, 2006; CHIARELLO *et al.*, 2008; HAYWARD, 2009), que ocorre principalmente devido ao fato de estes animais atacarem rebanhos de gado de muitos fazendeiros ocasionando prejuízos econômicos para os criadores, e outro motivo seria porque sua pelagem possui valor comercial no mercado, embora hoje a caça seja considerada uma atividade ilegal. Outra ameaça a esse grupo de animais é a morte acidental devido à colisão com veículos em estradas (COSTA *et al.*, 2005; CHIARELLO *et al.*, 2008). Conforme Morato *et al.* (2006), atualmente todas as dez espécies de felídeos neotropicais encontram-se ameaçadas pelos fatores descritos acima. Portanto, tendo em vista a crescente ameaça que a perda e fragmentação de habitats, juntamente com outras ações humanas, tem causado à sobrevivência de muitas espécies de carnívoros, é importante realizar estudos que forneçam informações sobre estas espécies, visando a embasar estratégias adequadas para a sua conservação.

Foi justamente no âmbito desta “crise” da biodiversidade mundial, ou seja, do grande número de espécies da flora e da fauna terrestre sofrendo ameaças diretas ou indiretas à sua sobrevivência, que surgiu uma nova disciplina: a biologia da conservação, que ao longo do tempo englobou várias áreas de pesquisa, como ecologia, genética, entre outras (PRIMACK; RODRIGUES, 2001; PEREZ-SWEENEY; RODRIGUES; MELNICK, 2003). A biologia da conservação procura, de modo geral, entender os efeitos das ações antropogênicas sobre as espécies e também quais medidas podem ser utilizadas para tentar evitar/prevenir a extinção destas, e quando for o caso, reintroduzir uma espécie no seu ambiente natural (PRIMACK; RODRIGUES, 2001).

Com o crescente desenvolvimento de técnicas moleculares nos últimos anos, houve o surgimento de uma nova área de pesquisa inserida neste campo da biologia da conservação, conhecida como genética da conservação (PEREZ-SWEENEY; RODRIGUES; MELNICK, 2003; EIZIRIK; JOHNSON;

O'BRIEN, 2006; FRANKHAM; BALLOU; BRISCOE, 2008). Esta disciplina tem por objetivo principal, em longo prazo, preservar espécies como entidades dinâmicas capazes de se adaptarem às mudanças ambientais (FRANKHAM; BALLOU; BRISCOE, 2008), e também preservar a continuidade dos processos ecológicos e evolutivos que ali atuaram e continuam atuando (EIZIRIK; JOHNSON; O'BRIEN, 2006).

As pesquisas nesta área incluem o uso de análises genéticas para elucidar aspectos da história e biologia das espécies relevantes para seu manejo e conservação (PEREZ-SWEENEY; RODRIGUES; MELNICK, 2003; EIZIRIK; JOHNSON; O'BRIEN, 2006; FRANKHAM; BALLOU; BRISCOE, 2008). Em conjunto com outras disciplinas, como a ecologia e a biologia de populações, a genética está se tornando uma ferramenta importante na determinação de *o que* conservar (sistemática molecular), *onde* focalizar os esforços de conservação (filogeografia) e *como* conservar a maior quantidade de diversidade genética nas populações, com o objetivo de manter o "potencial evolutivo" de uma espécie ou população (genética de populações) (PEREZ-SWEENEY; RODRIGUES; MELNICK, 2003).

Neste contexto, um exemplo da importância da genética é a definição de unidades evolutivamente significativas (UES ou ESU – *"Evolutionarily Significant Units"*) dentro de uma espécie, ou seja, a definição de unidades evolutivas infra-específicas. Neste conceito recente, criado nas décadas de 1980 e 1990, as ESU são definidas quando uma população (ou um grupo de populações de uma espécie) possui uma diferenciação genética alta em relação a outras populações, refletindo assim uma história de isolamento geográfico (EIZIRIK, 1996; EIZIRIK; JOHNSON; O'BRIEN, 2006; FRANKHAM; BALLOU; BRISCOE, 2008). Dessa maneira, por possuírem características próprias, estas UES deveriam ser consideradas como unidades independentes em termos conservacionistas (EIZIRIK, 1996; EIZIRIK; JOHNSON; O'BRIEN, 2006; FRANKHAM; BALLOU; BRISCOE, 2008).

Historicamente, o conceito de subespécie era e continua sendo utilizado para distinguir populações da mesma espécie, sendo que seu objetivo é de definir formas ou raças geográficas que se apresentam morfologicamente diferenciadas entre si. No entanto, pelo menos no caso dos felinos neotropicais, estas definições às vezes não levavam em conta certos aspectos

relevantes para a distinção real entre os grupos, como a variação individual intra-populacional. De fato, estudos utilizando marcadores moleculares com felinos já evidenciaram que muitas vezes as subespécies tidas como "clássicas", na realidade não correspondiam a unidades evolutivas reais (p. ex. Eizirik *et al.* 1998, 2001). Portanto, considerando que grande parte dos processos de avaliação de ameaças e estratégias de conservação envolve essas categorias subespecíficas, é de extrema importância que estas definições sejam re-consideradas à medida que novas informações são obtidas acerca dos padrões de subdivisão populacional geográfica observados nestas espécies (EIZIRIK; JOHNSON, 2006).

Outro aspecto interessante da utilização de ferramentas genéticas visando à conservação é o auxílio que esta pode trazer no entendimento da biologia das espécies. Por exemplo, o conhecimento dos padrões de dispersão e da história demográfica das espécies é freqüentemente crítico para assegurar a sobrevivência destas, e pode ser inferido através de análises genéticas (FRANKHAM; BALLOU; BRISCOE, 2008). Portanto, diante do que foi relatado até o momento, estudos como a filogeografia, por exemplo, podem contribuir significativamente para o planejamento de ações de manejo visando à conservação, visto que estes podem fornecer informações sobre o grau de estruturação entre as populações, sobre aspectos da biologia da espécie, entre outros, todos os quais são úteis no entendimento da história evolutiva de uma espécie.

1.3 A filogeografia

De modo geral, a maioria das espécies apresenta, naturalmente, algum nível de estruturação geográfica ou diferenciação genética, pois muitas vezes se formam subpopulações que acabam habitando locais diferentes, e ao longo das gerações, isso ocasiona alguma diferença na freqüência de alelos distintos em diferentes pontos do seu genoma (HARTL; CLARK, 1997; BALLOUX; LUGON-MOULIN, 2002). Esta estruturação pode ser influenciada por muitos fatores, como barreiras ambientais, processos históricos de isolamento e histórias de vida (ex. sistema de reprodução) diferentes, os quais podem, em

algum nível, “moldar” a diferenciação genética encontrada nas populações (BALLOUX; LUGON-MOULIN, 2002). Ainda segundo estes autores, como a distribuição geográfica das espécies é tipicamente mais ampla do que a capacidade de dispersão de um indivíduo, geralmente as populações são geneticamente diferenciadas através do isolamento por distância, ou seja, populações mais próximas são geneticamente mais similares entre si do que populações mais distantes.

Nesse contexto, a filogeografia é a área da ciência que investiga a distribuição geográfica de linhagens genealógicas dentro de uma espécie (ou comparando espécies próximas), e procura explicar quais são os principais fatores ou barreiras ambientais que influenciaram na distribuição da diversidade encontrada nas diferentes populações. Para a análise e interpretação da distribuição das linhagens, geralmente são necessárias informações ou abordagens provenientes da genética molecular, genética de populações, etologia, demografia, análise filogenética, paleontologia, geologia, e geografia histórica (AVISE, 2000). Outro aspecto interessante da filogeografia é a possibilidade de definir regiões geográficas prioritárias para a conservação, onde existam UES de várias espécies diferentes (MORITZ, 1994; MORITZ; FAITH, 1998). Assim, estudos filogeográficos podem contribuir para o planejamento da conservação, revelando rompimentos históricos entre populações e espécies, ou ainda áreas com alta divergência genética e endemismo (PEREZ-SWEENEY; RODRIGUES; MELNICK, 2003).

Em estudos de espécies neotropicais, a filogeografia tem se mostrado uma ferramenta bastante útil e capaz de revelar o papel que eventos históricos complexos possuem sobre a distribuição atual da diversidade e a estrutura genética das espécies. Como salientado acima, o conhecimento desta estruturação é de grande relevância para o manejo e a conservação (FRANKHAM; BALLOU; BRISCOE, 2008).

1.4 Marcadores moleculares

Diante do que foi relatado, nota-se como a utilização de técnicas moleculares tem auxiliado na realização de estudos populacionais, visando a fornecer informações adicionais sobre uma determinada espécie, as quais

seriam mais difíceis de obter por meio de observações diretas (FRANKHAM; BALLOU; BRISCOE, 2008). Nas últimas décadas, com os crescentes avanços em técnicas laboratoriais e estudos envolvendo o material genético da mitocôndria, o DNA mitocondrial (mtDNA) animal passou a ser amplamente utilizado em estudos populacionais e evolutivos, em especial na filogeografia (AVISE, 2000). O mtDNA é herdado maternalmente e é relativamente abundante, uma vez que existem muitas mitocôndrias por célula, o que o torna de fácil purificação ou análise por amplificação direcionada (FRANKHAM; BALLOU; BRISCOE, 2008). Em estudos sobre os carnívoros neotropicais, marcadores moleculares com base em variação no mtDNA têm sido amplamente utilizados visando a determinar padrões filogeográficos (p.ex. JOHNSON *et al.*, 2001; EIZIRIK; JOHNSON; O'BRIEN, 2006).

Segundo Avise (2000), algumas características particulares ao mtDNA têm feito com que este seja muito utilizado em estudos filogeográficos, como: herança materna (mencionada anteriormente), alta variabilidade intra-específica (entre indivíduos da mesma espécie) e ausência de recombinação entre diferentes moléculas. A alta variação intra-específica é conseqüente da alta taxa de evolução (mutação) do mtDNA quando comparado aos genes nucleares. Algumas hipóteses têm sido levantadas para tentar explicar essa alta taxa de evolução, como: (i) relaxamento da constrição funcional, visto que o mtDNA não codifica para proteínas envolvidas diretamente no seu processo de replicação ou transcrição; (ii) um mecanismo de reparo de DNA ineficiente e a alta exposição a agentes mutagênicos, como os radicais livres da cadeia oxidativa da respiração celular; e (iii) o fato de o mtDNA estar "nu", já que não é complexado com as histonas, e assim estaria menos protegido do que o genoma nuclear.

Recentemente, além da utilização de mtDNA, tem crescido o número de estudos que incluem outros tipos de marcadores moleculares em suas análises, como por exemplo, marcadores nucleares. Hare (2001), e Emerson e Hewitt (2005), destacaram a importância de se analisar mais de um tipo de marcador, incluindo um ou mais marcadores nucleares, pois estes podem mostrar diferentes aspectos da história evolutiva do grupo sob estudo.

Os microssatélites, também conhecidos como seqüências simples repetidas (SSR – *Simple Sequence Repeats*), são seqüências de DNA de 1 a 6

nucleotídeos repetidas em *tandem* (ou seja, em seqüência contínua) no genoma nuclear. Esses marcadores têm sido amplamente utilizados em estudos genéticos por serem altamente informativos, já que são co-dominantes e muitas vezes multi-alélicos, permitindo assim a identificação de genótipos individuais. Os microssatélites amplificados via PCR (*Polymerase Chain Reaction* - Reação em Cadeia da Polimerase) possuem uma alta reprodutibilidade e resolução, facilitando assim o seu uso em testes laboratoriais (OLIVEIRA *et al.*, 2006). Ainda conforme estes autores, inicialmente o uso deste marcador era limitado pelo alto custo de se isolar microssatélites na espécie em estudo. Contudo, é comum adotar uma medida alternativa que torna o estudo mais viável, a chamada transferibilidade, na qual *primers* desenvolvidos para uma determinada espécie podem ser utilizados em espécies filogeneticamente próximas (mesmo gênero ou até mesma família) (OLIVEIRA *et al.*, 2006).

De modo geral, os microssatélites são bastante polimórficos, abundantes e similarmente distribuídos ao longo das regiões eucromáticas do genoma (SCHLÖTTERER, 2004). Devido a essas características, este tipo de marcador tem sido amplamente utilizado em estudos populacionais, pois permite inferir a diversidade genética de uma espécie e os níveis de diferenciação genética entre grupos, detectar migrantes, fluxo gênico, entre outros aspectos (FRANKHAM; BALLOU; BRISCOE, 2008). Nesse aspecto, os microssatélites têm se mostrado marcadores nucleares úteis para determinar a estruturação genética e a história populacional de uma espécie, tornando-se, portanto, complementações interessantes ao mtDNA em estudos filogeográficos (SUNNUCKS, 2000; RICHARD; THORPE, 2001).

