UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

HIBRIDAÇÃO E INTROGRESSÃO ENTRE ESPÉCIES DE FELÍDEOS NEOTROPICAIS (MAMMALIA, CARNIVORA)

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"Olhe no fundo dos olhos de um animal e, por um momento, troque de lugar com ele. A vida dele se tornará tão preciosa quanto a sua e você se tornará tão vulnerável quanto ele. Agora sorria, se você acredita que todos os animais merecem nosso respeito e nossa proteção, pois em determinado ponto eles são nós e nós somos eles."

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Apresentação da Tese

O interesse pelo estudo da hibridação entre espécies selvagens, particularmente entre espécies de pequenos felídeos, surgiu em nosso grupo de pesquisa há muito tempo, ainda na década de 90. Nesta época, o Dr. Eduardo Eizirik, iniciou a coleta de amostras de felídeos, principalmente no estado do Rio Grande do Sul, com o objetivo de formar um banco de amostras para análises genéticas. A partir destas coletas, começou a ser definido um padrão de segregação espacial bem demarcado entre *Leopardus tigrinus* e *Leopardus geoffroyi* no estado, com a primeira espécie restrita basicamente ao norte do estado e a segunda ao sul. Juntamente a este padrão de distribuição, alguns indivíduos coletados apresentaram-se de difícil identificação devido à presença de características aparentemente intermediárias entre as duas espécies. Apesar da suspeita de que a hibridação entre as duas espécies pudesse estar ocorrendo, o projeto não teve andamento naquele momento.

Em 2001 iniciei meu Mestrado pela UFRGS, sob orientação do Dr. Thales de Freitas e co-orientação do Dr. Eduardo Eizirik, sobre estrutura de populações e níveis de variabilidade genética em populações de *L. tigrinus* no Brasil. Além das amostras de *L. tigrinus,* algumas amostras de *L. geoffroyi* foram incluídas no estudo para comparar níveis de variabilidade genética interespecíficos. Os primeiros resultados genéticos, sugestivos de hibridação, começaram então a aparecer, nos levando a ampliar as investigações sobre o assunto. Primeiramente, publicamos na revista Cat News, o trabalho de distribuição das duas espécies no estado, com a evidência de padrões de pelagem atípicos em alguns indivíduos e, posteriormente, iniciamos análises genéticas mais específicas buscando a confirmação dos eventos de hibridação. A partir destas análises conseguimos confirmar a ocorrência destes eventos, além de corroborar a hibridação de *L. tigrinus* com *L. colocolo*, previamente sugerida por um trabalho anterior, de 1999, realizado por outros pesquisadores com a participação do Dr. Eduardo Eizirik. O trabalho gerado foi recentemente aceito para publicação na revista Molecular Ecology e incluído como anexo no final desta tese. A partir dos primeiros resultados obtidos neste trabalho, foram desenhados os objetivos desta tese, e por isso sugerimos a leitura inicial deste trabalho que antecede os resultados aqui apresentados.

Esta tese foi elaborada com o principal objetivo de ampliar os conhecimentos existentes sobre os eventos de hibridação e introgressão existentes entre *Leopardus tigrinus*, *L. geoffroyi* e *L. colocolo,* incluindo a quantificação e caracterização destes processos. Além disso, procuramos conduzir avaliações morfológicas e ecológicas de *L.*

geoffroyi e *L. tigrinus* no estado do Rio Grande do Sul, onde a hibridação entre estas espécies foi detectada, de maneira a ampliar informações sobre estas em suas zonas de contato. Para tal, o presente estudo foi organizado em cinco capítulos, o primeiro apresentando uma introdução geral ao assunto e o último uma discussão geral sobre os resultados obtidos. Os Capítulos II, III e IV são referentes aos três artigos científicos (a serem submetidos) produzidos como resultado desta tese.

O Capítulo II apresenta o manuscrito em preparação que caracteriza os diferentes marcadores moleculares utilizados e suas respectivas variabilidades em cada uma das três espécies de felídeos envolvidas. Os padrões de variação genética encontrados foram avaliados com o objetivo de inferir o caráter da hibridação entre *L. tigrinus vs. L. colo*colo e *L. tigrinus vs. L. geoffroyi* e suas possíveis causas e conseqüências demográficas e evolutivas.

No Capítulo III, os marcadores moleculares descritos no Capítulo II foram utilizados para análises mais específicas e detalhadas do processo de hibridação entre *L. tigrinus* e *L. geoffroyi* no estado do Rio Grande do Sul. Neste capítulo foi definida, em maior detalhe, a extensão do processo de hibridação entre estas espécies em uma escala local. Análises morfológicas de indivíduos das duas espécies são apresentadas, com o objetivo de testar o nível de diferenciação morfológica entre estas, além de avaliar a correlação entre as identificações genéticas e morfológicas com base nas sugestões prévias da existência de morfologias ambíguas ou intermediárias no estado.

E por fim, no Capítulo IV é apresentada uma análise de segregação ecológica entre *L. tigrinus* e *L. geoffroyi* no estado do Rio Grande do Sul, onde a zona híbrida foi detectada. Padrões de associação diferencial de habitat, sobreposição de nicho alimentar entre as duas espécies e distribuição espacial dos híbridos identificados geneticamente são apresentados.

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Resumo

A hibridação entre espécies ou populações pode propiciar uma excelente oportunidade de estudo das relações genéticas, ecológicas e demográficas entre táxons relacionados, e dos processos evolutivos envolvidos na manutenção da distinção entre estes. Neste estudo, procuramos avaliar diferentes aspectos biológicos envolvidos nos processos de hibridação entre três espécies de felídeos Neotropicais: *Leopardus tigrinus*, *L. geoffroyi* e *L. colocolo*. A investigação genética, realizada por meio da análise combinada de diferentes marcadores moleculares como DNA mitocondrial, locos de microssatélite e segmentos dos cromossomos Y e X nas três espécies, revelou dois padrões diferenciados de hibridação para *L. tigrinus vs. L. colocolo* no centro e nordeste brasileiro e para *L. tigrinus vs. L. geoffroyi* no sul do Brasil. A hibridação entre *L. tigrinus* e *L. colocolo* foi inferida como um processo antigo, estando atualmente ausente ou restrita à ocorrência de eventos esporádicos na região central brasileira. Por outro lado, a hibridação entre *L. tigrinus* e *L. geoffroyi* no sul do país aparece praticamente restrita ao estado do Rio Grande do Sul como uma das mais intensas zonas atuais de hibridação já documentada em carnívoros, com cerca de 60% da população local considerada como de origem híbrida. Uma ampla variedade de tipos recombinantes foi identificada para este último par de espécies, indicando à produção de híbridos férteis aparentemente capazes de cruzar entre si e com ambas as espécies parentais, levando, assim, à homogeneização genética das populações locais das duas espécies. Esta zona híbrida pareceu ainda apresentar uma assimetria na direção de *L. geoffroyi*, podendo indicar a existência de pressões seletivas que favoreçam o retrocruzamento com esta espécie. Análises morfológicas e ecológicas foram também realizadas em *L. tigrinus* e *L. geoffroyi* no estado do Rio Grande do Sul com o objetivo de caracterizar estas espécies em suas áreas de contato. Apesar da intensidade da hibridação, as duas espécies aparentemente mantêm uma diferenciação morfológica no que diz respeito ao tamanho corporal. No entanto, variações nos padrões de pelagem parecem estar associadas, em parte, a origens híbridas. Da mesma maneira, análises de distribuição, associação com habitats e nicho trófico no estado sugerem a existência de uma segregação ecológica entre estes dois felídeos nesta região.

Abstract

Hybridization between species and populations may provide an excellent opportunity to study genetic, demographic and ecological relationships between closely related taxa, and the evolutionary processes involved in the maintenance of the species distinctness. At the present study, we aim to evaluate the different biological aspects involved on the hybridization processes between three Neotropical felids: *Leopardus tigrinus*, *L. geoffroyi* and *L. colocolo*. Genetic surveys, performed with a combined analysis of different molecular markers such as mitochondrial DNA, microsatellite loci and Y and X chromosomes segments on the three felid species, revealed two different patterns of hybridization between *L. tigrinus* and *L. colocolo* at central and northeastern Brazil and between *L. tigrinus* and *L. geoffroyi* at southern Brazil. The hybridization between *L. tigrinus* and *L. colocolo* was inferred as an ancient event, being, currently, absent or restricted to esporadic events in the central Brazilian region. On the other hand, hybridization between *L. tigrinus* and *L. geoffroyi* in southern Brazil seemed to be nearly restricted to Rio Grande do Sul state as one of the most intensive hybridization events documented in carnivores, in which approximately 60% of the local population carry a hybrid origin. A wide variety of recombinant types was identified for this pair of species indicating the production of fertile hybrids apparently able to cross with each other and also with both parental species, leading to the genetic homogenization of the local populations of the two species. This hybrid zone seemed to be also assimetric in *L. geoffroyi* direction, suggesting the existence of selective pressure that favours backcrossings with this species. Morphological and ecological analyzes was also performed at *L. tigrinus* and *L. geoffroyi* from Rio Grande do Sul state aiming to characterize both species in their contact zones. Despite the extensive hybridization, both species apparently keep the morphological differentiation related to body size. However, variations on the patterns of pelage seem to be associated in part to a hybrid origin. The same way, analyses of geographic distributions, habitat association and trophic niche in the state suggest the existence of an ecological segregation between these two felids in this region.

CAPÍTULO I

INTRODUÇÃO GERAL

Hibridação, introgressão e zonas híbridas entre espécies

A hibridação entre espécies tem sido vista, há várias décadas, pela maioria dos botânicos, como um evento extremamente comum em plantas e como uma importante fonte de novas variações e novas espécies. No entanto, para os zoólogos, apenas recentemente, com o avanço das técnicas moleculares, a hibridação passou a ser vista como uma importante fonte de novas variações, novas adaptações e até mesmo de novas espécies (Harrison 1993, Arnold 1992, Barton & Hewitt 1985, Dowling & Secor 1997, Allendorf *et al*. 2001). Anteriormente, os zoólogos acreditavam que a hibridação entre espécies animais era um evento extremamente raro e geralmente resultante na produção de híbridos estéreis, sem maiores conseqüências sobre as espécies parentais. O avanço das técnicas moleculares, por outro lado, permitiu a verificação da ampla ocorrência destes eventos em grupos animais, freqüentemente resultando na produção de híbridos férteis capazes de cruzar entre si e com uma ou ambas as espécies parentais (Harrison 1993).