1.5 A família Felidae e a espécie *Puma yagouaroundi*

A família Felidae pertence à ordem Carnivora, sendo a única linhagem da subordem Feliformia a ocorrer na região Neotropical (WONZENCRAFT, 2005). De modo geral, as espécies da família Felidae possuem o corpo compacto, musculoso e flexível. Estes animais são ágeis escaladores, nadam com facilidade e possuem uma audição e visão acurada. Possuem uma

pelagem macia e lanosa, sendo que a coloração predominante dos pêlos varia de cinza a avermelhado, de amarelado a marrom, acrescida na maioria das espécies de marcações escuras como faixas/listras, pintas ou rosetas. Por esse motivo, certas espécies de felinos são caçadas propositalmente, devido ao valor comercial de sua pelagem. Este grupo de animais possui um hábito alimentar altamente predatório, caçando principalmente outros mamíferos, mas também aves, répteis e peixes. Quanto ao habitat, ocorrem em diversos tipos de ambiente, incluindo áreas de vegetação densa como florestas tropicais ou temperadas, bem como campos abertos e regiões alagadiças (NOWAK, 1999).

A espécie *Puma yagouaroundi* (É. Geoffroy Saint-Hilaire 1803) é conhecida popularmente como gato-mourisco, gato-preto ou também jaguarundi, e é uma das dez espécies nativas de felinos presentes na região Neotropical (NOWAK, 1999; EISENBERG; REDFORD, 1999; WONZENCRAFT, 2005). Esta espécie apresenta duas 'fases' de coloração (ou morfotipos), uma que varia de preto a cinza/marrom (mais escura), e outra que varia de avermelhado a castanho (mais clara) (NOWAK, 1999); diferentemente das outras espécies de felinos, o jaguarundi não apresenta qualquer listra ou mancha em seu corpo, como rosetas ou pintas, em nenhuma fase da vida (OLIVEIRA, 1998; EISENBERG; REDFORD, 1999). Esta não é a única diferença que essa espécie possui em relação às outras da mesma família, tratando-se talvez do gato que possui menos características comumente associadas aos felinos (OLIVEIRA, 1998). O jaguarundi (Figura 1) distingue-se por seu corpo alongado e delgado, a sua cabeça pequena e achatada, por possuir orelhas pequenas e arredondadas, as pernas curtas e sua cauda longa, com aproximadamente dois terços do comprimento do corpo (cf. OLIVEIRA, 1998; NOWAK, 1999; EISENBERG; REDFORD, 1999; SUNQUIST; SUNQUIST, 2002). Por possuir um padrão corpóreo diferente de outros felinos, algumas vezes o jaguarundi é comparado com uma lontra ou também com uma irara (NOWAK, 1999; SUNQUIST; SUNQUIST, 2002).

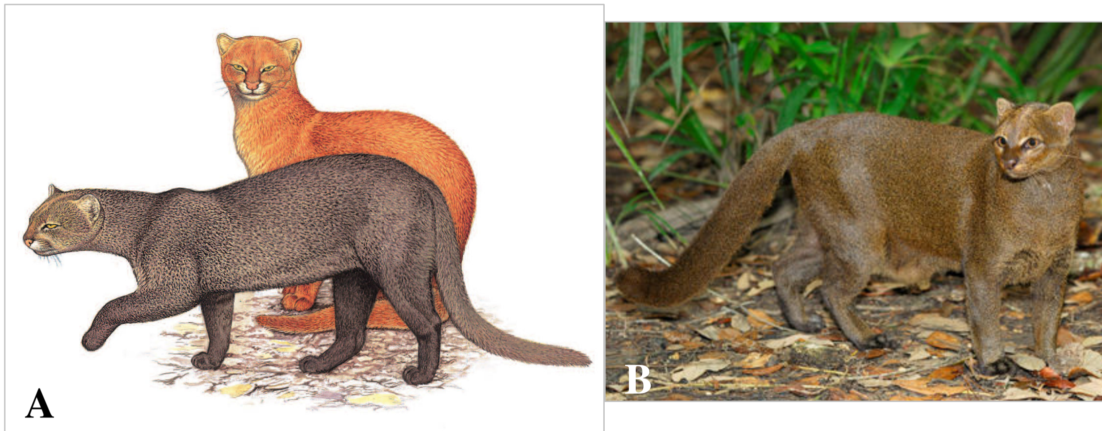

Figura 1: A. Desenho esquemático de *Puma yagouaroundi* em suas diferentes 'fases' de coloração, usualmente denominadas 'avermelhada' e 'cinza-escura'; B. Fotografia de um jaguarundi cinza/marrom-escuro. (FONTE: A - MASSOIA; CHEBEZ; BOSSO, 2006; B - http://www.wildearthguardians.org).

São poucos os estudos focados na espécie *Puma yagouaroundi*, sendo que a maioria dos trabalhos aborda o seu hábito alimentar ou padrões de movimentação (NOWAK, 1999). Desta forma, pouco se conhece ainda sobre o jaguarundi, sendo muito relevante qualquer informação adicional sobre esta espécie, especialmente porque os estudos com a dieta ou a extensão de locomoção geralmente são realizados com poucos indivíduos (OLIVEIRA, 2006).

Recentemente, um trabalho realizado por Tófoli, Rohe e Setz (2009) sobre a dieta de *P. yagouaroundi* em um mosaico da Mata Atlântica secundária e também silvicultura no Estado de São Paulo revelou que o jaguarundi possui uma dieta generalista, mas que possui preferência por pequenos vertebrados terrestres. Entre os itens alimentares observados nas fezes de 26 amostras analisadas, estavam: pequenos mamíferos (com a maior porcentagem), seguido de aves, invertebrados, répteis, grandes mamíferos (provavelmente consumo de carniça, ou indivíduos jovens), e também foi registrada a presença de uma serpente da família Viperidae. Guerrero e colaboradores (2002), analisando 36 fezes de jaguarundi no sul do México, também encontraram uma dieta similar ao trabalho descrito acima. Estes autores também observaram a importância de pequenos vertebrados para a sua alimentação, e encontram também itens alimentares como peixes e vegetais. Neste último caso, observou-se que durante a estação úmida, a freqüência de vegetais diminuiu e a de mamíferos e insetos aumentou, indicando assim que o material vegetal foi um complemento à sua alimentação (GUERRERO *et al.*, 2002). Em um estudo

realizado por Konecny (1989), na Colômbia, o mesmo padrão geral foi encontrado (a partir de 46 amostras de fezes): pequenos mamíferos (em maior porcentagem), aves, invertebrados (artrópodes), e ainda foram encontradas sementes de frutas em 11,3% das fezes analisadas.

Quanto ao período de atividade, de modo geral o jaguarundi possui uma atividade predominantemente diurna (KONECNY, 1989; EISENBERG; REDFORD, 1999; MAFFEI; NOSS; FIORELLO, 2007), o que coincide com o período de atividade de suas presas (TÓFOLI; ROHE; SETZ, 2009). No entanto, esta espécie também pode apresentar alguma atividade crepuscular (EISENBERG; REDFORD, 1999).

Em relação a estudos genéticos com esta espécie, um trabalho realizado por Moreno *et al.* (2006) estimou o conteúdo de informação polimórfica (PIC – *Polymorphic Information Content*) de quatro *loci* de microssatélites (originalmente desenhados para gato doméstico) testados em *Puma yagouaroundi* e outras duas espécies de felinos neotropicais. Utilizando 36 amostras de jaguarundi obtidas em zoológicos brasileiros, estes autores obtiveram valores de PIC entre 0.73 e 0.92, indicando um alto grau de polimorfismo e, portanto, o alto poder informativo destes marcadores para estudos genéticos.

De modo geral, esta espécie possui uma ampla distribuição geográfica, habitando desde o sudeste do Texas (Estados Unidos) até a Argentina (NOWAK, 1999). No entanto, há indícios de que esta espécie provavelmente esteja extinta no Texas, habitando, portanto, desde regiões com baixas altitudes no México, até a região central da Argentina (Figura 2) (SUNQUIST; SUNQUIST, 2002; CASO *et al.*, 2008). Ainda conforme estes autores, o jaguarundi habita tanto regiões abertas como fechadas, sendo encontrado em áreas de restinga, mangue, matas e cerrado. A espécie também é vista em habitats ribeirinhos (KONECNY, 1989; NOWAK, 1999) e florestas modificadas/alteradas (que estão em sucessão secundária), bem como florestas mais maduras (KONECNY, 1989). Diante desta ampla capacidade de habitar diferentes regiões, incluindo habitats abertos, *P. yagouaroundi* é tido como um felino com certa tolerância a distúrbios humanos (CASO *et al.*, 2008).

Figura 2: Distribuição geográfica atual aproximada de *Puma yagouaroundi* ao longo da região Neotropical (área demarcada em vermelho) (FONTE: IUCN, 2010)

Apesar da ampla distribuição geográfica, tem-se observado que o jaguarundi é uma espécie com baixa densidade populacional e incomum em certos locais, e a tendência inferida por pesquisadores em campo é que suas populações estejam diminuindo com o tempo (CASO *et al.*, 2008). Apesar disto, o *status* atual de ameaça de *Puma yagouaroundi* permaneceu classificado pela IUCN em sua última revisão como de Baixa Preocupação (LC – Least Concern). Entretanto, devido à falta de informação sobre esta espécie, é possível de que ela já deva ser considerada como de outra categoria, a de Quase Ameaçada (NC - Near Threatened) (CASO *et al.*, 2008). A avaliação mais precisa do atual *status* da espécie depende de uma investigação mais ampla de sua biologia e ecologia em face às atuais ameaças antrópicas, bem como a delimitação de unidades demográficas históricas (como subespécies ou ESUs) que requeiram estratégias próprias para a sua conservação.

Conforme Oliveira (1998), ao longo de sua distribuição geográfica, o jaguarundi possui oito subespécies descritas (Figura 3), sendo estas: 1 - *P. yagouaroundi ameghinoi* (Argentina), 2 - *P. yagouaroundi cacomitli* (litoral leste do México), 3 - *P. yagouaroundi eyra* (Paraguai e sul do Brasil), 4 - *P. yagouaroundi fossata* (sul do México), 5 - *P. yagouaroundi melantho* (leste do Peru), 6 - *P. yagouaroundi panamensis* (Panamá), 7 - *P. yagouaroundi tolteca* (litoral oeste do México), e 8 - *P. yagouaroundi yagouaroundi* (Guiana e região amazônica do Brasil). É importante ressaltar que estas classificações clássicas de subespécies de felinos foram produzidas com base em aspectos morfológicos e critérios bastante subjetivos, muitas vezes empregando tamanhos amostrais pequenos. Assim sendo, como mencionado anteriormente, esta divisão em oito subespécies pode não refletir realmente a estruturação filogeográfica da espécie, e requer uma avaliação crítica empregando diferentes conjuntos de dados e abordagens analíticas.

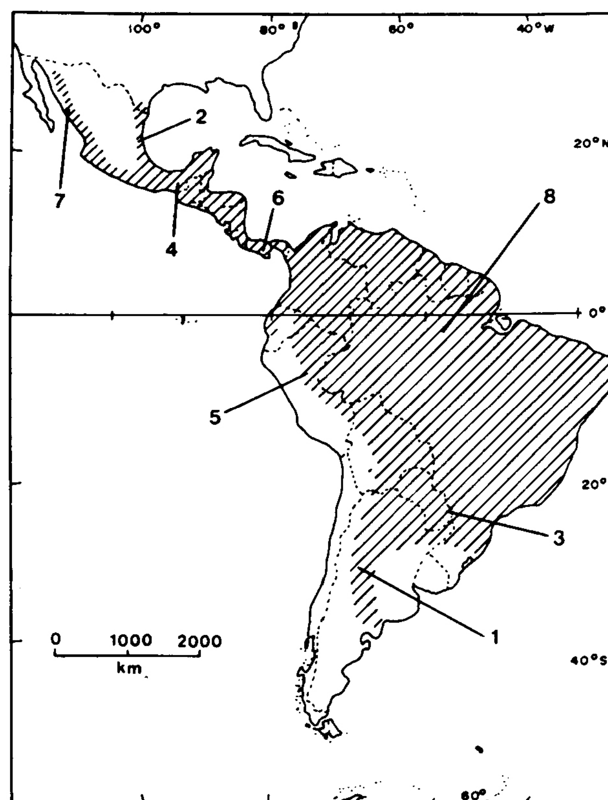

Figura 3: Mapa indicando a distribuição geográfica das oito subespécies descritas para *P. yagouaroundi* (FONTE: OLIVEIRA, 1998).

Em relação à taxonomia, das oito grandes linhagens de felinos existentes, o jaguarundi está atualmente inserido na linhagem do Puma (Figura

4) (JOHNSON; O'BRIEN, 1997; JOHNSON *et al.*, 2006). Contudo, devido à distinta aparência física desta espécie quando comparada à de outros gatos, conforme descrito anteriormente, sua classificação foi considerada historicamente confusa (SUNQUIST; SUNQUIST, 2002). *Puma yagouaroundi* já foi inserido no gênero *Felis*, no subgênero *Herpailurus* dentro do gênero *Felis*, ou mesmo em um gênero próprio, *Herpailurus* (WONZENCRAFT, 2005). No entanto, estudos recentes mostraram que o jaguarundi forma um grupo monofilético com o puma (*Puma concolor*), e o guepardo (*Acinonyx jubatus*), posicionando-se como grupo irmão do primeiro (JOHNSON; O'BRIEN, 1997; WONZENCRAFT, 2005; JOHNSON *et al.*, 2006). Como consequência, foi proposto que o gênero desta espécie mudasse de *Herpailurus* para *Puma* (WONZENCRAFT, 2005; JOHNSON *et al.*, 2006).