A hibridação interespecífica pode caracterizar-se pela ocorrência de eventos esporádicos entre espécies simpátricas (comum em plantas), pela formação de zonas híbridas estreitas entre táxons com efetiva distribuição parapátrica, ou até mesmo por um intenso processo de miscigenação entre as populações parentais (Harrison 1993). Segundo Barton & Hewitt (1985) e Harrison (1993) os principais aspectos de investigação em zonas híbridas envolvem a definição dos processos demográficos, comportamentais e evolutivos envolvidos na origem, manutenção e destino destas. A principal questão envolvendo a origem das zonas híbridas envolve a definição dos eventos históricos e demográficos e das forças evolutivas que propiciaram a origem destes processos. No geral, dois cenários são propostos: o primeiro indica a geração de uma zona híbrida como resultado de contato secundário entre populações que se diferenciaram em alopatria, enquanto que o segundo sugere o surgimento das zonas *in situ* em resposta direta a pressões de seleção variantes espacialmente (gradientes ambientais). Freqüentemente, sugere-se que as zonas híbridas são conseqüências diretas de distúrbios ou mudanças ambientais. Assim, a investigação de aspectos ecológicos, como seleção ambiental, e processos históricos, como dispersão e expansões populacionais, assim como de mudanças ambientais propiciadas por ações

humanas, são extremamente importantes para a definição da origem de uma zona híbrida (Harrison 1993, Hewitt 2001). A avaliação destes eventos pode trazer importantes informações que auxiliarão na definição de uma origem natural ou antropogência para uma zona de hibridação, o que se apresenta de fundamental importância para a conservação das espécies (Allendorf *et al*. 2001). A hibridação ocorrendo naturalmente não constitui uma ameaça à conservação das espécies envolvidas, sendo, neste caso, considerada como parte de suas histórias evolutivas (Arnold 1992). No entanto, esta se torna um problema para a conservação se for propiciada por mudanças no habitat ou na composição das espécies provocadas por ações humanas, requerendo nestes casos medidas urgentes de manejo para impedir um comprometimento das histórias evolutivas e integridades genéticas das espécies envolvidas (Huxel 1999, Allendorf *et al*. 2001, Rhymer & Simberloff 1996).

A análise de padrões de hibridação entre espécies envolve um amplo espectro de investigações. No entanto, o primeiro e crucial passo na investigação de hibridação e introgressão entre organismos reside na adequada identificação dos híbridos para futuros aprofundamentos nos padrões evolutivos envolvidos neste processo. Até a década de 1960, a detecção de indivíduos híbridos, detinha-se no exame de características morfológicas, onde se assumia que os indivíduos híbridos seriam fenotipicamente intermediários às espécies parentais. No entanto, freqüentemente, esta morfologia intermediária não é detectada, Dentre outros fatores, isto pode ocorrer porque indivíduos híbridos que contenham grande parte de seus genes de uma das espécies parentais serão, com freqüência, morfologicamente indistinguíveis desta. Deste modo, a inclusão da análise de marcadores moleculares em estudos de hibridação tem simplificado e aprimorado a identificação e descrição de populações hibridizantes. Os recentes avanços nas técnicas moleculares, especialmente o desenvolvimento da PCR "*Polymerase Chain Reaction*", têm aumentado o número de locos que podem ser usados para a detecção de eventos de hibridação e identificação precisa de indivíduos híbridos e puros, incluindo a identificação de híbridos morfologicamente crípticos (Allendorf *et al*. 2001).

A partir da identificação precisa dos híbridos, os marcadores moleculares nos permitem um amplo expectro de investigações sobre os padrões de hibridação e introgressão existentes em cada caso específico, e suas possíveis conseqüências na estrutura genética e evolução das espécies envolvidas. Um dos principais aspectos que deve ser investigado em uma zona de hibridação involve a avaliação da viabilidade dos híbridos e conseqüente existência de introgressão genética em uma ou ambas as espécies parentais. Alguns marcadores moleculares, por exemplo, nos permitem definir as

diferentes categorias híbridas existentes em uma zona de hibridação (F1, F2 e retrocruzamentos), gerando assim informações importantes sobre a viabilidade dos híbridos e as conseqüências genéticas, demográficas e evolutivas dos cruzamentos interespecíficos sobre as espécies envolvidas. Em zonas híbridas onde somente indivíduos híbridos do tipo F1 são encontrados (ou seja, os híbridos são estéreis), as conseqüências do cruzamento interespecífico podem envolver somente a perda de esforços reprodutivos para as populações parentais sem nenhuma modificação genética. Por outro lado, a produção de híbridos F1 férteis com possibilidade de cruzamento entre si (F2) e de retrocruzamento com uma ou ambas as espécies parentais pode levar a produção de "Hybrid swarms", onde a população será constituída basicamente por uma ampla variedade de tipos recombinante (Harrison 1993, Jiggins & Mallet 2000, Allendorf *et al.* 2001). Este segundo caso de zona híbrida permite a introgressão de componentes genéticos de uma população parental para a outra podendo apresentar diferentes conseqüências. A produção de híbridos férteis com diversas possibilidades de cruzamento e adaptados às condições locais pode levar, após algumas gerações, a uma população na qual essencialmente todos os indivíduos sejam de origem híbrida. Esta população híbrida pode evoluir até o fusionamento total das duas espécies parentais, formando uma única espécie, assim como, à eventual extinção de apenas um dos táxons parentais dependendo da direção e intensidade da introgressão, ou até mesmo a uma extrema diferenciação da população híbrida com a evolução de barreiras ao cruzamento e conseqüente formação de uma espécie distinta (Arnold 1992, Arnold 1993, Harrison 1993, Allendorf *et al.* 2001). Por outro lado, uma zona híbrida pode apresentar-se estável, sendo mantida por milhares de anos em uma determinada área geográfica, sob a influência de algum processo seletivo, sem afetar profundamente as espécies parentais. Estas zonas geralmente representam situações de equilíbrio mantido pelo balanço entre seleção e dispersão (Barton & Hewitt 1985). Este balanço pode envolver seleção contra híbridos F1, F2 ou retrocruzamentos independente do ambiente, como uma redução da fertilidade ou viabilidade das formas híbridas (seleção endógena); ou uma seleção habitat-dependente (seleção exógena) onde diferentes combinações genéticas podem ser favorecidas em diferentes ambientes (Arnold 1992, Barton 2001). A seleção exógena é vista por alguns autores como a principal mantenedora de zonas híbridas estáveis e ocorre, geralmente, em limites de diferentes habitats ou gradientes ambientais onde os híbridos tornam-se melhor adaptados a ambientes heterogêneos dentro deste gradiente do que as espécies parentais (Arnold 1992, Barton 2001, Hewitt 2001).

Apesar dos marcadores moleculares constituírem ferramentas extremamente úteis na identificação de híbridos, e na caracterização dos padrões de hibridação e introgressão existente em cada caso específico, diversos estudos adicionais devem ser associados à análise genética, como uma avaliação morfológica, com descrição morfométrica das espécies parentais e de seus possíveis híbridos, e ecológica com a definição da ocorrência geográfica exata de cada uma das espécies, da amplitude das áreas de sobreposição entre elas, dos padrões de uso de habitat, dieta, comportamento e aspectos competitivos nas áreas de contato assim como fora destas. Este tipo de análise multidisciplinar, com certeza, irá propiciar uma melhor compreensão dos padrões e processos evolutivos presentes em cada zona de hibridação detectada na natureza (Wayne 1996, Allendorf *et al*. 2001, Daniels *et al*. 2001, Wayne & Brown 2001).

Marcadores Moleculares em estudos de hibridação

Atualmente, os marcadores moleculares mais utilizados para a análise e investigação genética de relações evolutivas entre espécies, como eventos de hibridação, incluem locos nucleares altamente polimórficos como os de microssatélite (Beaumont *et al*. 2001, Randi & Lucchini 2002), bem como seqüências de DNAmt (Lehman *et al*. 1991, Gottelli *et al*. 1994, Johnson *et al*. 1999), introns de genes ligados ao cromossomo Y (Johnson *et al*. 1999, Vilà *et al*. 2003) e ao cromossomo X (Roca *et al.* 2004), além de introns autossômicos (Pacheco *et al*. 2002, Macholán *et al*. 2006, Vallender *et al.* 2006).

A vantagem dos locos de microssatélite neste tipo de estudo reside no fato de que estes marcadores genéticos biparentais apresentam-se altamente polimórficos (Schlotterer 1998), sendo, usualmente, variáveis o suficiente para permitir a identificação inequívoca de todos os espécimes amostrados e a realização de inúmeras análises estatísticas de associação dos indivíduos às suas populações de origem (Hansen *et al*. 2000). A utilização de seqüências de DNAmt, e introns do cromossomo Y, X e locos autossômicos também apresenta vantagens neste tipo de estudo devido às diferentes propriedades evolutivas destes marcadores. Por exemplo, segmentos do DNAmt e cromossomo Y apresentam herança matrilinear e patrilinear, respectivamente, e ambos possuem um tamanho efetivo populacional quatro vezes menor do que segmentos equivalentes em autossomos (Hare 2001). Desta maneira, apresentam sensibilidades diferentes a processos demográficos históricos (p.ex. simetria de fluxo gênico ou hibridação entre machos e fêmeas), e a comparação de seus padrões evolutivos permite uma reconstrução mais detalhada da

história dos organismos em questão. Os introns de genes autossômicos e de cromossomo X têm sido, ainda, pouco utilizados em estudos de hibridação, mas apresentam-se como importantes candidatos para futuros estudos, pois, geralmente, apresentam taxas evolutivas (substituição nucleotídica) mais lentas do que locos hipervariáveis como os microssatélites, permitindo, assim, com maior facilidade, a identificação de monofilias recíprocas entre espécies (Slatkin 1995, Murray 1996, Culver *et al*. 2001, Hare 2001, Pacheco *et al.* 2002).