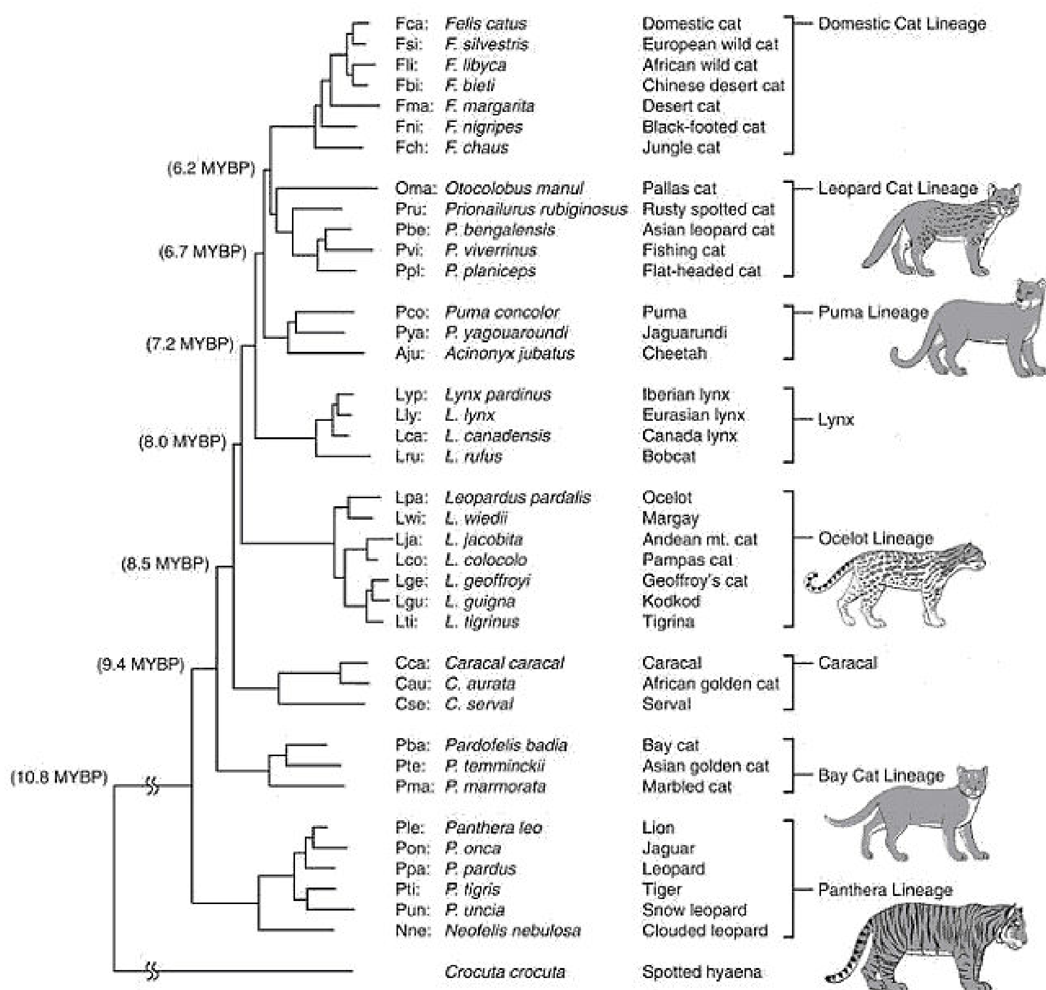

Figura 4: Relações filogenéticas entre as 38 espécies de felinos atuais, as quais formam as oito linhagens de felinos. As datas nos nós são estimativas do período de divergência entre as diferentes linhagens em milhões de anos. Árvore construída com base no trabalho de Johnson *et al.*, 2006 (FONTE: CULVER, 2009).

2. Objetivos

O presente trabalho tem por objetivos (i) estimar a diversidade genética e o padrão filogeográfico de *Puma yagouaroundi* (É Geoffroy Saint-Hilaire 1803), incluindo inferências sobre sua estrutura populacional atual, sua história demográfica, e a validade das unidades previamente propostas como subespécies para este felídeo; (ii) relacionar os resultados obtidos a informações disponíveis sobre paleogeografia (incluindo processos históricos associados a possíveis barreiras ao fluxo gênico entre diferentes populações), com a finalidade de compreender sua influência sobre a distribuição atual da variabilidade genética nesta espécie.

3. Apresentação do artigo

Esta dissertação de mestrado é apresentada no formado de artigo científico, intitulado “Phylogeography and evolutionary history of the jaguarundi (*Puma yagouaroundi*) (Mammalia, Felidae)”, a ser submetido para o periódico *Journal of Heredity*. Com intuito de facilitar a leitura do texto, as legendas das tabelas e figuras foram inseridas junto das mesmas.

Phylogeography and evolutionary history of the jaguarundi (*Puma yagouaroundi*) (Mammalia, Felidae)

CARLA B. PIRES[1], TADEU G. DE OLIVEIRA[2,3], WARREN E. JOHNSON[4], STEPHEN J. O'BRIEN[4], EDUARDO EIZIRIK[1,3]

[1] Laboratório de Biologia Genômica e Molecular, Faculdade de Biociências, PUCRS, Avenida Ipiranga, 6681, prédio 12C, sala 172, Porto Alegre, RS 90619-900, Brazil.

[2] Departamento de Biologia, UEMA, Cidade Universitária Paulo VI, Caixa Postal 09, São Luis, MA 65055-970, Brazil.

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

[4] Laboratory of Genomic Diversity, National Cancer Institute (NCI), Frederick, MD 21702-1201, USA.

Corresponding authors:

Carla B. Pires and Eduardo Eizirik

E-mails: carla.pires@gmail.com and eduardo.eizirik@pucrs.br

Running title: Jaguarundi phylogeography

ABSTRACT

Jaguarundis (*Puma yagouaroundi*) are small cats widely distributed across the Neotropics, ranging from Mexican lowlands to central-southern Argentina. So far no study has assessed the extent of its present genetic diversity, as well as its population structure, demographic history and phylogeographic patterns. We have addressed these issues by analyzing 1191 bp of the mitochondrial DNA (mtDNA) and eight microsatellite loci in up to 95 individuals, covering a large portion of the species' geographic range. MtDNA results showed low to moderate levels of genetic diversity, while the microsatellites exhibited moderate to high variability. Contrary to the eight subspecies classically recognized for the jaguarundi, mitochondrial analyses revealed the existence of two major phylogeographic groups (Northern and Southern) that appear to be at least in part influenced by a historical barrier such as the Amazon river. The microsatellites suggested a similar pattern but with a lower degree of differentiation, possibly due to male-biased gene flow. No evidence of significant demographic changes through time was observed, indicating that the species has maintained rather stable population size in the past. The results presented here may be useful in developing conservation strategies on behalf of this intriguing and poorly studied wild cat.

Keywords: population structure, evolution, conservation, *P. yagouaroundi*, mitochondrial DNA, microsatellites.

INTRODUCTION

Understanding the population history of a species and also its current genetic structure can reveal which evolutionary processes (e.g. barriers to gene flow, isolation by distance, among others) may have played a role in the distribution of its present genetic variability (Avise 2000; Frankham et al. 2002). This knowledge is important given that it can indicate the existence of ESUs (*Evolutionarily Significant Units*) or MUs (*Management Units*), which in turn are useful in management actions (Diniz-Filho et al. 2008; Eizirik et al. 2006; Moritz 1994). Moreover, understanding phylogeographic patterns and population structure is of great value not only from the conservation point of view, but also because it provides important knowledge on the species' biology, since it may be used to infer the evolutionary processes that have shaped the observed pattern, and their relationship to life-history traits (Avise 2000; Diniz-Filho et al. 2008; Eizirik et al. 2006; Frankham et al. 2002).

Even though Brazil's biodiversity is considered to be very high in terms of the number of species, with estimates ranging from 9.5% to 13.1% of the world's total species, many of these species and even higher taxonomic levels remain poorly known (Brandon et al. 2005; Lewinsohn and Prado 2005; Schipper et al. 2008). This is the case of many Neotropical carnivores, for which even basic information is lacking, considering that few robust and thorough studies have been performed so far (Oliveira 2006). Although this scenario has changed to some extent in the last few years, with studies addressing aspects such as population history and phylogeography of some species, (e.g. Culver et al. 2000; Eizirik et al. 1998, 2001; Haag et al. 2010; Tchaicka et al. 2007; Trinca et al. [in press]), the majority of studies published so far involves food habits, often employing limited sample size. Hence, information on the ecology, natural history, population structure and phylogeographic patterns of many Neotropical carnivores remains scarce (Eizirik et al. 2006, 2012; Oliveira 2006).

The jaguarundi (*Puma yagouaroundi*) is a wild cat that fits the scenario described above, as it is one of the Neotropical felids with the least available information (Eizirik et al. 2006; Oliveira 2006). Nearly all studies conducted so far with the jaguarundi addressed aspects of its diet, which is considered to be generalist since it mainly consists in small mammals, birds and reptiles (Silva-Pereira et al. 2011; Guerrero et al. 2002; Konecny 1989; Tófoli et al. 2009), although fish (Guerrero et al. 2002), arthropods and plants might also be found at a lower frequency (Konecny 1989; Sunquist and Sunquist 2002; Tófoli et al. 2009).

This curious animal, considered to be a cat with few feline features due to its unique appearance, has a broad geographic distribution (Fig 1) ranging from the eastern and western lowlands of Mexico to central-southern Argentina (Nowak 1999; Oliveira 1998; Sunquist and Sunquist 2002). As a result, it is found in a variety of open and closed habitats such as tropical rainforests, semideciduous forests, scrub savannas and swampy grasslands, indicating that it is quite flexible ecologically (Caso et al. 2008; Eisenberg and Redford 1999; Oliveira 1998). Unlike many other cats, *P. yagouaroundi* seems to have a diurnal activity (Di Bitetti et al. 2010; Konecny 1989; Maffei et al. 2007; Oliveira 1998; Sunquist and Sunquist 2002), which is possibly why it is often seen by field researchers. This fact may induce the false impression that this felid is common and abundant, which is at odds with recent findings indicating that it is actually an uncommon, low-density species (Caso et al. 2008). In accordance with the most recent molecular phylogeny of the Felidae (Johnson et al. 2006), the jaguarundi diverged from its sister species (the puma [*Puma concolor*]) *ca.* 4.2 million years ago (MYA). Consequently, one may hypothesize that this split may have occurred in North America, prior to the Great American Biotic Interchange (GABI) in the Pliocene, and followed by independent colonization of South America by each species (Eizirik 2012). However, the estimated credibility estimated leaves room for an alternative hypothesis, i.e. that their speciation occurred in South America after the invasion by a common ancestor during the GABI. Regarding intra-specific genetic issues, only two published studies included this species: Eizirik et al. (2003) discovered a 24-bp deletion in the *MC1R* gene that affects coat color variation in this species, while Moreno et al. (2006) evaluated the Polymorphism Information Content (PIC) of four microsatellite loci originally described for the domestic cat. Still, none of these studies focused exclusively on the jaguarundi, nor revealed aspects of its range-wide population structure and demographic history.

In this study, we addressed the evolutionary history of the jaguarundi using biological samples that span most of the species' distribution. We performed several analyses based on two distinct data sets, one comprising two mitochondrial DNA (mtDNA) segments and the other consisting of eight microsatellite loci. On the basis of these data, we make inferences on phylogeographic patterns, population structure and demographic history of this elusive felid.

MATERIALS AND METHODS

Sample collection and laboratory methods

A total of 71 biological samples such as blood and/or tissue was obtained for this study. The majority of blood samples was collected from wild specimens caught by collaborators in field ecology studies, or from captive individuals of known geographic origin. Tissue samples were obtained mostly from road-killed individuals, and a few samples came from museums. Blood samples were preserved in a saturated salt solution (100 mM Tris, 100 mM EDTA, 2% SDS), and tissue samples in 96% ethanol. Genomic DNA extraction was performed using a commercial kit (DNeasy Blood & Tissue - Qiagen) following the manufacturer's guidelines. After this procedure, the total DNA was quantified in a 1% agarose gel stained with GelRed® (Biotium Inc.) using a molecular marker of known concentration such as the LowMass DNA Ladder (Invitrogen), and the samples were subsequently diluted to a 5ng/µL concentration. In addition to the samples mentioned above, 33 additional samples that were also included in this study already had their DNA extracted and quantified previously. Thus, a total of 104 biological samples of *P. yagouaroundi* was included in the study, covering most of the species' range (Fig. 1, Table 1).

DNA amplification of both mitochondrial and nuclear (microsatellite) markers was carried out by the polymerase chain reaction (PCR). Two different mitochondrial DNA (mtDNA) fragments were amplified: (i) the 5' portion of the *NADH dehydrogenase subunit 5* (*ND5*) gene, using primers reported by Trigo et al. (2008); and (ii) a partial fragment of the *cytochrome b* (*cyt-b*) gene, using primers described by Irwin et al. (1991) [L15162 and H15915]. Each PCR reaction (20 µL) contained 10 – 15 ng of DNA, 0.2µM of each primer, 1X Buffer (Invitrogen), 1.5 – 2.0 mM of $MgCl_2$, 200 µM of each dNTP and 0.2 U of Platinum® *Taq* Polymerase (Invitrogen). Negative controls (all reagents but no DNA) were included in every PCR reaction. Thermocycling conditions for the *ND5* segment included an initial denaturing step of 94°C for 3min, followed by 10 touchdown cycles of 94°C for 45s, 60-51°C for 45s (-1°C per cycle), 72°C for 1.5 min; and 30 additional cycles of 94°C for 45s, 50°C for 45s, 72°C for 1.5 min; and a final extension step of 72°C for 3 min. The thermocycling profile for the *Cytb* segment was similar to the one described above, differing in the touchdown step (5 cycles of 94°C for 45s, 55-50°C for 45s [-1°C per cycle], 72°C for 1.5 min), in the number of additional cycles (40 instead of 30), and in the final extension step

(72°C for 30 min). PCR amplification was then confirmed and quantified in a 1% agarose gel stained with GelRed®, and the products were purified using the enzymes Exonuclease I and Shrimp Alkaline Phosphatase. After this step, PCR products were sequenced using the *DYEnamic ET Dye Terminator Sequencing Kit* (GE Healthcare), and analyzed in a MegaBACE 1000 automated sequencer (GE Healthcare).

Regarding the microsatellite data, eleven loci originally described for the domestic cat (Menotti-Raymond et al. 1999) were tested for *Puma yagouaroundi*. A test with different annealing temperatures for each locus was performed in order to optimize the use of these *primers* in jaguarundi samples. Taking into account the results obtained in this test, eight microsatellite loci where selected based on amplification efficiency and yield. Two of them contained trinucleotide repeats (F98 and F146) and six comprised tetranucleotide repeats (F124, F42, FCA441, FCA391, FCA453 and FCA424). For all loci, every forward primer had a M13 tail (5'-CACGACGTTGTAAAACGAC-3') at their 5' end, allowing the paring of a third complementary primer (M13) that was dye-labeled (Boutin-Ganache et al. 2001). PCR reactions were performed separately for each locus in a final 10 µL volume, containing 10 – 20ng of DNA, 0.013 µM of the M13-tailed forward primer, 0.2µM of the reverse primer and a fluorescent M13 primer (6-FAM, NED or HEX), 1X Buffer (Invitrogen), 1.5 – 2.0 mM of $MgCl_2$, 200 µM of each dNTP and 0.1 U of Platinum® Taq Polymerase (Invitrogen). Again, negative controls (all reagents but no DNA) were included in all PCR reactions. Thermocycling conditions were the same for all loci (except for the annealing temperatures, which varied across loci - Table 5), and consisted of an initial denaturing step at 94ºC for 5 min, followed by 30 cycles of 94ºC for 45s, 53 - 60ºC (see Table 5) for 45s, 72ºC for 1 min, and a final extension at 72ºC for 15 min. After PCR amplification, all samples (including the negative control) were diluted 1:5, loci with different fluorescent dyes were pooled together in a multiplex set (see Table 5), and then genotyped using a MegaBACE1000 (GE Healthcare) automated sequencer with the internal size standard ET550-R. The software Genetic Profiler 2.2 was used to analyze the results.

Sequence Analysis

DNA sequence electropherograms were visualized and checked using FinchTV 1.4.0 (http://www.geospiza.com/Products/finchtv.shtml), and then aligned using the Muscle algorithm (Edgar 2004) implemented in the software MEGA 5 (Tamura et al. 2011). The DNA alignment was manually checked and corrected when necessary. During the first step

described above, we found evidence of mtDNA heteroplasmy in some individuals, where high quality sequences (high peaks and low baseline) sometimes presented double “clean” peaks on both strands at a specific nucleotide site. Some of these samples were re-sequenced in order to verify the ambiguity at that position; if confirmed, the ambiguous site was scored as missing information and excluded from subsequent analyses.

Summary statistics such as haplotype (Hd) and nucleotide (π) diversity, and the number of variable and parsimony-informative sites, were estimated for each mitochondrial segment separately, as well as the concatenated data set, using Arlequin 3.5 (Excoffier et al. 2005) and MEGA 5. Only samples that had both segments (*ND5* and *Cytb*) sequenced were included in the mtDNA concatenated data set, which was then used for all subsequent analyses.

We performed phylogenetic analyses of the concatenated mtDNA data using three different approaches: (i) maximum parsimony (MP) using the software TNT (Goloboff et al. 2008), (ii) maximum likelihood (ML) using the online version of PhyML 3.0 (http://www.atgc-montpellier.fr/phyml/) and (iii) Bayesian inference (BI) using Beast v. 1.6.2 (Drummond and Rambaut 2007). For the latter two, we initially determined the model of DNA evolution that best represented our data using the Akaike Information Criterion (AIC) implemented in the software JModeltest (Posada 2008). For the MP phylogenetic reconstruction, we used heuristic searches with 1.000 replicates of random starting trees (random addition sequence-Wagner trees) followed by the tree bisection-reconnection (TBR) branch swapping algorithm. A similar search profile was used for the ML method, with heuristic searches followed by TBR, where the starting trees used the BIONJ algorithm (Gascuel 1997). In both analyses, the confidence on the results was evaluated by performing 1.000 bootstrap pseudoreplicates. The BI phylogenetic analysis was carried out using a Markov Chain Monte Carlo (MCMC) algorithm that ran for 5,000,000 generations, sampling trees every 500 steps, and discarding the first 500,000 generations as burn-in. A coalescent tree prior was used in this analysis. The program Tracer v.1.5 (Rambaut and Drummond 2007) was used to verify if the estimated parameters generated by the MCMC run were stabilized, and then posterior probabilities were calculated. Additional DNA sequences of *Puma concolor* (GenBank accession numbers: AY598493 and AY598487), the sister species of the jaguarundi (Johnson et al. 2006), were used as outgroups in all phylogenetic analyses.

To further evaluate the evolutionary relationships among the *P. yagouaroundi* haplotypes, we generated a haplotype network for the concatenated mtDNA data set using the median-joining algorithm (Bandelt et al. 1999) implemented in the software Network 4.6.1.0

(www.fluxus-engineering.com). In addition, we investigated the demographic history underlying the current haplotype diversity by performing neutrality tests (Tajima's *D* [Tajima 1989] and Fu's *Fs* [Fu 1997]) and a mismatch distribution analysis, as implemented in Arlequin.

We also estimated a Bayesian Skyline Plot (Drummond et al. 2005), using the concatenated data set and the software Beast, to infer the dynamics of population size through time, and to assess if there was any evidence of demographic change in the past. For this purpose, we initially estimated a substitution rate for a subset of the ingroup samples using a uniform prior distribution for the age of the split between the jaguarundi and the puma, whose boundaries were set by the 95% credibility interval reported by Johnson et al. (2006) for this divergence (3.16 to 6.01 million years ago [MYA]). We assumed a Yule Process for the tree prior and an uncorrelated lognormal relaxed molecular clock, and performed a MCMC of 50 million steps (burn-in of 10%). We then performed a second Beast run for the Bayesian Skyline analysis, assuming a strict molecular clock and the substitution rate estimated in the previous run, along with a Skyline piecewise-constant tree prior. The MCMC was run for 100,000,000 generations, with the first 10% discarded as burn-in. The software Tracer was used to verify and analyze the estimated parameters.

To assess if there was any population structure and differentiation among geographic groups, we estimated frequency-based fixation indices such as Φ_{ST}, and carried out an Analyses of Molecular Variance (AMOVA) (Excoffier et al. 1992) using Arlequin, with 10,000 permutations. Different population structure scenarios were tested, including some suggested by the phylogenetic and network analyses, and also by subdividing South American individuals into groups sampled in the major biomes found on this continent. Several distance measures were used during each analysis, and the results reported here are from a Kimura 2P + G distance (the closest model to the one estimated as a best-fit to this data set) and the proportion of differences (p-distance). Unlike conventional *F*-statistics, these measures also take into account the molecular distance among haplotypes, and thus the information of how much a haplotype is different or similar to another is incorporated. Finally, we used the software Alleles In Space (AIS) (Miller 2005) to perform a Mantel test (Mantel 1967), assessing the correlation between genetic and geographic distances among samples, which would indicate the occurrence of isolation by distance in the jaguarundi. The significance of the estimated correlation was assessed with 10,000 permutations.

Microsatellite data analyses

The program Convert (Glaubitz 2004) was used to facilitate the conversion between different input files required by the population genetic software. Indices of genetic diversity were estimated by calculating the number of alleles per locus, the polymorphic information content (PIC), and the observed (H_o) and expected heterozygosity (H_e) using Cervus 3.0.3 (Kalinowski et al. 2007) and Arlequin. The latter program was also used to test for Linkage Disequilibrium (LD) between loci and to detect departures from Hardy-Weinberg equilibrium at each locus. The software HP-Rare (Kalinowski 2005) was used to compute the allelic richness and the number of private alleles for each population (see Results for further information regarding defined groups) using the rarefaction approach, which compensates for differences in sample size. The number of gene copies considered was set based on the number of diploid individuals of the smallest population sample being compared.

In order to assess the presence of genetically differentiated groups and to assign individuals to their respective group, we employed the Bayesian clustering approach implemented in Structure 2.3.3 (Pritchard et al. 2000). We performed an MCMC run with 1,000,000 steps (after a burn-in of 100,000 generations), varying the values of K (number of assumed genetic clusters) from 1 to 5. Five replicates were run for each value of K. The analysis was performed without any prior information regarding the sampling location of the individuals. The estimates of log likelihoods of each replicate were checked to verify that there was little variation within each assumed K, indicating that the MCMC runs had converged. Otherwise, five additional runs for each value of K were performed with longer MCMC runs and longer burn-in lengths as well. If necessary, the number of K values was also increased. The population structure inferred with this approach was further tested in the subsequent analyses.

To evaluate how the microsatellite diversity is distributed among populations, we used the software Arlequin to estimate the F_{ST} index (Wright 1978), based on the number of different alleles, and also to perform an Analyses of Molecular Variance (AMOVA). As in the mtDNA analyses, we tested different scenarios of population structure to assess which subdivision best explained the observed variation in allele frequencies. To assess the existence of an isolation-by-distance pattern, we performed a Mantel test (10,000 permutations) using the program AIS.

RESULTS

Mitochondrial markers

The estimated statistics of mtDNA diversity (Table 2) showed a similar pattern of variability for the two analyzed segments, with the number of variable and parsimony-informative sites varying from 22 (*Cytb*) to 25 (*ND5*), and 17 (*Cytb*) to 18 (*ND5*), respectively. The nucleotide diversity (π) indices were low to moderate, and indicated a slightly higher value of overall diversity in the *Cytb* segment (0.0053) than in *ND5* (0.0050). The haplotype diversity was moderate to high for both segments, ranging from 0.8151 (*ND5*) to 0.8849 (*Cytb*). The concatenated data set comprised an alignment that contained 1191 bp, 42 variable sites (32 of which were parsimony-informative), 21 different haplotypes, and a moderate to high level of haplotype diversity (Hd = 0.87). Although this data set included fewer samples than the individual segments, most of the variability found in each separate segment was maintained in this concatenated data set.

The haplotype network (Fig 2, see also Table 3) showed that most mtDNA sequences from South America were a least six mutational steps away from the Central America and Mexico haplotypes, thus suggesting the existence of geographic structure in jaguarundi matrilines. Interestingly, the haplotype found in Venezuela was identical to the one found in Mexico. On the other hand, individuals from Guatemala were more closely related to the South America haplotypes than to the Central America ones, suggesting some historical female gene flow between these regions. Another interesting feature was the presence of one highly frequent haplotype (Py-H3), shared across different areas in the southern part of South America, whereas other haplotypes differed from this central one by only one or two mutations (Fig 2, Table 3). Also within this southern region, we found two additional haplotypes (Py-H1 and Py-H2) with relatively high frequency, one encompassing most individuals from Rio Grande do Sul and Paraná states (Py-H1) in Brazil, and the other (Py-H2) with a broader range including samples from other areas such as southeastern and northeastern Brazil. This indicates that, although some haplotypes appear to be geographically restricted, others are shared among different regions within Brazil.

The model of DNA substitution that best fit the data according to the AIC was the 3-parameter-model (K3P) (Kimura 1981) with unequal base frequencies and a gamma distribution (G) of rate variation among sites. The second best model was a very similar one, HKY+G (gamma shape = 0.1980), which has the same attributes as the first one except for

the two rates regarding the transversions, and had an AIC value only slightly higher (ΔAIC = 0.6411) than the K3P. Burnham and Anderson (2002) suggested that models having ΔAIC (difference between AIC values of two models) values within 1 or 2 of the best model have strong support and should also be considered. Therefore, considering that the analytical packages used for ML and BI phylogenetic analyses have the second model as an implemented option, but not the K3P, we employed the HKY+G model in all analyses.