Em geral, a combinação destes diferentes marcadores com suas diferentes características constitui o padrão ideal para o estudo de aspectos complexos das relações evolutivas entre espécies, como eventos de hibridação.

Casos de Hibridação em Carnívoros Selvagens

Diversos casos de hibridação entre espécies de carnívoros têm sido documentados, variando desde a identificação de eventos esporádicos até a extensa homogeneização entre populações, em alguns casos, com extrema relevância para a conservação, por ocorrerem entre espécies selvagens e introduzidas.

Dentro da família Canidae alguns casos são documentados entre espécies selvagens, como entre coiotes (*Canis latrans*) e lobos cinza (*Canis lupus*) (Lehman *et al*. 1991, Roy *et al*. 1994) e coiotes e lobos vermelhos (*Canis rufus*); além de casos entre espécies selvagens e domésticas como entre lobos cinza, coiotes e chacais (*Canis simensis*) com cães domésticos (*Canis familiares*) (Randi & Lucchini 2002, Adams *et al*. 2003, Verardi *et al*. 2006). A origem destes eventos de hibridação tem sido associada, principalmente, a diferenças nas densidades relativas entre as espécies envolvidas, que, em muitos casos, são favorecidas ou provocadas pela ação do homem (Vilá & Wayne 1999). No caso da hibridação com cães domésticos, por exemplo, o atual decréscimo dos tamanhos populacionais das espécies selvagens devido à fragmentação de habitats e programas de controle a predadores, tem gerado pequenas populações em próximo contato com humanos e cães domésticos e, assim, favorecido a hibridação, que, nestes casos, pode levar a um comprometimento da integridade genética das espécies selvagens ameaçando a sua sobrevivência (Wayne & Brown 2001).

Casos de hibridação entre mustelídeos selvagens e introduzidos também têm sido documentados, como entre a marta Européia (*Martes martes*) e a espécie introduzida da América do Norte (*M. americana*) (Kyle *et al*. 2003), e os conhecidos “ferrets” (*Mustela furo*), utilizados como animais de estimação, com a espécie nativa da Europa (*M. putorius*)

(Davison *et al*. 1999). Assim como na hibridação entre cães selvagens e cães domésticos, estes exemplos apresentam-se de fundamental importância para a conservação das espécies selvagens ou nativas, por representarem casos de hibridação de origem antropogênica, com possíveis conseqüências negativas sobre estas.

Eventos de hibridação entre integrantes da Família Felidae também são documentados, incluindo o cruzamento entre diferentes espécies selvagens e entre felídeos selvagens e domésticos. Dentre os exemplos incluindo táxons selvagens, está a hibridação entre duas espécies de linces da América do Norte, o lince Canadense (*Lynx canadensis*) e o "bobcat" (*L. rufus*), que parece constituir-se basicamente de eventos esporádicos com produção apenas de híbridos F1 (Schwartz *et al*. 2004). Por outro lado, um dos casos de hibridação mais estudado entre carnívoros, inclui as subespécies de gatos selvagens da Europa, Ásia e África (*Felis silvestris silvestris*, *F. s. ornata* e *F. s. lybica*) e o gato doméstico (*F. s. catus*), onde as taxas de hibridação e introgressão são extremamente elevadas em determinadas regiões de ocorrência das variedades selvagens, constituindo uma séria ameaça à integridade genética destas (Beaumont *et al*. 2001, Daniels *et al*. 2001, Randi *et al*. 2001, Pierpaoli *et al*. 2003, Lecis *et al*. 2006). A intensidade da hibridação entre estes táxons é favorecida, principalmente, por sua proximidade filogenética. No entanto, a elevada destruição e fragmentação dos habitats naturais das subespécies selvagens, que acabam por reduzir suas densidades populacionais, juntamente com a alta densidade de gatos domésticos introduzidos, que se tornaram selvagens em determinadas localidades de ocorrência dos táxons nativos, são fatores importantes que também favorecem as altas taxas de hibridação entre estes grupos (Nowell & Jackson 1996). Dois casos adicionais de hibridação entre felídeos foram previamente documentados e incluem as espécies alvo deste estudo, sendo descritos detalhadamente a seguir.

Família Felidae e a Linhagem da Jaguatirica

A família Felidae (Mammalia, Carnivora) compreende atualmente 36 espécies (Wozencraft 2005) distribuídas por todo o planeta, à exceção dos pólos, Austrália, Nova Zelândia, Madagascar e Caribe (Nowak 1999). Este grupo caracteriza-se por uma variedade de espécies altamente especializadas ao hábito carnívoro, que sofreram uma diversificação relativamente rápida e recente em sua história evolutiva, iniciada há cerca de 10 milhões de anos na Eurásia no final do Mioceno (Martin 1989, Kitchener 1991, Mattern & McLennan 2000). Este padrão de diversificação, associado à grande quantidade de

paralelismos e convergências registrados na história evolutiva desta família (Martin 1989), acabou por dificultar a classificação sistemática e evolutiva de seus integrantes.

Uma maior elucidação das relações filogenéticas entre os felídeos começou a ser alcançada, principalmente, a partir dos recentes avanços tecnológicos na área da genética molecular (Collier & O'Brien 1985, Wayne *et al*. 1989, Pecon-Slatterry *et al*. 1994, Johnson *et al*. 1996, Masuda *et al*. 1996, Johnson & O'Brien 1997, Pecon-Slatterry & O'Brien 1998, Mattern & McLennan 2000). Grande parte destes estudos apresenta uma subdivisão da família em oito diferentes linhagens monofiléticas. No entanto, somente com o recente trabalho conduzido por Johnson *et al*. (2006), a ordem cronológica do surgimento de cada linhagem, suas relações e exata composição de espécies foi resolvida.

Dentre as oito linhagens definidas por alguns destes autores, encontra-se a Linhagem da Jaguatirica que parece ter divergido há cerca de 8,0 milhões de anos (ma). Esta linhagem apresenta-se como endêmica da Região Neotropical e inclui sete espécies de felídeos de pequeno e médio porte que aparentemente iniciaram sua diferenciação há cerca de 2,9 ma, durante e após a formação do istmo do Panamá que permitiu a colonização da América do Sul por grupos provenientes da América do Norte. As sete espécies constituintes desta linhagem são: a jaguatirica (*Leopardus pardalis*), o gato maracajá (*L. wiedii*), o gato-andino (*L. jacobita*), o gato-palheiro (*L. colocolo*), o gato-do-mato-grande (*L. geoffroyi*), o huiña (*L. guigna*) e o gato-do-mato-pequeno (*L. tigrinus*). Dentro desta linhagem, segundo Johnson *et al*. (2006) são reconhecidos dois clados monofiléticos principais, um com o agrupamento de *L. pardalis* e *L. wiedii* como espécies irmãs, e outro incluindo as cinco espécies restantes. Neste segundo clado, *L. colocolo* é posicionada como espécie irmã de *L. jacobita* e *L. geoffroyi* de *L. guigna*, estando *L. tigrinus* mais relacionada ao par *geoffroyi* - *guigna* do que ao par *colocolo – jacobita* (Figura 1).

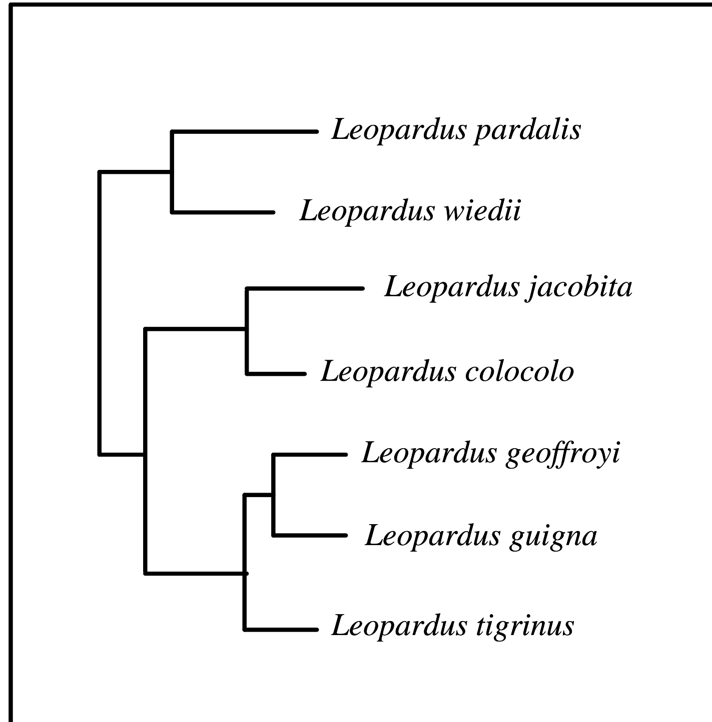


Figura 1 – Relações filogenéticas entre as espécies de felídeos pertencentes à Linhagem da Jaguatirica (fonte: Johnson *et al*. 2006).