The phylogenetic reconstruction obtained with the three different methods (Fig 3, and Supplementary Fig. S1) recovered most of the aspects observed in the network analysis. We found in all trees the presence of a highly supported clade containing most of the South America (SA) haplotypes. As in the network analysis, the Guatemala haplotype (Py-H6) was placed within this major SA clade. The maximum parsimony analysis found 12 trees with 186 steps, in which the most basal haplotype was Py-H14 followed by Py-H7, both from the North America (Mexico) + Central America (NCA) region. The strict consensus tree (Supplementary Fig S1) also depicted two derived clades in which almost all internal branches were collapsed, indicating the lack of structure within each group. The main difference between the topologies produced with the three approaches was that, whereas in the MP and ML trees the haplotypes from Mexico + Central America were in a basal position, suggestive of a north-south migration of the jaguarundi, in the Bayesian approach there were two well-defined major clades (NCA and SA, both with posterior probabilities of 1.0), precluding any inference regarding which group arose first.

In order to test which population structure was most likely, taking into account the genetic differentiation among haplotypes, an Analysis of Molecular Variance (AMOVA) was performed and Φ_{ST} values were estimated for three different scenarios of population subdivision. The first one was based on the geographic origin of the individuals, but partially taking into account the separation between South and Central America observed in the network and phylogenetic analyses. That is, one group (NCA) comprised haplotypes from Mexico and Central America, while the other (SA) by all the samples from South America. The AMOVA analysis indicated that there was more genetic variation between these two major groups than within each of them. This was reflected in high and significant Φ_{ST} values: 0.5433 for Kimura 2P + G distance, and 0.5354 for the proportion of differences (both *p*-values < 0.001).

The second scenario of population structure that was assessed took into consideration that the haplotype found in the Venezuela specimens was identical to the one found in Mexico, and thus more likely belonged in the NCA group. Therefore, samples from regions

located to the north of the Amazon river (Northern group – Venezuela included) were compared with those originating from areas to the south of this river (Southern group). In this case, the inferred differentiation was even higher than in the first scenario, with an Φ_{ST} of 0.6124 for the Kimura 2P + G distance, and 0.6043 for the proportion of differences (both *p*-values < 0.001). The Mantel test (r = 0.3316, $p = 0.0009$) results indicated that part of this high genetic differentiation between these two groups was due to isolation by distance, as indicated by correlation between the genetic and geographic distances.

The third and last scenario was aimed at testing for further subdivision of South America into major biomes. On the basis of the results from first two scenarios, we hypothesized that the strongest population partition was between the Northern and Southern groups. We thus compared the Northern group with three regional subpopulations of the Southern group: the south-southeast (SSE) population, including samples from the southern portion of the Atlantic Forest and adjacent Pampas grasslands; the central-west (CW) population, encompassing mostly the Cerrado biome; and the northeast (NE) population with samples from the northern portion of the Atlantic Forest along with the dry Caatinga biome. The results showed that the highest Φ_{ST} values were obtained in all comparisons made with the Northern group (Φ_{ST} values between 0.5350 and 0.6120 for the K2P + G distance; all $p <$ 0.001), while when the southern subpopulations were compared to each other, the largest difference was between the NE and SSE groups, with a relatively high and significant Φ_{ST} of 0.2069 ($p < 0.01$) for the K2P + G distance (Table 4). Interestingly, when the CW population was compared to either one of the other southern sub-groups, none of the Φ_{ST} values was significant ($p > 0.05$) and one of the comparisons (CW *vs*. NE) showed a negative Φ_{ST}. This result could be an indication that part of the differentiation between the SSE and NE populations may be due to isolation by distance. This hypothesis was supported by a Mantel test performed for the Southern group alone, where a moderate but significant correlation was observed (r = 0.2615, $p = 0.0001$). Therefore, all the mtDNA results so far support the existence of two major phylogeographic groups, Northern and Southern, with some genetic structure within the latter. Further scenarios could not be tested due to the small sample size available for some regions, which would likely bias the results of some analyses.

With respect to the demographic history, our analyses did not reveal any clear signal indicating major demographic changes in the jaguarundi over the past several thousand years. Neither Tajima's *D* nor Fu's *Fs* test resulted in significant values (estimated for Northern and Southern groups separately; data not shown). Likewise, the mismatch distribution analysis showed a multimodal pattern (not shown). The Bayesian Skyline Plot (Fig 4) also failed to

reconstruct a clear pattern of demographic changes in the past. Although we observed a trend suggesting gradual increase in the past 150,000 years, followed by a recent drop and subsequent demographic recovery, these reconstructions were accompanied by broad credibility intervals, thus failing to reject a null hypothesis of constant population size.

Microsatellite markers

Of the 104 available *P. yagouaroundi* samples, we were able to successfully amplify most of the eight selected microsatellite loci for 76 individuals. Samples with too much missing data (i.e. > 50% of the total number of loci) were excluded from the data set and thus from all subsequent analyses. All loci but one (F146, with 5.26%) had less than 5% missing data. We found evidence that could suggest potential allelic size homoplasy for two loci (F124 and FCA391), since even though most of the adjacent alleles were four nucleotides apart, some differed by only three or two base pairs. This might be a result of insertion-deletion events surrounding the microsatellite region, or the existence of a complex repeat locus in the jaguarundi, relative to the original target species for marker development (domestic cat). Such factors could induce size homoplasy among the defined alleles, as reported previously for *Puma concolor* (Culver et al. 2001). Other than these two exceptions, no difference was found regarding the type of repeat (tetra or tri) between the domestic cat, (Menotti-Raymond et al. 1999, 2005) and the jaguarundi (Table 5). Still, taking into account the considerations mentioned above, we decided to perform the AMOVA analysis and F_{ST} estimates using only the number of different alleles as input information.

We identified a total of 79 alleles across all loci (Table 6), and the number of alleles per locus varied from 5 (FCA424) to 17 (F42). The former locus had the lowest polymorphic information content (PIC), 0.096, and locus F124 had the highest PIC value (0.883). The average number of alleles per locus was 9.88, and the average observed and expected heterozygosities was 0.596 and 0.739, respectively (see Table 6). Tests for deviations from Hardy-Weinberg equilibrium (HWE) and linkage equilibrium were performed for two major groups separately: NCA and South America (Venezuela included) (see below). After a Bonferroni correction, departures from HWE remained at 3 loci (F42, F98 and F146), and 2 pairs of loci (F124 and F42, FCA391 and FCA424) were in linkage disequilibrium (LD), both in the South American population. When Venezuela was placed with the NCA group (i.e. within the Northern group, as previously defined) the same results were obtained, but an additional pair of loci (F124 and FCA424) was found to be in LD. As the loci inferred to be

in LD are actually on different chromosomes of the domestic cat (Menotti-Raymond et al. 1999), these deviations from equilibrium may indicate further population structure that has not been captured, likely due to the small sample size available for some regions (see Fig 1, and Table 1 for further information).

Initial runs with the software Structure showed considerable variation in the estimated likelihood across replicates of each assumed *K* value. We thus performed more exhaustive runs in order to obtain more consistent estimates, and also increased the maximum value of *K* from five to seven. The final and more thorough MCMC run had a length of 5,000,000 steps (first 510,000 discarded as burn-in) and showed similar values of posterior probability within each value of *K*, indicating that the run had stabilized. The mean posterior values behaved as a plateau after k = 2 (Fig 5a). Thus, to infer the number of genetically distinct populations (*K*) in *P. yagouaroundi*, we used the ΔK approach suggested by Evanno et al. (2005), which indicated that $K = 2$ was the most likely number of groups for this data set (Fig 5b).

Although the membership (Q) values of individuals from the Southern group where mostly mixed between the two inferred genetic clusters, all the samples but one from Central America, Mexico (NCA) and also Venezuela showed high membership values (>0.85) in one specific cluster, thus suggesting that the two probable populations represent the Northern and Southern groups (as in the mtDNA). We performed an additional run (5,000,000 MCMC steps after 500,000 generations discarded as burn-in) setting *K* from 1 to 2 with 10 replicates each, to confirm this assignment of Venezuelan individuals to the Northern group. In all 10 replicates, Venezuelan samples were consistently assigned (Q > 0.93) to the same cluster as the NCA samples. The mixed assignment observed in other South American samples may be a result of many factors, such as different sample size among regions, as well as an influence of isolation by distance across different parts of the sampled range (Manel et al. 2005; Pritchard et al. 2010; Schwartz and McKelvey 2009).

As in the mtDNA data set, three different schemes of population structure were tested to evaluate the extent of genetic differentiation among groups. The AMOVA results for the first scenario (NCA *vs.* South America [Venezuela included]) showed that a small but significant portion of the detected genetic variation was located between these two groups (F_{ST} = 0.0597, $p < 0.001$). The second population structure tested (Northern *vs.* Southern regions, with Venezuela in the Northern one) resulted in a similar pattern, with a slightly lower value of F_{ST} (0.0552, $p < 0.001$). Although might indicate that individuals from Venezuela are more genetically similar to those found farther south in South America than to the NCA, it is important to note that only two samples of Venezuela were present in this data

set. Furthermore, a Mantel test (r = 0.2634, $p = 0.0000$) indicated that isolation by distance might be playing a role in the genetic differentiation of these two broad geographical areas.

Finally, the third scenario tested was the Northern group *vs.* the southern subpopulations. The only difference with respect to the mtDNA scenario is that, given the small sample size available for the Cerrado biome (5 individuals, i.e. 10 alleles), we decided to exclude this population from the comparisons, so that only two populations (SSE and NE) were tested. Also, since we did not have any strong evidence (significantly higher F_{ST} in one specific scenario) supporting the grouping of Venezuela in either major group, we chose to allocate Venezuela with the NCA samples for this third scenario, considering the geographical proximity of the regions compared to other sampling sites. The results from this final analysis (Table 7) showed that, again, the highest F_{ST} values were obtained when comparisons were made with the Northern group, varying from $F_{ST} = 0.0404$ to $F_{ST} = 0.0679$ with the NE and SSE populations, respectively (both $p < 0.01$). In contrast, the tests between the southern subpopulations (SSE *vs.* NE) were not significant. A weak correlation between geographic and genetic distances (r = 0.1520, $p = 0.0138$) was found when the Mantel test was performed using only the Southern samples.

DISCUSSION

When compared with other felid species, as well as other carnivores, the genetic diversity estimates obtained for the jaguarundi in this study can be considered to be low to moderate. The nucleotide diversity estimated for *ND5* ($\pi = 0.0050$) was higher than the ones estimated for other Neotropical carnivores, such as the small cats *Leopardus tigrinus* ($\pi = 0.0031$) and *Leopardus geoffroyi* ($\pi = 0.0039$) (Trigo et al. 2008; T. Trigo, pers. comm.); the crab-eating raccoon *Procyon cancrivorus* ($\pi = 0.00239$; Tsuchiya-Jerep 2009), a small procyonid with a somewhat similar distribution to *P. yagouaroundi*; and the puma, *Puma concolor* ($\pi = 0.00318$), the jaguarundi's sister species with a noteworthy low genetic diversity, albeit the latter study had also analyzed additional (partial) segments of the *16S* and *ATP8* mitochondrial genes, besides the partial *ND5* (Culver et al. 2000). But on the other hand, were considerably smaller than the leopard's estimated diversity ($\pi = 0.0121$), although, again, a partial fragment of another segment, the control region (116bp), was also included in that study (Uphyrkina et al. 2001). Similarly, a lower degree of diversity was also found in comparisons with other Neotropical carnivores, such as the Neotropical otter *L.*

longicaudis (π = 0.00741; Trinca et al. [in press]) for the *ND5* segment, and the lowland tapir with a π of 0.00932 (de Thoisy et al. 2010) and the giant otter (π = 0.015; Pickles et al. 2011) for the *Cytb* segment, in which the π estimated for jaguarundi using only *Cytb* was equal to 0.0053 (± 0.0013). However, it is relevant to state that the latter two studies sequenced almost the entire *Cytb* gene. Nevertheless, a similar pattern is found when comparisons are made using both segments, in which jaguarundi's diversity is quite lower than the observed in the brown-nosed coati (π = 0.01949 for *ND5* and π = 0.01771 for complete *Cytb*) (Tsuchiya-Jerep 2009), and smaller than that obtained for the small Neotropical cat *L. colocolo* (π = 0.00799 for *ND5* and π = 0.00807 for the *Cytb* portion that overlaps the fragment analyzed here; A. Santos, pers. comm.).

Unlike the values of the nucleotide diversity, which sometimes varied considerably between different species, the jaguarundi's haplotype diversity (Hd = 0.8151 for *ND5* and 0.8849 for *Cytb*) did not show such a discrepancy when compared with species of these same studies, whose values ranged from 0.759 to 0.86 for *ND5* and 0.783 to 0.988 for *Cytb*. Therefore, even though some of the comparisons made were not entirely equivalent mainly due to the fact of additional genes included in these studies, we still decided to present them since there was some overlap with the data set analyzed herein.

Regarding microsatellite variability, the estimated indices of genetic diversity obtained for the jaguarundi were similar to the ones reported for other felid species, such as the puma's South American population (Culver et al. 2000), jaguar (Eizirik et al. 2001; Haag et al. 2010), leopard (Uphyrkina et al. 2001), tiger (Luo et al. 2004), as well as the small Neotropical cats *Leopardus tigrinus* and *L. geoffroyi* (Trigo et al. 2008). The estimated levels of expected heterozygosity were also similar to the procyonid *Nasua nasua* and smaller than those found for *Procyon cancrivorus* (Tsuchiya-Jerep 2009).

The results from the mtDNA phylogenetic analyses inferred by the three different approaches consistently showed high statistical confidence for the Southern clade encompassing most of the South American (SA) individuals. A second clade, named the Northern group, was present only in the Bayesian inference analysis with a high posterior probability of 1 (Fig 3), and partially in the MP (Fig S1). Nevertheless, the high support in all trees for at least one of these clades (Southern) leaves room for inferring the existence of a group that is somehow divergent from the other individuals. The degree of this distinctiveness, however, seems to be quite recent in evolutionary terms, since one of these clades, the Northern group, was not completely monophyletic.

This two-group partition was also observed in the median-joining network, in which there is no haplotype sharing between samples from Northern and the Southern populations, although the Guatemala mtDNA sequences were more closely related to the southern samples. Interestingly, the phylogenetic relationships of the mtDNA haplotypes recovered with two of the methods (MP and ML) suggested paraphyly of the Northern group with respect to the Southern group, which would support a north-south route for the colonization of South America by the ancestors of present-day jaguarundi lineages. This postulated hypothesis is corroborated by comparisons of mtDNA genetic variation between these major groups. The Northern group displayed higher nucleotide diversity, larger number of variable sites and a proportionally greater number of haplotypes than the Southern one (see Table 8), which had a much larger sample size. Thus, given the somewhat unexpected lower variation of the Southern group, one could hypothesize that the Southern group was colonized by the Northern population. On the other hand, the microsatellites did not exhibit this difference, as these two groups had similar diversity estimates with these markers. This inferred scenario contrasts with those reported for other Neotropical cats such as the jaguar (Eizirik et al. 2001), ocelot and margay (Eizirik et al. 1998), and also the puma's extant North American population (Culver et al. 2000). However, this phylogenetic structure had only weak support (< 50% bootstrap) in the MP and ML trees, and was not present in the BI tree, thus warranting caution regarding this interpretation. Additional analyses including more mtDNA segments and more extensive geographic sampling, especially regarding in NCA and northern South America, will be necessary to further address this issue.

The finding that Venezuela's haplotype was identical to Mexico's brings up an interesting pattern that has been postulated for several other species, i.e. that the Amazon river is a potential historical barrier to gene flow. This was reported for two large mammals that are well-known swimmers: the jaguar (Eizirik et al. 2001) and the lowlad tapir, *Tapirus terrestris* (de Thoisy et al. 2011). It also has been suggested to be a barrier in other small Neotropical cats, such as *L. pardalis* and *L. wiedii* (Eizirik et al. 1998), and might as well be the case for jaguarundi, in which this river might have posed an important historical barrier to gene flow. An important caveat to this inference is that we still lack biological samples from a large portion on northern South America, including regions adjacent to the Amazon river. It is thus impossible at this point to specifically test the role of the river itself, relative to other possible barriers across that region. Still, given the observed phylogeographic pattern, we raise this hypothesis for this species as well, and expect that in the future it may be more rigorously tested with additional sampling across that region.

The partition in Northern *vs.* Southern groups was also corroborated by the mtDNA AMOVA analysis, in which the highest Φ_{ST} value (Φ_{ST} = 0.6124) was obtained when these two major phylogeographic populations were tested. The microsatellite results also showed a similar pattern, but with a lower level of differentiation, in which the F_{ST} values were maximized (F_{ST} = 0.0597) when the NCA *vs.* South America samples were compared, thus indicating that the distribution of the genetic variability is best explained by this two-group partition. Although at low level, isolation by distance seems to have played a role in limiting the gene flow between these two broad geographic areas, hence leading to a differentiation between them. Again, this observation should be further investigated once more complete sampling across Amazonia becomes available.

The disparity in the estimated values of population differentiation between these two types of molecular markers is likely due to the properties of each marker, since the mtDNA is inherited uniparentally and thus reflects the matrilineal aspect of the population history, whereas microsatellites are inherited biparentally (Avise 2000; Sunnucks 2000). As a consequence, this marker combination may reveal sex-biased dispersal, with microsatellites exhibiting less genetic differentiation when females are philopatric (Prugnolle and Meeus 2002; Sunnucks 2000). It is also important to note the temporal differences in terms of the evolutionary processes that each marker captures: whereas one mainly assesses deeper population history (mtDNA), the other (microsatellites) detects the contemporary distribution of the genetic variability (Wang 2010), which may also result in different levels of estimated differentiation. Nevertheless, these results corroborate the usual felid feature of females being more philopatric with respect to their birth place, while males are the predominant dispersers (Sunquist and Sunquist 2002), considering that a higher degree of structure was detected with the mtDNA data compared to the microsatellites. This pattern of female philopatry and male dispersal was also reported for other Neotropical carnivores such as the crab-eating fox (Tchaicka et al. 2007), the crab-eating raccoon (Tsuchiya-Jerep 2009), and the jaguar (Eizirik et al. 2001).

No sign of further substructure within each group was observed, nor any clear-cut sign of recent population expansion (Fig. 3 and S1). The only pattern suggested by the mtDNA on a regional scale was a weak but significant differentiation between the South and the Northeastern samples in South America, which seems to be at least in part a result of isolation by distance. The median-joining network also revealed this lack of internal structure by showing a common haplotype shared among very distant regions, especially within South America (see Fig. 2). In general these findings are in agreement with the species' biology.

Jaguarundis are found in a wide variety of habitats, ranging from sea level to elevations at least as high as 3.200m distributed across most of the Neotropical region, and thus inhabiting both open and closed areas. The feeding ecology of the jaguarundi is also quite broad, including mainly small mammals, birds, and reptiles, as well as medium-sized mammals, fish, arthropods, plants and even fruits (Guerrero et al. 2002; Konecny 1989; Oliveira 1998; Tófoli et al. 2009; Sunquist and Sunquist 2002). Even though little is known about the land use patterns of the jaguarundi, Konecny (1989) reported that the home ranges of two male individuals were around 100 Km^2, an unusually large area for a small cat. On the other hand, the female's home range was rather smaller than the former averaging 20 square kilometers and indicating hence that male jaguarundis have in fact a much broader dispersal activity than females. These observations fit our genetic results that suggest male-mediated gene flow as an explanation for the patterns of spatial structuring detected with the mtDNA and microsatellites.

Finally, the two-group partition inferred herein does not support the eight subspecies classically recognized for *P. yagouaroundi* (Oliveira 1998). Most of these subspecies were described during the nineteen century based on very few specimens (e.g. Mearns 1901), hence not taking into consideration the intraspecific variation of the species, which stresses the need for a revision of this classification (Eizirik et al. 2006). Even though our sampling covers a broad range of the jaguarundi's distribution, it does not encompass all geographical areas where some of the subspecies were initially described (e.g. Peru and Panama). Yet, we can state that the genetic data analyzed here do not corroborate most of the subspecies named for jaguarundi, indicating instead the existence of two distinct populations with no perceptible deep genetic structure.

CONCLUSIONS

In the present study, the analyses performed based on two mtDNA segments and eight microsatellite loci support the recognition of two distinct phylogeographic groups: a Northern (from regions located to the north of the Amazon river) and a Southern group (south of this same river). The degree of differentiation between these two populations, however, appears to be recent in evolutionary terms, considering that no complete reciprocal monophyly was obtained for the Northern clade, indicating the occurrence of gene flow between these broad regions until the recent past. We found no strong evidence of further substructure within each group, which is in agreement with the species' biology of being highly flexible and adaptable,

also seeming to have great dispersal ability. The differentiation between these two major phylogeographic groups was influenced at least in part by isolation by distance, as indicated by a correlation between the genetic and geographical distance. These results do not support the recognition of the eight classical jaguarundi subspecies, and prompt for a re-evaluation of this taxonomic scheme for use in conservation assessments. The suggested historical barrier (Amazon river) and the direction of migration of the jaguarundis remain to be further confirmed by a more comprehensive study involving more samples surrounding the Amazon river, as well as more genetic markers. Nevertheless, the genetic patterns presented here contribute to an improved understanding of the evolutionary history of the jaguarundi, and may be incorporated in future management strategies on behalf of this species.

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TABLES AND FIGURES

Table 1: Samples of *Puma yagouaroundi* analyzed in this study.

Individual ID	Geographic Location	Institution/Contact
bPya003[C,N,M] bPya004[C,N,M] bPya005[C,N,M] bPya006[C,N,M]	Cordoba, Argentina	Zoológico de Cordoba
bPya007[N,M]	Barão, Rio Grande do Sul State (RS), Brazil	Museu de Ciências e Tecnologia (MCT) / PUCRS
bPya013[C,N]	Unknown State, Brazil	CASIB / Zoológico de Goiânia
bPya014[C,N,M] bPya015[C,N,M] bPya016[C,N,M]	P.N. do Iguaçu, Paraná State (PR), Brazil	Projeto Carnívoros
bPya017[C,N,M]	Tenente Portela, Rio Grande do Sul State (RS), Brazil	Jan e Cibele
bPya018[N,M] bPya019[C,N,M]	Rio Grande do Sul State (RS), Brazil	Zoológico de Sapucaia do Sul
bPya020[N]	Cachoeira do Sul, Rio Grande do Sul State (RS), Brazil	Edson Salomão
bPya022[C,N,M] bPya023[C,N,M]	São Paulo State (SP), Brazil	Plano de Manejo Pequenos Felinos Brasileiros / Zoológico do Rio
bPya026[C,N,M]	Monte Alto, São Paulo State (SP), Brazil	Plano de Manejo Pequenos Felinos Brasileiros / Zoológico de Mogi Mirim
bPya028[N]	Sorocaba, São Paulo State (SP), Brazil	Plano de Manejo Pequenos Felinos Brasileiros / Zoológico de São José do Rio Pardo
bPya029[C,N,M]	São Paulo State (SP), Brazil	Plano de Manejo Pequenos Felinos Brasileiros / Zoológico de Campinas
bPya031[C,N]	Lajeado, Rio Grande do Sul State (RS), Brazil	Paulo Brack e Keithe Sofia R. Schimidt
bPya032[C,M]	Probably Rio Grande do Sul State (RS), Brazil	Zoológico de Sapucaia do Sul
bPya033[C,N]	Barão, Rio Grande do Sul State (RS), Brazil	Museu de Ciências e Tecnologia / PUCRS
bPya034[N,M]	Mato Grosso do Sul State (MS), Brazil	Zoológico da Cesp
bPya035[C,N,M]	Restinga Seca, Rio Grande do Sul State (RS), Brazil	Everton R. Behr
bPya036[C,N,M]	São Borja, Rio Grande do Sul State (RS), Brazil	Cibele Indrusiak
bPya037[N,M]	Ivoti, Rio Grande do Sul State (RS), Brazil	Cibele Indrusiak / FZB / Zoológico de Sapucaia do Sul
bPya038[C,M]	Unknown State, Brazil	Zoológico de Sapucaia do Sul
bPya039[C,N,M]	Feliz, Rio Grande do Sul State (RS), Brazil	Zoológico de Sapucaia do Sul
bPya042[C,N,M]	Lagoa Vermelha, Rio Grande do Sul State (RS), Brazil	E. Eizirik, C. Indrusiak, M. Rodrigues / Arca de Noé, Criadouro Conservacionista de Morro Reuter

bPya043[C,N,M]	Pinto Bandeira, Rio Grande do Sul State (RS), Brazil	Silvana Frankenberger, Cibele Indrusiak / IBAMA
bPya045[C,N,M] bPya046[C,N,M] bPya047[C,N,M] bPya048[C,N,M] bPya050[C,N,M] bPya051[C,N,M] bPya052[C,N,M] bPya055[M]	Ceará State (CE), Brazil	Tadeu Gomes de Oliveira
bPya056[C,N,M]	Northern Central America	Guatemala Zoo
bPya057[C,N,M]	Guatemala	Auto Safari Chapin / W. Johnson
bPya058[N,M]	Argentina	Buenos Aires Zoo
bPya059[C,N]	Mexico	Leon Zoo
bPya060[C,N,M]	Mexico	LGD / W. Johnson
bPya061[N]	Mexico	Auto Safari Chapin / W. Johnson
bPya063[N]	Venezuela	Leslie Pantin Zoo
bPya064[C,N,M]	Venezuela	Barquisimento Zoo
bPya065[C,N,M]	Anaurilândia, Mato Grosso do Sul State (MS), Brazil	Dênis Sana
bPya066[C,N,M]	Santa Rita do Pardo, Mato Grosso do Sul State (MS), Brazil	Dênis Sana / Zoológico CESP
bPya067[C,N,M]	Espírito Santo State (ES), Brazil	Andreas Kierbusch
bPya069[N,M] bPya070[M]	Buíque, Pernambuco State (PE), Brazil	Tadeu Gomes de Oliveira
bPya071[C,N,M]	Carazinho, Rio Grande do Sul State (RS), Brazil	Tatiane Trigo / Zoológico da Universidade de Passo Fundo (RS)
bPya072[C,N,M]	Taquara, Rio Grande do Sul State (RS), Brazil	Melissa Vilcher / FZB
bPya073[C,N,M]	Cerrito, Rio Grande do Sul State (RS), Brazil	Fábio Mazim / FZB
bPya074[C,N,M]	Rio Grande do Sul State (RS), Brazil	Batalhão Ambiental / FZB
bPya075[C,N,M]	Machadinho, Rio Grande do Sul State (RS), Brazil	FZB
bPya076[C,N,M]	Rio Grande do Sul State (RS), Brazil	Sofia Zank / FZB
bPya077[C,N,M]	Campo Belo do Sul, Santa Catarina State (SC), Brazil	Carlos Benhur Kasper, Marina Piccoli / BAESA
bPya078[C,N,M]	Foz do Iguaçu, Paraná State (PR), Brazil	Fernando Azevedo
bPya080[C,N,M]	Humaitá, Rio Grande do Sul State (RS), Brazil	Tatiane Trigo, Flávia Tirelli, Alexsandra Schneider, Fernanda Pedone / Arca de Noé, Criadouro Conservacionista de Morro Reuter
bPya081[C,N,M] bPya082[C,M]	Seberi, Rio Grande do Sul State (RS), Brazil	Carlos Benhur Kasper
bPya083[C,N,M]	Antonina, Paraná State (PR), Brazil	Fernanda Braga / Museu de História Natural Capão da Imbuia