Leopardus tigrinus, L. geoffroyi e L. colocolo

O gato-do-mato-pequeno (*Leopardus tigrinus*), o gato-do-mato-grande (*L. geoffroyi*) e o gato-palheiro (*L. colocolo*) são espécies de pequenos felídeos neotropicais proximamente relacionadas segundo dados moleculares (Johnson *et al.* 2006). Morfologicamente, as três espécies apresentam-se como felídeos de pequeno porte: *L. tigrinus* com um comprimento total variando entre 71 a 93,6 cm e peso entre 1,75 e 3,5 Kg; *L. geoffroyi* medindo de 69 a 125 cm e peso variando de 2,2 a 7,8 Kg; e *L. colocolo* com comprimento total variando de 66,3 a 106 cm e peso entre 1,7 e 3,65 Kg (Sunquist & Sunquist 2002). Quanto aos padrões de pelagem, *L. colocolo* é a espécie mais diferenciada com uma coloração muito variável, podendo ir do cinza-amarelado ao cinza escuro ou marrom-avermelhado, geralmente sem a presença de pintas. Suas principais características diagnósticas são a pelagem mais longa e áspera, principalmente na região do dorso, e a presença de listras largas e escuras nas patas anteriores e posteriores. *L. tigrinus* e *L. geoffroyi* são espécies pintadas, que se diferenciam pelo padrão de pintas e coloração de fundo. *L. tigrinus* apresenta pelagem geralmente ocre com manchas formando rosetas de

bordas negras e centros castanhos. A coloração da pelagem de *L. geoffroyi* varia do cinza ao amarelado e difere-se das outras espécies de felídeos pintados da Região Neotropical por apresentar o único padrão de pintas sólidas e negras sem a formação de rosetas (Oliveira 1994, Oliveira & Cassaro 1999, Eisenberg & Redford 1999, Nowell & Jackson 1996; ver Figura 2).

L. tigrinus e *L. geoffroyi* apresentam distribuições basicamente parapátricas na Região Neotropical, com *L. tigrinus* ocorrendo do sul da Costa Rica até o sul do Brasil (norte do estado do Rio Grande do Sul) e nordeste da Argentina e *L. geoffroyi* desde a Bolívia e o chaco paraguaio até o sul do Chile, cobrindo praticamente toda a Argentina, o Uruguai e sul do Rio Grande do Sul (Oliveira 1994, Eisenberg & Redford 1999, Eizirik *et al.* 2006). *L. colocolo* apresenta uma distribuição basicamente simpátrica com *L. geoffroyi* ocorrendo desde o Chile, cobrindo praticamente toda Argentina, Paraguai e Uruguai até regiões da Bolívia, Equador e partes do Brasil central, onde sua distribuição encontra-se com a de *L. tigrinus* (Oliveira 1994, Nowell & Jackson 1996; ver Figura 3).

Quanto aos habitats em que ocorrem, *L. tigrinus* parece ocupar predominantemente áreas de floresta úmida tropical e subtropical e *L. geoffroyi* e *L. colocolo*, áreas mais abertas de cerrado, campos e mosaicos de vegetação, incluindo matas pouco densas (Nowell & Jackson 1996, Nowak 1999). No entanto, para as três espécies, existem registros nos mais variados ambientes (Ximenez 1975, Bisbal 1989, Johnson & Franklin 1991, Olmos 1993, Oliveira 1994, Nowell & Jackson 1996, Oliveira & Cassaro 1999). Estas informações constituem-se basicamente em registros de ocorrência, sendo a existência de estudos mais aprofundados, sobre o uso de habitat por cada uma das espécies, extremamente escassa. *L. tigrinus* e *L. colocolo* apenas recentemente começaram a ser estudadas em ambiente selvagem (p.ex. B. Kasper, L. Silveira, dados não publicados), havendo pouquíssimas informações a respeito de suas distribuições em escala local, requerimento e padrão de uso de habitats e relações com outras espécies de felídeos simpátricos (Oliveira 2006). Quanto a *L. geoffroyi*, apenas três estudos baseados em rádio-telemetria abordaram aspectos de uso de habitat e definição de áreas de vida em populações do sul do Chile (Johnson & Franklin 1991) e Argentina (Manfredi *at al*. 2006, Pereira *et al*. 2006).

Em relação à alimentação, poucos estudos através da análise de fezes e conteúdos estomacais (com tamanhos amostrais extremamente pequenos) foram realizados até o momento. Para *L. tigrinus*, os estudos existentes foram realizados em áreas da Caatinga no Estado do Piauí (Olmos 1993), na Costa Rica (Gardner 1971) e nordeste do Brasil

(Ximenez 1982), indicando a predominância de mamíferos e répteis na dieta desta espécie. Os dados existentes para a dieta de *L. geoffroyi* envolvem áreas da Patagônia (Johnson & Franklin 1991, Novaro *et al*. 2000), Uruguai e Rio Grande do Sul (Ximenez 1982) e Argentina (Manfredi *et al*. 2004) e também indicam os mamíferos como os principais componentes da dieta desta espécie, além de uma relação próxima com a água representada pela presença de espécies de anfíbios, peixes e aves semi-aquáticas. Os dados para a dieta de *L. colocolo* são os mais escassos, incluindo apenas observações de predação sobre pequenos mamíferos e ovos e filhotes de pingüins na Patagônia (Nowel & Jackson 1996, Sunquist & Sunquist 2002).

Assim como a maioria das espécies de felídeos, *L. tigrinus*, *L. geoffroyi* e *L. colocolo* estão incluídas em inúmeras listas internacionais, nacionais e regionais da fauna selvagem ameaçada de extinção (UNEP-WCMC 2004, IBAMA 2003, IUCN/SSC Cat Specialist Group 2003, Marques *et al*. 2002). Apesar de constarem em várias listas de espécies ameaçadas, é difícil dizer ao certo as principais ameaças atuais para estas espécies e seu verdadeiro *status* na natureza, visto que muito pouco é conhecido sobre elas. A ausência do conhecimento de aspectos básicos e específicos da biologia destas espécies acaba por dificultar a elaboração e efetivação de estratégias de manejo e conservação (Nowell & Jackson 1996).

A) Foto: Tadeu Gomes de Oliveira. Fonte: Oliveira & Cassaro (1999)

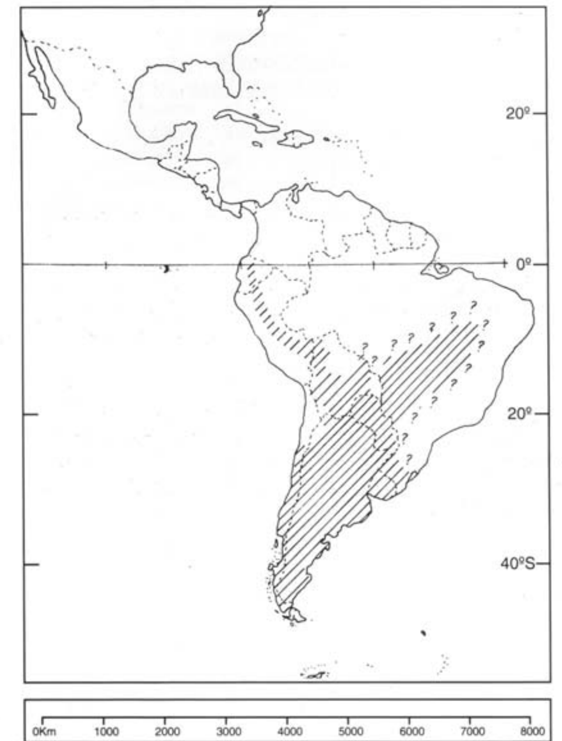
B) Foto: Thales R. O. de Freitas

C) Foto: Francisco Erize. Fonte: Nowell & Jackson (1996)

Figura 2. Exemplares de *Leopardus tigrinus* (A), *L. geoffroyi* (B) e *L. colocolo* (C).

(A)

(B)



(C)

Figura 3. Distribuição geográfica de *Leopardus tigrinus* (A), *L. geoffroyi* (B) e *L. colocolo* (C). Mapas retirados de Oliveira & Cassaro (1999).

Evidências prévias de hibridação entre *Leopardus tigrinus*, *L. geoffroyi* e *L. colocolo*

A primeira evidência de hibridação entre felídeos Neotropicais foi documentada por Johnson *et al*. (1999), entre indivíduos de *Leopardus tigrinus* e *L. colocolo*. A detecção dos híbridos foi realizada pela análise de seqüências do DNA mitocondrial (DNAmt) e do gene *ZFY* do cromossomo Y. Neste estudo, indivíduos identificados morfologicamente como *L. tigrinus*, apresentaram seqüências de DNAmt específicas de *L. colocolo* e seqüências do gene *ZFY* específicas de *L. tigrinus*, implicando em uma origem híbrida proveniente do cruzamento entre machos de *tigrinus* e fêmeas de *colocolo*. Estudo posterior, realizado por Trigo *et al*. (2008), corroborou a existência destes eventos na região central do Brasil pela análise do DNAmt, identificando cinco indivíduos híbridos, incluindo alguns dos mesmos indivíduos identificados por Jonhson *et al*. (1999). Estes dois estudos documentam a primeira evidência da ocorrência de eventos de hibridação entre *L. tigrinus* e *L. colocolo*, no entanto, restringem-se apenas à identificação de híbridos, sem maiores investigações nos padrões, causas e extensão destes eventos.

O segundo caso de hibridação entre felídeos Neotropicais foi também documentado pela primeira vez por Trigo *et al*. (2008) entre *L. tigrinus* e *L. geoffroyi* no sul do Brasil pela análise de DNAmt e locos de microssatélite. As primeiras evidências da possibilidade de hibridação entre estas espécies foram documentadas por Mazim *et al*. (2004) e Eizirik *et al*. (2006) através da identificação de indivíduos com características morfológicas ambíguas (porte de *L. geoffroyi* ou de *L. tigrinus* com padrões intermediários na formação das pintas características de cada espécie). Eizirik *et al*. (2006) avaliaram a distribuição espacial das duas espécies no estado do Rio Grande do Sul (RS), registrando uma aparente segregação entre estas, com *L. tigrinu*s restringindo-se à região norte do estado, onde predominam áreas com formações florestais, e *L. geoffroyi* à região sul, caracterizada por formações vegetais mais abertas, como campos e savanas com matas de galeria. A zona de contato entre as duas espécies demonstrou-se extremamente restrita, e coincidente com a Depressão Central do estado, caracterizada pela convergência das diferentes formações vegetais de seu entorno. Exatamente nesta região de contato, os autores indicaram a predominância de indivíduos com características morfológicas ambíguas. Por outro lado, Mazim *et al*. (2004) registraram indivíduos de *L. geoffroyi* com padrões de pelagem intermediários com *L. tigrinus* na região sudeste do RS. Estes últimos autores argumentam que estes padrões poderiam estar mais relacionados a uma variação individual dentro da espécie do que a uma possível origem híbrida com *L. tigrinus*, visto

que esta última espécie não apresentava registros de ocorrência na área em questão. A possibilidade de hibridação acabou sendo confirmada pelos dados moleculares, onde pelo menos 14 indivíduos de uma amostra total de 61 *L. tigrinus* e 41 *L. geoffroyi* foram identificados como híbridos, sendo estes em sua maioria provenientes da região central do RS, onde Eizirik *et al*. (2006) previamente registraram o contato entre as espécies e a existência de indivíduos morfologicamente intermediários. Além da identificação dos híbridos, um gradiente de diferenciação genética foi encontrado entre *L. geoffroyi* e diferentes populações de *L. tigrinus,* sendo identificada uma maior similaridade entre as populações das duas espécies próximas à área de contato, provavelmente em decorrência aos eventos de hibridação e introgressão detectados nesta região. Segundo este mesmo estudo molecular, a formação da zona híbrida entre *L. tigrinus* e *L. geoffroyi* pode ter sido propiciada por uma expansão demográfica de *L. tigrinus* que teria levado ao contato com *L. geoffroyi* e favorecido, assim, a ocorrência dos cruzamentos interespecíficos. Da mesma maneira, a hibridação com *L. colocolo* no Brasil central pode ter sido facilitada pelo mesmo evento.