bPya084[N]	Foz do Iguaçu, Paraná State (PR), Brazil	Fernanda Braga / Museu de História Natural Capão da Imbuia
bPya085[N,M]	Diamante do norte, Paraná State (PR), Brazil	Fernanda Braga / Museu de História Natural Capão da Imbuia
bPya086[N,M]	Paraná State (PR), Brazil	Fernanda Braga / Museu de História Natural Capão da Imbuia
bPya087[C,N,M]	Santa Cruz, Rio Grande do Sul State (RS), Brazil	Igor P. Coelho
bPya088[C,N,M]	Cruzeiro do Sul, Rio Grande do Sul State (RS), Brazil	Carlos Benhur Kasper
bPya089[C,N,M]	Mendoza, Argentina	Gabriela Fernandez, Mariano Merino
bPya090[C,N]	Floriano, Piauí State (PI), Brazil	Carlos Benhur Kasper
bPya091[N,M]	Bom Jesus, Rio Grande do Sul State (RS), Brazil	Carolina, Vinícius / BAESA
bPya092[C,N]	Putinga, Rio Grande do Sul State (RS), Brazil	LBGM/PUCRS
bPya093[C,N]	Bagé, Rio Grande do Sul State (RS), Brazil	Felipe Peters / ULBRA
bPya094[C,N,M]	Entrerios do Sul, Rio Grande do Sul State (RS), Brazil	Fábio Vilela / ULBRA
bPya095[C,N,M]	Pinheiro Machado, Rio Grande do Sul State (RS), Brazil	Fábio Mazim; MCT/ PUCRS
bPya096[C,N,M]	Martinho Campos, Minas Gerais State (MG), Brazil	Fabrício Santos / UFMG
bPya303[C,N,M]	Rebio-Guaribas, Paraíba State (PB), Brazil	Flávio Rodrigues / UFMG, Inst. Pró-Carnívoros
bPya304[C,N,M]	Colatina, Espírito Santo State (ES), Brazil	José de Penha Rodrigues, Sandra Giselda Paccanguella, Vinicius de seixas Queiroz – CENAP / ICMBio
bPya306[C,N,M]	Mineiros, Goiás State (GO), Brazil	Leandro Silveira, Anah Jacomo, Mariana Furtado, Cynthia Kashivakura / Instituto Onça-pintada
bPya307[N]	P.N do Iguaçu, Paraná State (PR), Brazil	Fernando Azevedo / CENAP
bPya327[N]	P.N. Grande Sertão Veredas, Minas Gerais State (MG), Brazil	Edsel A. Moraes Jr. / Inst. Biotrópicos
Hya001[C,N]	Mexico	LGD / W. Johnson
Hya010[N,M]	Guatemala	Costa Rica Zoo / W. Johnson
Hya012[C]	Probably Guatemala	Auto Safari Chapin / W. Johnson
Hya014[C,N,M] Hya015[C,N]	Guatemala	Auto Safari Chapin / W. Johnson
Hya018[N]	Vera Cruz Province, Mexico	LGD / W. Johnson
Hya020[C,N,M] Hya025[N,M] Hya027[C,N,M]	Mexico	LGD / W. Johnson
Hya024[C,N,M]	Tamaulipas Province, Mexico	LGD / W. Johnson
Hya026[C,N,M]	Mexico	Aragon Zoo / W. Johnson
Hya028[C,N,M] Hya029[C,N]	Tabasco Province, Mexico	LGD / W. Johnson

Hya032[C,N]	Bahia State, Brazil	LGD / W. Johnson
Hya036[C,N]	Paraná State, Brazil	Matelândia Zoo / W. Johnson
Hya040[N,M] Hya041[C,N] Hya042[C,N]	Cordoba, Argentina	LGD / W. Johnson
Hya046[C,N,M]	Zulia state, Venezuela	LGD / W. Johnson
Hya047[N]	Lara state, Venezuela	LGD / W. Johnson
Hya050[C,N]	Bolivia	LGD / W. Johnson
Hya058[N]	Paraná State, Brazil	LGD / W. Johnson
Hya059[N]	French Guiana	Jean-Christophe vie / LGD / W. Johnson

[C] = samples typed for the *cytochrome b* gene;
[N] = samples typed for the *ND5* gene;
[M] = samples typed for the microsatellites;

Table 2: Summary statistics of nucleotide and haplotype diversity estimated for the mitochondrial segments of *P. yagouaroundi* analyzed in this study.

Segments	N	Length (bp)	V	Pi	H	Hd (S.D.)	π (S.E.)
ND5	95	558	25	18	16	0.8151 (0.0288)	0.0050 (0.0012)
CYTB	76	633	22	17	17	0.8849 (0.0201)	0.0053 (0.0013)
ND5 + CYTB	72	1191	42	31	21	0.8720 (0.0254)	0.0050 (0.0009)

N = number of sequences used; V = number of variable sites; Pi = number of parsimony-informative sites;
H = number of haplotypes; Hd = haplotype diversity and its standard deviation (S.D.);
π = nucleotide diversity and its standard error (S.E.).

Table 3: List of the haplotypes found in *P. yagouaroundi* and the individuals belonging to each haplotype, as well as the geographic distribution and frequency (Freq.) of these mtDNA haplotypes.

Haplotype ID	Individuals	Freq.	Geographic Distribution
Py-H1	bPya014, 017, 031, 035, 043, 072, 076, 081, 088, 092	10	Paraná state, Rio Grande do Sul state, Brazil;
Py-H2	bPya016, 013, 026, 029, 045, 046, 047, 048, 051, 052, 090, 096	12	Paraná state, probably from Goiás state, São Paulo state, Ceará state, Piauí state, Minas Gerais state, Brazil;
Py-H3	bPya036, 003, 004, 005*, 006, 019, 023, 033, 039, 065, 066, 067, 074, 075, 080, 089, 093, 304; and Hya036, 041, 042	21	Argentina; Rio Grande do Sul state, São Paulo state, Mato Grosso do Sul state, Espírito Santo state, Brazil;
Py-H4	bPya042, 078, 083, 094	4	Rio Grande do Sul state, Paraná state, Brazil;
Py-H5	bPya056	1	North Central America;
Py-H6	bPya057; and Hya014, 015	3	Guatemala;
Py-H7	bPya059	1	Mexico;
Py-H8	bPya071	1	Rio Grande do Sul state, Brazil;
Py-H9	bPya073, 095	2	Rio Grande do Sul state, Brazil;
Py-H10	bPya077	1	Santa Catarina state, Brazil;
Py-H11	bPya087	1	Rio Grande do Sul state, Brazil;
Py-H12	bPya303	1	Paraíba state, Brazil;
Py-H13	bPya306, 050	2	Goiás state, Brazil;
Py-H14	Hya001	1	Mexico;
Py-H15	Hya020	1	Mexico;
Py-H16	Hya024	1	Mexico;
Py-H17	Hya026, 046; and bPya064	3	Mexico and Venezuela
Py-H18	Hya027	1	Mexico;
Py-H19	Hya028, 029; and bPya060	3	Mexico;
Py-H20	Hya032	1	Bahia state, Brazil;
Py-H21	Hya050	1	Bolivia;

* This sample was also identical to the Py-H10 haplotype, because it had an ambiguity in the only site that distinguished these two haplotypes and thus, was excluded from the analyses performed in Arlequin software, since we could not correctly assign it to either haplotype.

Table 4: Pairwise and overall F_{ST} values estimated for the populations of Jaguarundi with the mitochondrial concatenated data set. Two measures of distance was used, the Kimura2 + G (lower diagonal), and the proportional number of differences (upper diagonal).

Groups		Northern group (+ Venezuela)	Southern group populations[a]		
			NE	CW	S, SE
Northern group (+ Venezuela)		---	0.5913**	0.5312**	0.6052**
Southern group populations[a]	NE	0.5973**	---	-0.0118	0.2069*
	CW	0.5350**	-0.0115	---	0.0298
	S, SE	0.6120**	0.2071**	0.0295	---
Overall F_{ST} among groups		0.4855**			

* Significant value of F_{ST} ($P < 0.01$); ** Significant value of F_{ST} ($P < 0.001$);

[a] Biomes regarding each South Amazon populations: NE = North of Mata Atlântica + Caatinga; CW = Cerrado; S, SE = South of Mata Atlântica + Pampa;

Table 5: Characteristics of the microsatellite loci analyzed in this study, including PCR amplification conditions and allelic size range observed in the jaguarundi.

Locus	Dye	Multiplex set	Annealing Temperature	Repeat type (bp)[a]	Size range
F124	FAM	A	53 ºC	Tetra[b]	218 – 264
F42	NED	A	60 ºC	Tetra	254 – 318
F98	HEX	A	58 ºC	Tri	189 – 204
FCA441	FAM	B	53ºC	Tetra	142 – 166
F146	NED	B	60 ºC	Tri	159 – 180
FCA391	FAM	C	60 ºC	Tetra[c]	216 – 264
FCA453	NED	C	60 ºC	Tetra	204 – 240
FCA424	HEX	C	58 ºC	Tetra	172 – 196

[a] = repeat type found in *Felis catus* and also in the present study with Jaguarundi, exceptions in two locus (see [b] and [c]);

[b] = although most alleles were varying four nucleotides apart, two of these alleles found varied only three sites apart from the other alleles within this locus;

[c] = another case where three alleles varied only two bases apart from others alleles, but differed four nucleotides apart from each other, behaving like a tetra within them.

Table 6: Genetic diversity indices obtained for the eight microsatellite loci analyzed in *P. yagouaroundi*.

Locus	N	A	PIC	H_o	H_e
F124	75	13	0.883	0.613	0.898
F42	74	17	0.870	0.500	0.887
F98	75	6	0.730	0.680	0.764
FCA441	73	7	0.722	0.534	0.761
F146	72	8	0.753	0.750	0.789
FCA391	75	14	0.877	0.840	0.894
FCA453	73	9	0.804	0.753	0.832
FCA424	73	5	0.091	0.096	0.093
Overall mean	---	9.88	0.716	0.596	0.739

N = number of individuals used; A = number of alleles per locus; PIC = Polymorphic Information Content; H_o = Observed heterozygosity; H_e = Expected heterozygosity.

Table 7: Pairwise and overall F_{ST} values estimated for the populations of Jaguarundi with the eight microsatellite loci. The F_{ST} estimates were obtained based only on the number of different alleles and its frequencies.

Groups		Northern group (+ Venezuela)	Southern group[a]	
			NE	S, SE
Southern group populations[a]	NE	0.0404*	---	---
	S, SE	0.0679**	0.0150	---
Overall F_{ST} among groups			0.0374**	

* Significant value of F_{ST} ($P < 0.01$); ** Significant value of F_{ST} ($P < 0.001$);

[a] Biomes regarding each South Amazon populations: NE = Northern Atlantic Forest + Caatinga; S, SE = Southern Atlantic Forest + Pampa.

Table 8: Comparative genetic diversity indices for both mtDNA segments (concatenated data set of 1091bp) and eight microsatellite loci estimated separately for the Northern and Southern group, as well as for the entire population.

	mtDNA				Microsatellites				
	Nº of indiv. mtDNA/ µsat	Nº of variable sites	Nº of haplotypes	Nucleotide diversity (π) (S.E)	H_o (S.E)	H_e (S.E)	Mean nº of alleles per loci	Allelic Richness*	Nº of private alleles*
Total	72/76	42	21	0.0050 (0.0009)	0.596	0.739	9.88	9.85	9.85
Northern group (+ Venezuela)	15/13	23	9	0.00543 (0.0012)	0.60797 (0.184)	0.71333 (0.202)	6.6	6.22	2.11
Southern group	57/63	21	12	0.00269 (0.0007)	0.59427 (0.260)	0.73079 (0.275)	8.5	6.25	2.14

Nº of indiv. = Number of individuals;
S.E = Standard error.
* Values calculated using the rarefaction method, with a minimum sample size of 8 diploid (heterozygous) individuals.