A partir dos primeiros trabalhos identificando a existência de hibridação entre *L. tigrinus* e ambas as espécies *L. geoffroyi* e *L. colocolo*, inúmeros outros estudos tornam-se necessários para o entendimento da real extensão destes eventos, das causas, conseqüências e processos evolutivos relacionados a estes, incluindo estudos moleculares, morfológicos e ecológicos.

Objetivos

Este trabalho tem como objetivo geral gerar novas informações sobre as relações genéticas, evolutivas e ecológicas entre três espécies de felídeos Neotropicais, principalmente, no que diz respeito à ocorrência de eventos de hibridação e introgressão genética entre estas.

Como objetivos específicos, o presente trabalho envolve:

1) Descrever a variabilidade genética em *Leopardus tigrinus*, *L. geoffroyi* e *L. colocolo,* utilizando os seguintes marcadores moleculares: locos de microssatélite, segmentos do DNA mitocondrial e introns ligados aos cromossomos X e Y;

2) Utilizar o conjunto total de marcadores moleculares para confirmar, quantificar e caracterizar a existência de eventos de hibridação e introgressão genética entre as três espécies de felídeos descritas acima;
3) Utilizar o conjunto total de marcadores moleculares para quantificar e caracterizar localmente a zona de hibridação entre *L. tigrinus* e *L. geoffroyi* no estado do Rio Grande do Sul;
4) Caracterizar e avaliar a variação morfológica existente entre *L. tigrinus* e *L. geoffroyi* no estado do Rio Grande do Sul a partir de biometria e padrões de pelagem;
5) Avaliar a distribuição geográfica e a associação a diferentes categorias fitofisionômicas das duas espécies de felídeos citadas acima e híbridos identificados geneticamente no estado do RS;
6) Avaliar o nível de sobreposição dos nichos tróficos de *L. tigrinus* e *L. geoffroyi* no estado do RS;
7) Ampliar os conhecimentos da biologia destas espécies e contribuir para a elaboração de programas de manejo e conservação em campo e em cativeiro.

CAPÍTULO II

MANUSCRITO EM PREPARAÇÃO

Contrasting patterns of genetic introgression among three hybridizing Neotropical cats (*Leopardus tigrinus*, *L. geoffroyi* and *L. colocolo* – Carnivora Felidae) revealed by multiple molecular markers

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Running title: hybridization among three Neotropical cats

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Abstract

Hybridization among three small Neotropical cats (*Leopardus tigrinus*, *L. geoffroyi* and *L. colocolo*) has been recently documented through the analysis of mitochondrial DNA and microsatellite markers. In this study we extend these analyses by including a larger sample size of these species, and by combining the use of mtDNA sequences and microsatellite loci with Y and X chromosome intron sequences to genetically characterize the processes of hybridization and introgression among these cats. We characterize the variability of these markers in the three species, and employ this genetic information to infer the evolutionary and demographic patterns underlying the hybridization events. The hybrid zone previously detected between *L. tigrinus* and *L. geoffroyi* appears to be concentrated in Rio Grande do Sul, the southernmost Brazilian state, where the ranges of these two species meet. Several genetic combinations of the different markers were found in both species, suggesting the existence of extensive ongoing or recent hybridization, which results in bidirectional and partially asymmetric genetic introgression. In contrast, our data indicate a pattern of older, unidirectional or strongly asymmetric hybridization between *L. tigrinus* and *L. colocolo* in central and northeastern Brazil.

Introduction

Natural introgressive hybridization is currently considered by many biologists to be an evolutionary process with broad relevance to adaptation and speciation (Arnold 1992, Harrison 1993, Allendorf *et al*. 2001, Barton 2001). Although this phenomenon was until recently recognized as most important in plants, there is a growing recognition of the relevance of introgressive hybridization for the origin of evolutionary novelties and diversification in sexually reproducing animals (Dowling & Secor 1997). Various patterns of introgression may occur in animal hybrid zones (Harrison 1993) and in consequence multiple outcomes are possible, with different levels of importance for the evolutionary history of the implicated species. In the extreme cases, introgression may lead to complete admixture of the hybridizing forms, or to the reinforcement of reproductive barriers through selection for conspecific mating. Several possibilities are intermediate between these extremes, such as the formation of stable hybrid zones or the production of variably fit introgressed genotypes allowing the expansion of the introgressed form into a novel habitat (Barton & Hewitt 1985, Arnold 1992, Arnold 1993, Harrison 1993). Considering these scenarios, attempts to identify and characterize patterns of introgression in different animal taxa constitute an important component of the investigation of the evolutionary history of hybridizing species.

In recent years, the use of different molecular markers to investigate hybrid zones has generated new and important insights on the introgressive patterns present in animal species. Such genetic analyses may detect different magnitudes and directionalities of introgression in different pairs of interacting species (Lehman *et al*. 1991, Gotelli *et al*. 1994), as well as among loci undergoing different modes of inheritance (*e.g.* Helbig *et al*. 2001, Cianchi *et al*. 2003, Helbig *et al*. 2005) and is also useful to elucidate the relative rate of current hybridization *vs.* past introgression events (*e.g.* Cianchi *et al*. 2003). The characterization of these genetic patterns is especially important to allow the inference of possible selective pressures acting on the different loci and species in a particular hybrid zone.

Many recent genetic studies with different types of molecular markers have documented the occurrence of hybridization and introgression in carnivore species (*e.g.* Roy *et al*. 1994, Kyle *et al*. 2003, Norén *et al*. 2005, Verardi *et al*. 2006). However, few cases have been described in the family Felidae, as the hybridization between the Canadian lynx (*Lynx canadensis*) and the bobcat (*Lynx rufus*) (Schwartz *et al*. 2004), hybridization

between wildcats (*Felis silvestris silvestris* and *Felis s. libyca*) and domestic cats (*Felis s. catus*) (Beaumont *et al*. 2001, Daniels *et al*. 2001, Randi *et al*. 2001, Pierpaoli *et al*. 003, Lecis *et al*. 2006, Driscoll *et al*. 2007) and between three species of Neotropical cats (Johnson *et al.*1999, Trigo *et al.* 2008). In the Neotropical Region, the occurrence of hybridization between males of the little spotted cat (*Leopardus tigrinus*) and female pampas cats (*Leopardus colocolo*) in central Brazil, where the two species are sympatric, was first reported by Johnson *et al*. (1999). That observation was based on mitochondrial DNA (mtDNA) and intron sequences of a Y-chromosome marker, and was subsequently corroborated by our previous study including samples from the same area and employing mtDNA and autosomal microsatellite markers (Trigo *et al*. 2008). However, both of those studies only revealed isolated cases of hybridization between these two species, with no further investigation on the extent and history of this process. In addition to those initial observations with respect to this pair of felid species, we reported that *L. tigrinus* also hybridizes with *L. geoffroyi* in southern Brazil, where a hybrid zone between them seems to exist (Trigo *et al*. 2008). Our first detailed study involving the investigation of the hybridization between these three Neotropical cats revealed some interesting patterns. The hybridization between *L. tigrinus* and *L. geoffroyi* was detected mostly in the vicinity of a restricted geographic contact zone between the two cats in Rio Grande do Sul, the southernmost Brazilian state (Eizirik *et al*. 2006), whereas the hybridization between *L. tigrinus* and *L. colocolo* was detected in central Brazil, where these two species are sympatric and *L. geoffroyi* is absent (see Figure 1A). In these two areas, different selective pressures or interaction histories may have led to different patterns of introgression between each pair of species: while the *L. tigrinus vs*. *L. colocolo* hybridization seems to be sporadic, with only few individuals identified as hybrids, and resulting in basically unidirectional introgression of mtDNA haplotypes from *L. colocolo* into *L. tigrinus* individuals, the *L. geoffroyi vs*. *L. tigrinus* hybridization appears to be frequent and possibly bidirectional, with introgression of mtDNA haplotypes into both parental species.