Fig 1: Map showing the current geographic distribution (dark grey) of *P. yagouaroundi* (modified from IUCN, 2011 < http://www.iucnredlist.org/apps/redlist/details/9948/0>), approximate sample collection sites (circles), and number of samples obtained from each site (larger circles; [small circles indicate a single sample]). Black circles indicate all the samples analyzed for the concatenated mtDNA data set and most of the samples analyzed for the microsatellites, whereas white circles indicate additional samples typed only for the latter marker type (see Table 1 for further information on samples typed for the microsatellites). Larger black circles with a white underline indicate the presence of one sample that was typed only for the microsatellites. Dotted ellipses indicate the phylogroups (Northern and Southern) identified in this study.

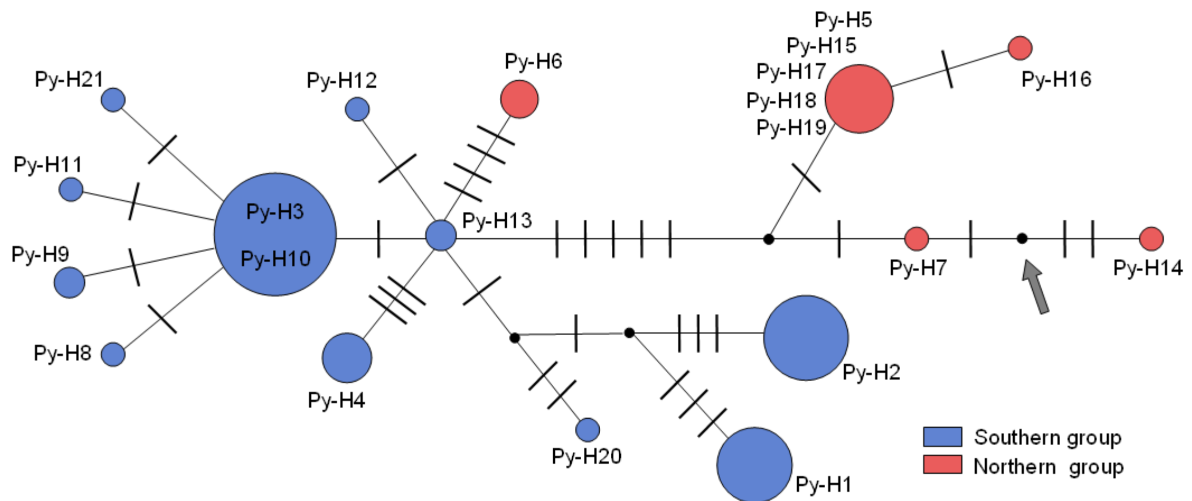

Fig 2: Haplotype network generated with the median-joining algorithm, showing the relationship among the mtDNA sequences found for *P. yagouaroundi*. Haplotype names are indicated next to each circle (see Table 3 for details). The size of each circle is proportional to the haplotype frequency in the analyzed samples. Bars placed on connecting lines represent mutational steps. Circles containing more than one associated name indicate that sequences were collapsed into a single haplotype due to the elimination of sites containing missing data (complete deletion option) for this analysis. Small black circles indicate median vectors, i.e., hypothesized (possibly unsampled) sequences required to connect observed haplotypes. The gray arrow indicates where the outgroup *P. concolor* is connected.

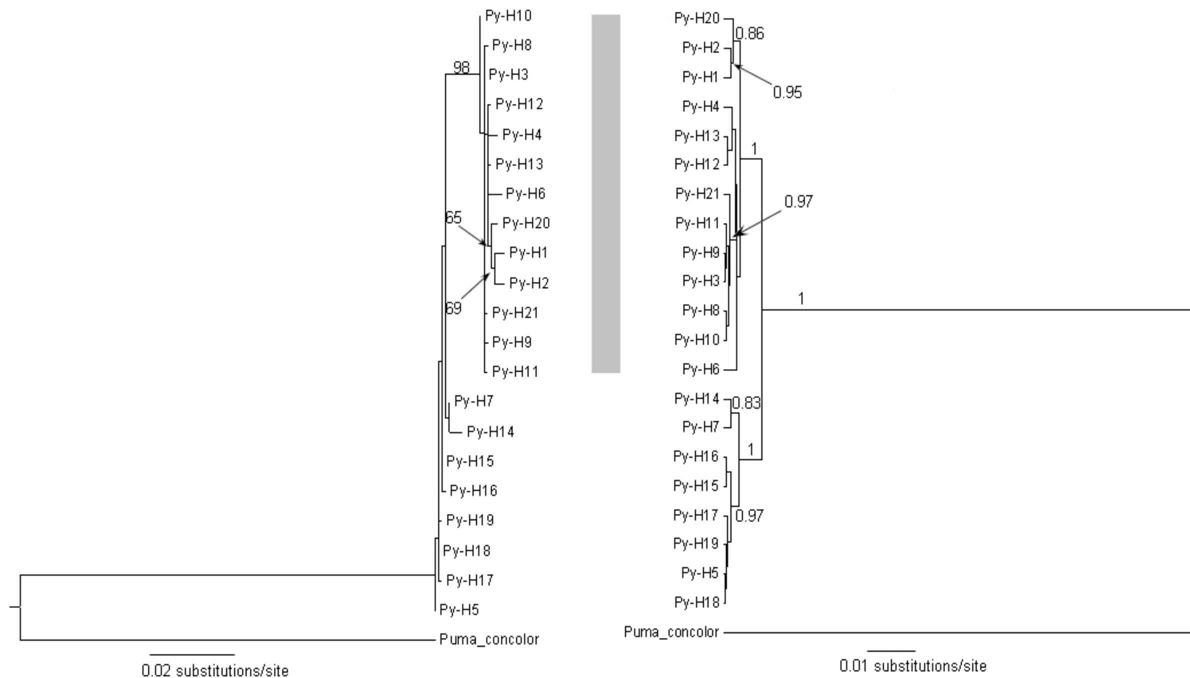

Fig 3: Maximum Likelihood (Log-Likelihood = -2408.42010; left tree) and Bayesian inference (Posterior Median = -2370.6887; right tree) trees found using the concatenated mtDNA data set of Jaguarundi. Labels on the tips of the branches represent the 21 haplotypes that were included in the analyses (see Table 3 for further information). Both phylogenetic inference methods assumed the HKY + G (gamma shape = 0.1980) model of sequence evolution (see text for details). The gray bar indicates a well supported clade containing most of the South American individuals. Values above or below nodes (or connected by an arrow) refer to the bootstrap support observed with the Maximum Likelihood approach, or the clade posterior probability derived from the Bayesian Inference. Only values higher than 50% are shown. Scales bars at the bottom indicate the proportion of substitutions per site along the branches.

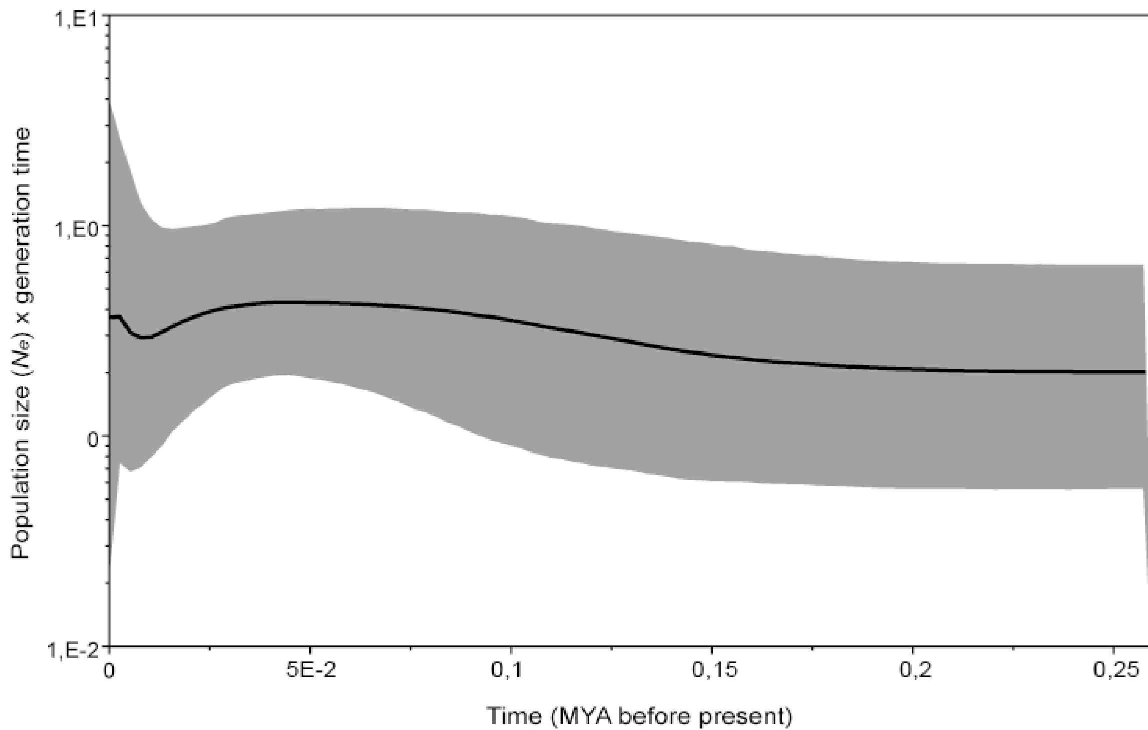

Fig 4: Bayesian skyline plot showing population size (y-axis) variation over the past 250,000 years (*x*-axis in million years ago [MYA]). The thick black line indicates the median estimate of the population size ($N_e.\tau$) and the gray shaded area represents the 95% credibility (Highest Posterior Density) interval. The substitution rate was estimated from the concatenated mtDNA data set, and applied on a scale of MYA. Therefore, assuming a generation time of five years for the jaguarundi, the y-axis values should be multiplied by 200.000 in order to obtain interpretable values of *Ne*.

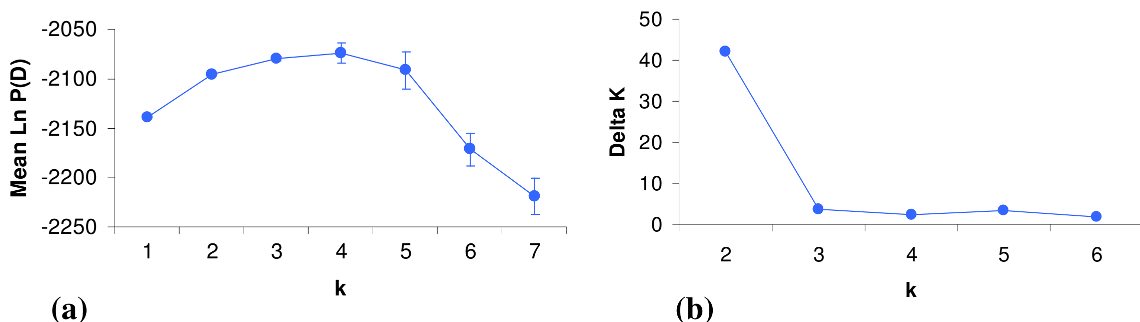

Fig 5: Number of genetically distinct population clusters in *P. yagouaroundi* estimated from the microsatellite data set. **(a)** Mean values of the estimated posterior probability (Ln P[D]) and its standard deviation found in 5 independent MCMC runs with *K* varying from 1 to 7. **(b)** ΔK plot indicating the most likely number of clusters (highest value of ΔK).

Supplementary Material

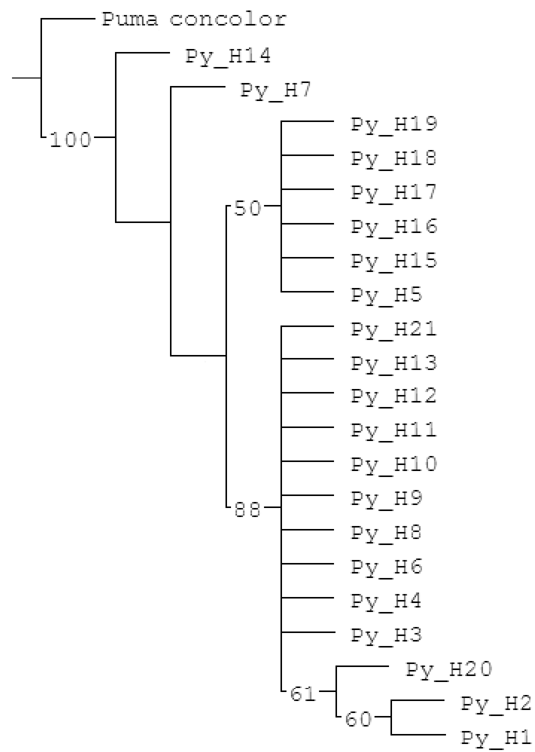

Fig S1: Maximum parsimony strict consensus tree obtained from the twelve most parsimonious trees found for *P. yagouaroundi*, based on the concatenated mtDNA data set. The values placed on the branches represent percent support estimated from 1,000 bootstrap pseudoreplicates. Only values equal to or higher than 50% are shown.

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