Studies concerning introgressive hybridization often require the analysis of a variety of genetic markers. The great majority of studies performed so far have employed only mitochondrial DNA combined with microsatellite loci (*e.g.* Randi *et al*. 2001, Lancaster *et al*. 2006, Gay *et al*. 2007, Trigo *et al*. 2008). As mtDNA is inherited primarily in a matrilineal fashion, it can be used to identify the maternal species; however, it fails to detect male-mediated introgression that may be detected using biparentally inherited markers such as microsatellites. These latter markers are useful to study hybridization due

to the possibility to assess a large number of hypervariable loci, which can be analyzed with several newly available statistical tools for individual-based inference. However, microsatellite markers usually suffer with the occurrence of homoplasy (Jarne & Lagoda 1996), often saturating these loci as informative markers. Additionally, in hybridization cases where rates of introgression are high and parental species are closely related and do not posses clearly diagnostic alleles, the detection of hybrids may be extremely difficult even with an extensive number of microsatellite loci (Boecklen & Howard 1997, Anderson & Thompson 2002, Lancaster *et al*. 2006, Vähä & Primmer 2006). In our previous study on the hybridization between *L. tigrinus*, *L. geoffroyi* and *L. colocolo* (Trigo *et al*. 2008), the use of nine microsatellite loci and three mtDNA segments revealed that these markers are capable of detecting introgression in this system, but were somewhat limited in their power to characterize this process in detail, as well as to precisely diagnose hybrid animals. This has led us to screen for additional molecular markers so as to expand the available knowledge about the hybridization/introgression patterns among these three Neotropical cats. Nuclear intron sequences, which are potentially powerful species-specific markers for studies of hybridization (Pacheco *et al*. 2002), were evaluated here to assess their utility for this type of analysis. Therefore, this present study was designed aiming to meet the following objectives: 1) to identify and characterize novel molecular markers, especially linked to the X and Y chromosomes, to allow for in-depth multi-locus analyses of the *L. tigrinus*/*L. geoffroyi*/*L. colocolo* hybridization; 2) to analyze a comprehensive suite of molecular markers (including mtDNA, autosomal microsatellites, and X- and Y-linked intronic sequences) in a broad sample of individuals aiming to assess the magnitude and symmetry of introgression among species; 3) to compare the inferred patterns of introgression among markers presenting different inheritance modes, with the goal of clarifying the hybrid combinations that gave rise to the present populations; and 4) to investigate the rates of current hybridization *vs.* past introgression events for each pair of hybridizing species. Overall, we aimed to combine these inferences in an attempt to enhance the knowledge on the past history and evolutionary significance of the hybridization among these three Neotropical wild cats.

Material and Methods

Sample collection, molecular markers and laboratory procedures

Sample Collection

The samples used in this study include almost all individuals (*Leopardus tigrinus*, n = 60; *L. geoffroyi*, n = 40; *L. colocolo*, n = 7) analyzed in our previous study (Trigo *et al*. 2008), along with additional material collected from all three species in various geographic areas, totaling 119 *L. tigrinus*, 78 *L. geoffroyi* and 10 *L. colocolo* (see Supplementary Material for a full list of samples). These specimens include captive, road killed and wild-caught animals. Two samples of *Leopardus guigna* and one each of *Leopardus pardalis* and *L. wiedii* were also included for comparison. Samples of *L. tigrinus* originated from Paraguay and different regions of Brazil, while *L. geoffroyi* and *L. colocolo* were sampled in different countries of South America including Brazil, Uruguay, Argentina, Bolivia and Chile (Figure 1B and Supplementary Material). Blood samples were preserved in a salt saturated solution (100mM Tris, 100mM EDTA, 2% SDS), and other tissue samples were stored in 70% ethanol. DNA extraction was performed using standard phenol/chloroform protocols (Sambrook *et al*. 1989).

Mitochondrial DNA analyses

In our previous study on *L. tigrinus*/*L. geoffroyi*/*L. colocolo* genetics we utilized three mitochondrial segments: 1) the 5' portion of the mtDNA control region; 2) a segment of the *ND5* gene, and 3) the *ATP8* gene and adjacent fragments (Trigo *et al*. 2008). Based on the informative content for each segment observed in that study, for this extended analysis we selected only the control region and *ND5* fragments, given their better performance over *ATP8*. DNA sequences for these two segments were generated for the new samples following the same methods described previously, and subsequently integrated with the data sets reported by Trigo *et al*. (2008).

Initial sequence comparisons and measures of variability were performed using MEGA 3.2 (Kumar *et al*. 2004) for the concatenated data set given the linked inheritance of non-recombining mtDNA. Haplotype networks were generated using the median-joining approach (Bandelt *et al*. 1999) implemented in NETWORK 4.1.0.8 (www.fluxus-engineering.com). For each of the identified clades we estimated the gene diversity (*h*, the probability that two randomly chosen mtDNA lineages were different in the sample) and nucleotide diversity (π per nucleotide site, the probability that two randomly chosen

homologous nucleotides are different in the sample) using the software ARLEQUIN 3.11 (Excoffier *et al*. 2005). In order to infer the occurrence of past events of population expansion, we performed mismatch distribution analyses (Rogers & Harpending 1992) with DNASP 4.0 (Rozas *et al*. 2003) and neutrality tests such as Tajima's D (Tajima 1989) and Fu's Fs (Fu 1997) with ARLEQUIN.

Nuclear intron analyses

Analyses of nuclear introns included one segment located on the X chromosome (*PLP1*) and two intronic segments of Y-linked genes (*ZFY* and *SMCY3*). The second intron of the *Proteolipid Protein 1* X-linked gene (*PLP1*) segment was amplified and sequenced using the primers described by Murphy *et al*. (1999), and selected for use in this study given its high levels of variation observed in previous investigations of carnivore genetics (*e.g*. Johnson *et al*. 2006, Tchaicka *et al*. 2007). To select informative Y-linked segments, we initially performed a screen for variation in multiple loci that had been previously employed in felids, but whose diversity in this Neotropical lineage was not known. We tested intronic segments of five different Y-linked genes: 1) *ZFY*, using primers described by Pecon-Slattery & O´Brien (1998); 2) *SMCY3*, *DBY4* and *DBY7*, using primers described by Hellborg & Ellegren (2003); and 3) *UBE1Y*, using primers described by Pecon-Slattery *et al*. (2004). To identify variable sites in these five segments within or between the focal species of this study, we initially amplified and sequenced them in five individuals each of *L. geoffroyi* and *L. tigrinus*. This was a conservative survey, as these two species are more closely related to each other than they are to *L. colocolo* (Johnson *et al.* 2006), so that it would be expected to be more difficult to find variation that would distinguish this particular pair. All five segments could be amplified and sequenced, totaling 1,694 base pairs (bp) of Y chromosome fragments surveyed (*ZFY*: 399 bp, *UBE1Y*: 463 bp, *SMCY3*: 425 bp, *DBY4*: 164 bp and *DBY7*: 243 bp). Very little genetic diversity was observed in these segments, and the only identified variable sites were located in the *ZFY* and *SMCY3* introns. We thus focused our efforts on these two variable introns, which were subsequently concatenated for analysis given the linked inheritance of non-recombining Y chromosome loci.

All the nuclear introns were amplified by the Polymerase Chain Reaction (PCR; Saiki *et al*. 1985) in reactions of 20 μL final volume containing 1.5 – 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 U of Taq DNA polymerase (Invitrogen) and 0.2 μM each of the forward and reverse primers. Thermocycling conditions for all segments began with 10 cycles of

touchdown, each including a 45s denaturing step at 94ºC, 45s annealing at 60-51ºC, and a 1.5 min extension at 72 ºC; this was followed by 30 – 35 cycles of 45s denaturing at 94ºC, 45s annealing at 50ºC and 1.5 min extension at 72ºC. PCR products were then purified using the enzymes exonuclease I and shrimp alkaline phosphatase. Purified PCR products were sequenced using the DYEnamic ET terminator kit (Amersham) and subsequently analyzed in a MegaBACE 1000 automated sequencer. Sequence electropherograms were verified and corrected by eye using CHROMAS (http://www.thecnelysium.com.au/ chromas.html), and then aligned using the CLUSTALW algorithm implemented in MEGA; the alignment of each segment was checked and edited by hand separately.

For the X-linked *PLP1* segment, the initial step of analysis was the identification of heterozygote females. Heterozygote sites were identified when two different nucleotides were present at the same position in the electropherograms of both strands, with the weakest peak reaching at least 25% of the strongest signal. As few heterozygous sites were found (see results), the haplotypes were defined by hand.

Median-joining networks were generated using NETWORK to assess the frequency and relationships between haplotypes from both types of introns (X and Y linked). This analysis was also utilized to infer the species-specific haplotypes and then evaluate its utilization at hybridization analysis. The gene and nucleotide diversity were calculated with ARLEQUIN.

Microsatellite analyses

In addition to the analyses based on mtDNA and nuclear sequences, we include in this study the nine microsatellite markers employed in our previous paper (Trigo *et al*. 2008), along with two additional loci, totaling 11 STRs (FCA391, FCA424, FCA441, FCA453, FCA723, FCA742, F42, F53, F98, F124, F146). Each microsatellite locus was amplified individually by PCR, using primers described for the domestic cat (*Felis silvestris catus*) by Menotti-Raymond *et al*. (1999, 2005) and under the same conditions applied by Trigo *et al*. (2008). PCR products for microsatellite loci generated here were genotyped using fluorescent detection in a MegaBACE1000 automated sequencer, using the software GENETIC PROFILER 1.5. Five individuals genotyped in our previous work were included in all runs performed with the automated sequencer to precisely calibrate allele sizes.

The genetic diversity in *L. tigrinus*, *L. geoffroyi* and *L. colocolo* was evaluated by the average number of alleles and observed and expected heterozygosity using GENEPOP

3.1d (Raymond & Rousset 1995) and ARLEQUIN. Departures from Hardy-Weinberg Equilibrium (HWE) were tested with ARLEQUIN using the exact test of Guo & Thompson (1992). Linkage equilibrium (LE) for each species was assessed with GENEPOP. The sequential Bonferroni correction was applied to adjust the significance levels of HWE and LE, taking into account multiple comparisons on the same data set (Rice 1989). The genetic differentiation among species was assessed with an Analysis of Molecular Variance (AMOVA, Excoffier *et al.* 1992) performed with ARLEQUIN, using an Fst analog (Reynolds *et al.* 1983) and Rst (Slatkin 1995). Statistical significance of the observed values was tested using 10,000 permutations.

We also used the Bayesian approach implemented in STRUCTURE (Pritchard *et al*. 2000) to define the number of genetically differentiated groups in our total sample and to simultaneously assign individuals to source populations. This Bayesian approach assumes HWE and LE within populations, and utilizes departures from these to split groups into subpopulations. By using this method we defined the number of populations (K) that best fit these assumptions. Each individual sampled was then assigned probabilistically to one of these populations or simultaneously to more than one population if its genotypes indicated that it was admixed. For this purpose we used the option of the admixture model, unlinked loci, correlated frequencies (Falush *et al*. 2003) and no previous information of phenotypic or geographic population of origin, *i.e.* the number of populations was defined only by the genetic data. We tested K = 1 - 10 and performed five independent runs for each K to evaluate the consistency and variation of the probability of the data. We chose the value of K with the highest likelihood as the best model of population structure for explaining the data (Pritchard *et al*. 2000) and then evaluated the species and geographic classification of the individuals. For the selected K values, we assessed the average proportion of membership (Q) of the sampled populations (*L. tigrinus*, *L. geoffroyi*, *L. colocolo*) to the inferred clusters, and the proportion of membership of each sampled individuals (q). The STRUCTURE analysis was performed using a Markov Chain Monte Carlo (MCMC) of 500,000 iterations following a burn-in period of 200,000 iterations.

Multi-locus analyses

To integrate the information gleaned from the different types of molecular markers, we used the software LAMARC (Kuhner 2006) to estimate demographic parameters such as theta (θ) and migration rates for each of the three cat species. These parameters were

estimated simultaneously for the sequence-based markers (mtDNA, *PLP1* intron and *ZFY*/*SMCY3* Y-linked introns), including corrections for different mutation rates and different effective populations sizes for each genomic region, thus allowing for a direct comparison of the inferred patterns among them. The estimation of these demographic parameters allowed us to evaluate the patterns of gene flow among the three species and also among molecular markers undergoing different modes of inheritance, which may be very informative to investigate the process of genetic introgression in the two focal hybrid zones. The number of migrants per generation was calculated from the estimated migration rate parameter "M" in LAMARC incorporating the mean θ of the recipient population to correct for variation in the mutation rate among segments (as suggested in the program documentation). The data was subjected to three independent runs of a Bayesian search strategy including one long chain of 1,600,000 steps with a sampling increment of 40 (resulting in a total of 40,000 sampled trees), following a burn-in period of 4,000 sampled genealogies.

Results

Mitochondrial DNA

We sequenced 225 base pairs (bp) of the mtDNA control region and 567 bp of the *ND5* gene, resulting in a mtDNA data set totaling 792 bp. The final data set comprised 78 *L. geoffroyi*, 111 *L. tigrinus*, nine *L. colocolo*, two *L. guigna*, one *L. pardalis* and one *L. wiedii*, including sequences generated by Trigo *et al*. (2008). This data set contained 146 substitutions (136 transitions and 10 transversions) and two indels (insertions or deletions), defining a total of 89 unique haplotypes.

The median-joining network of haplotypes (Figure 2A) defined three monophyletic groups, one for each of the three Neotropical cats addressed here (*L. tigrinus*, *L. geoffroyi* and *L. colocolo*) including 37, 28 and 17 haplotypes respectively (a slightly higher number of haplotypes is shown in Table 1 for *L. geoffroyi* and *L. colocolo,* because the network analysis considers only sites with no missing information; three additional haplotypes [sampled in one individual each] were distinguishable by sites which were lost due to the presence of missing data in some other individual). Interestingly, the two *L. guigna* haplotypes formed a distinct cluster which was nested within the *L. geoffroyi* clade, supporting the hypothesis that the former species arose as an isolated population of the latter.

The highest gene and nucleotide diversity were found in the *L. colocolo* clade, contrasting with the results we published previously (Trigo *et al*. 2008) that reported the lowest gene diversity for this group, with a smaller sample size. The *L. tigrinus* clade showed the second highest gene diversity, but the lowest nucleotide diversity and lowest average number of pairwise differences (Table 1, Figure 2A).

The detailed evaluation of the network revealed several misplaced individuals and the sharing of haplotypes between species (Figure 2A). Four haplotypes placed in the *L. geoffroyi* clade were identified in *L. tigrinus* individuals, leading to the identification of seven misplaced animals representing genetic introgression from the *L. geoffroyi* into the *L. tigrinus* population. Among the seven misplaced individuals, five were from Rio Grande do Sul (RS) state while two were from São Paulo state. On the other hand, ten different haplotypes were introgressed in the opposite direction (*L. tigrinus* mtDNA haplotypes found in *L. geoffroyi* individuals) resulting in 15 misplaced *L. geoffroyi*, all from RS state. Surprisingly, the highest number of 'misplaced' animals was observed in the *L. colocolo* clade, which included 12 different haplotypes sampled in 22 *L. tigrinus* individuals. These misplaced specimens included the entire *L. tigrinus* sample from the northeastern Brazilian region, 66.7% (4/6) of the central Brazilian region, and one (bLti88) from Paraná state in the southeastern Brazilian region. Interestingly, 11 of the 12 'swapped' *L. colocolo* haplotypes were only sampled in *L. tigrinus* individuals, with only case of haplotype sharing between the two species, involving the individual Lco13, from central Brazil. The unique case of introgression in the opposite direction (*L. tigrinus* mtDNA sampled in a *L. colocolo* individual) remains the same captive individual (Lco02) reported in our previous study (Trigo *et al*. 2008). No case of haplotype introgression between *L. colocolo* and *L. geoffroyi* was detected.

The demographic expansion pattern previously detected for *L. tigrinus* is affirmed and extended by this expanded data set, which shows two frequent haplotypes connected by short branches to multiple rarer sequences (Figure 2A). The network analysis also showed some interesting patterns of structuring in the *L. colocolo* clade. While haplotypes carried by *L. colocolo* individuals presented some genetic structuring, with a stronger differentiation among sequences, the misplaced *L. tigrinus* individuals showed a shallower ramification with a similar suggestive pattern of demographic expansion to that detected in the *L. tigrinus* clade. The mismatch distribution of this clade presented a bimodal pattern of population growth (Figure 3A), however the two neutrality tests yielded significantly negative values (Tajima's D = -1.97155, p = 0.013; Fu's Fs = -10.75877, p =

0.000). This detected pattern led us to further investigate the existence of a recent demographic expansion recorded by the haplotypes represented by *L. tigrinus* from central and northeastern Brazilian regions (CNE). The mismatch distribution and neutrality tests were then performed subdividing the *L. colocolo* clade into two subpopulations: one included only *L. colocolo* individuals (n = 7) and another contained the *L. tigrinus* from the CNE region, including Lco13 which shared a haplotype with these individuals (n = 23). These analyses showed negative values for the *L. colocolo* group, but devoid of statistical significance for both neutrality tests (Tajima's D = -0.22754, p = 0.46; Fu's = -2.23734, p = 0.068) and a clearly bimodal distribution in the mismatch analysis (Figure 3B). On the other hand, the mismatch distribution was clearly unimodal for the CNE *L. tigrinus*, and one of the neutrality tests was significantly negative for this group (Fu's = -5.14854, p = 0.003; Tajima'D = -1.03947, p = 0.168) (Figure 3C).

Nuclear intronic segments

We sequenced a total of 808 bp for the *PLP1* intron from 56 *L. geoffroyi* [20 females (F) and 36 males (M)], 62 *L. tigrinus* (21F, 41M) and three *L. colocolo* (1F, 2M). Sequences of one individual each of the species *L. guigna*, *L. pardalis* and *L. wiedii* were obtained and used for comparison. These sequences comprised ten haplotypes defined by 14 variable sites, comprising ten transitions and four transversions (Table 1, Figure 2B). The majority of the females sampled were homozygous for one of the ten haplotypes, with only few observed heterozygotes (6 *L. tigrinus* and one *L. geoffroyi* individuals; see Supplementary Material).

The network analysis indicated four haplotypes as more distinct from the others (Figure 2B). Three of them (Hp8, Hp9, Hp10) were found only in *L. colocolo* individuals and in one *L. tigrinus* specimen from central Brazil (bLti81), which was also misplaced in the mtDNA data set and previously recognized as a hybrid between these two species (Trigo *et al*. 2008). The fourth haplotype (Hp7) was found only in *L. pardalis* and *L. wiedii* individuals. The remaining six haplotypes, sampled only in *L. tigrinus*, *L. geoffroyi* and *L. guigna* individuals, were more closely related to each other (distinguished by only one to three sites). Hp5 was sampled only in *L. guigna* and was closely related to Hp3, predominantly assigned to *L. geoffroyi*, along with Hp4. The three remaining haplotypes (Hp1, Hp2 and Hp6) were predominantly sampled in *L. tigrinus* individuals, with Hp6 being sampled only in individuals from the CNE region. The observed genealogical relationships among haplotypes were in agreement with the current understanding of the

phylogeny of this Neotropical felid lineage, which defines *L. pardalis* + *L. wiedii* and *L. geoffroyi* + *L. guigna* as pairs of sister species, with *L. tigrinus* more closely related to the latter pair and *L. colocolo* in an intermediate position between the two groups (Johnson *et al*. 2006). Considering these first observations, the *PLP1* haplotypes recognized here may be inferred to be species-specific, despite the higher intraspecific than interspecific level of differentiation detected in haplotypes associated to *L. tigrinus* and *L. geoffroyi*.

The same sharing of haplotypes detected in the mtDNA data set was observed with the *PLP1* segment. While Hp1 and Hp2 were predominantly assigned to *L. tigrinus*, they were also sampled in eleven *L. geoffroyi* individuals. In turn, Hp3 was mostly sampled in *L. geoffroyi*, but also recorded in three *L. tigrinus* individuals. Since these two species are closely related, this haplotype sharing might be due to recent ancestry, making it more difficult to employ this segment for the assessment of hybridization. However, all the individuals implicated in this haplotype sharing were from RS state, where hybridization between these species has been reported, and four of them had been previously identified as putative hybrids with microsatellite and mtDNA data (Trigo *et al*. 2008). Likewise, five of these 14 individuals had introgressed mtDNA haplotypes according to our present analysis. Therefore, we consider it likely that the observed inter-species haplotype sharing in this X-linked intron is largely (if not exclusively) due to hybridization between these two felids. Assuming that they were originally species-specific, we can hypothesize, based on their observed frequencies, that three haplotypes (Hp8, Hp9 and Hp10) were originally diagnostic for *L. colocolo*, two (Hp3 and Hp4) for *L. geoffroyi*, and three (Hp1, Hp2 and Hp6) for *L. tigrinus*. Assuming that this hypothesis is correct, we performed an analysis of genetic diversity independently for each inferred species-specific haplotype cluster (including allele copies sampled in a different species), and found higher diversity estimates for *L. colocolo* and *L. tigrinus* (Table 1).

The third sequence-based data set included the two Y-linked introns (*ZFY* and *SMCY3*) spanned a total of 824 bp, and was sampled from 36 *L. geoffroyi*, 53 *L. tigrinus*, five *L. colocolo* and one *L. wiedii*. These sequences comprised seven haplotypes defined by ten polymorphic sites (including six transitions and four transversions). The Y-chromosome haplotype network (Figure 2C) revealed a genealogical pattern that was strongly congruent with the *PLP1* data set, and consequently with the recognized phylogenetic relationships among these species. Two haplotypes were observed in *L. colocolo* individuals (Hy5 and Hy6), with no sharing with *L. tigrinus.* One haplotype (Hy1) was predominantly assigned to *L. geoffroyi*, while three other haplotypes (Hy2, Hy3 and

Hy4) were almost exclusively found in *L. tigrinus* individuals. Interestingly, two of these *L. tigrinus* haplotypes (Hy3 and Hy4) were unique to samples from CNE region. Two haplotypes were shared between *L. tigrinus* and *L. geoffroyi*, leading to the identification of two *L. tigrinus* and ten *L. geoffroyi* individuals that were 'misplaced', all of which were collected in RS state. Of these 12 individuals, six were also misplaced with the mtDNA data and seven with the *PLP1* intron.

Microsatellites

Eleven autosomal microsatellite loci were analyzed for 78 *L. geoffroyi*, 119 *L. tigrinus* and 10 *L. colocolo*. All loci were polymorphic for the three cat species, with the highest average number of alleles per locus recorded in the *L. tigrinus* population (Table 2). The observed heterozygosity was very similar among the three species, and only slightly higher in the *L. colocolo* population. *L. tigrinus* had the highest number of loci presenting departures from HWE (five in comparison to two and one in *L. geoffroyi* and *L. colocolo*, respectively), and was the only species showing significant deviations from LE for some pairs of loci (FCA424 *vs.* FCA441, FCA424 *vs.* F98, FCA441 *vs.* F98 and FCA424 *vs.* FCA742; significance level adjusted with a Bonferroni correction for 55 comparisons).

The STRUCTURE analysis conducted to assess the number of genetically distinct populations (K) contained in our total sample, and to simultaneously assign individuals to their source population, showed the highest mean probability of the data for K = 5 (-7635.84) followed closely by K = 4 (-7639.22). The individual associations with K = 4 and K = 5 were extremely similar, with the only difference between them being the subdivision of *L. geoffroyi* into two populations in the latter case. However, the individual assignments to the two putative populations of *L. geoffroyi* were roughly symmetrical, with almost no individuals strongly assigned to either one of the clusters. As the correlated allele frequencies model seems to overestimate K in such settings, it is possible that the assignment pattern observed with K = 5 may reflect deviations from random assortment that are not caused by genuine population subdivision (Falush *et al.* 2003). Considering this, we chose to interpret the results assuming K = 4. Examining this set of results, the three species were predominantly associated to one of the four clusters inferred by STRUCTURE [*L. geoffroyi*: cluster 1 ($Q1$ = 0.847), *L. tigrinus*: cluster 2 ($Q2$ = 0.707) and *L. colocolo*: cluster 4 ($Q4$ = 0.943)]. While *L. colocolo* was poorly associated to the other three clusters (proportions lower than 0.05), the two other species showed relatively higher

associations. In addition to cluster 1, *L. geoffroyi* was associated to cluster 2 (*L. tigrinus*) with a proportion of membership of $Q2 = 0.111$, while the *L. tigrinus* population was also associated to cluster 1 (*L. geoffroyi*) and to cluster 3 with a proportion of membership of $Q1 = 0.106$ and $Q3 = 0.168$, respectively.

Fifty individuals of *L. geoffroyi* were associated to cluster 1 (*L. geoffroyi* group) with $q1 \geq 0.90$ (Figure 4). All the individuals with probabilities < 0.90 presented the second highest association predominantly to cluster 2 (inferred to correspond to *L. tigrinus*), followed by cluster 3. Of these, 71.4% (20/28) were from RS state, in southernmost Brazil. Evaluating the *L. tigrinus* assignments, we observed that $q2 \geq 0.90$ was achieved for 72 individuals. Among the individuals with probabilities < 0.90, 19 were associated with high probabilities to cluster 3 (ranging from $q3 = 0.828$ to 0.992) and two with intermediate probabilities ($q3 = 0.347$ and 0.424). Remarkably, all of these individuals were from CNE (with the exception of the one sample [bLti88] from Paraná state, southeastern Brazil) and matched exactly the same animals which had introgressed mtDNA haplotypes from *L. colocolo*, as well as *PLP1* and *ZFY*/*SMCY3* haplotypes that were distinct from the *L. tigrinus* samples from the southern and southeastern Brazilian regions (SSE). Only one *L. tigrinus* individual with $q2 < 0.90$ (bLti81) was strongly assigned to the *L. colocolo* cluster ($q4 = 0.988$). Almost all other *L. tigrinus* individuals assigned to their phenotype-related cluster with probabilities < 0.90 were also connected to the *L. geoffroyi* cluster, and originated predominantly from southern Brazil. All the *L. colocolo* individuals were associated to cluster 4 with $q4 \geq 0.90$, excepted for one individual (Lco02; $q4 = 0.655$), which were also associated to the *L. tigrinus* cluster ($q2 = 0.328$) and showed evidence of hybridization based on the mtDNA data with this same species (see Trigo *et al*. [2008] for a discussion on this specific individual).

Considering a *q*-value of 0.9 as a plausible threshold to distinguish purebred individuals from hybrids, as has been proposed in several similar studies (*e.g.* Flamand *et al*. 2003, Lancaster *et al*. 2006, Vähä & Primmer 2006), we identified *ca.* 24.8% (49/197) of our total sample from *L. geoffroyi* and *L. tigrinus* as hybrids between these two species, in contrast to only two hybrids between *L. tigrinus* and *L. colocolo*. According to these data, although *L. tigrinus* and *L. geoffroyi* may be recognized as two genetically distinct groups, a high level of admixture between them was detected. In contrast, *L. colocolo* appears to be a genetically isolated population with very little evidence of admixture with the other two species. The triangle plot generated with STRUCTURE (Figure 5) illustrates this pattern, with the three species predominantly associated to one of the triangle points,

but depicting a genetic continuum between *L. tigrinus* and *L. geoffroyi* samples, as opposed to an essentially segregated position of *L. colocolo*.

Finally, given the microsatellite-based inference that the *L. tigrinus* samples from CNE are genetically distinct from the remaining individuals, we decided to test the magnitude of differentiation among the four populations defined by STRUCTURE, using Fst and Rst indices. All comparisons yielded higher values of Rst than Fst, suggesting that the divergence between these four groups cannot be explained solely by different distributions of allele frequencies, but also by shifts in mean allele length (Slatkin 1995). The highest values of differentiation for both indices were detected between *L. colocolo* and the CNE population of *L. tigrinus*, in stark contrast to the evidence of rampant hybridization between these two groups obtained with the mtDNA data. On the other hand, the lowest values for both indices were estimated between *L. geoffroyi* and SSE *L. tigrinus*, in agreement with the high level of admixture between these species detected with all molecular markers. Interestingly, these values were lower than those observed between the two subpopulations of *L. tigrinus* (CNE *vs.* SSE) (Table 3).

Multi-locus analyses

When the *L. geoffroyi* and *L. tigrinus* populations were compared, we could identify individuals of both species with several combinations of introgressed haplotypes. Some of these individuals had clearly intermediate assignment values based on microsatellite analysis, while others showed high (> 0.90) or low (<0.20) association to their phenotype-based population. Additionally, some individuals exhibiting intermediate genetic compositions based on the microsatellite data presented no evidence of introgression based on the other molecular markers. A very different pattern of introgression was observed between *L. tigrinus* and *L. colocolo*, when the different types of markers were compared. Although all *L. tigrinus* samples from northeastern Brazil (and almost all of those from central Brazil) had introgressed mtDNA haplotypes originating from *L. colocolo*, very little evidence of hybridization between these species was detected with the other markers. Table 4 summarizes the genetic combinations found in *L. tigrinus*, *L. geoffroyi* and *L. colocolo* individuals assessed simultaneously for all the molecular markers included in this study.

The estimates of migration rates among the three cats, expressed as the number of migrants per generation, revealed asymmetric patterns of gene flow among species and also among markers (Table 5). A first observation was that of lack of evidence for any

gene flow between *L. geoffroyi* and *L. colocolo* for all three types of markers, in agreement with our previous results (Trigo *et al*. 2008). With respect to the case of *L. tigrinus* and *L. geoffroyi*, gene flow between them was inferred with all markers, with a minimum point estimate of 0.16 migrants per generation (and all credibility intervals excluding zero at their lower bound). Higher values of introgression from *L. tigrinus* into *L. geoffroyi* than in the opposite direction were also inferred with all markers, mainly with the mtDNA. Asymmetry was also suggested among markers, with consistently higher migration rates inferred with the mtDNA data than those observed with the Y and X segments. Considering *L. tigrinus* and *L. colocolo*, migration rates indicated higher sequence introgression from *L. colocolo* into *L. tigrinus* individuals than in the opposite direction, represented basically by mtDNA introgression. As visualized in the network analysis, the only evidence of introgression from *L. tigrinus* into *L. colocolo* was that of mtDNA for Lco02, a captive animal whose history is uncertain. The inferred migration detected with the X chromosome segment from *L. colocolo* into *L. tigrinus* in this analysis is possibly related to the single sample of *L. tigrinus* which had an *L. colocolo* haplotype (bLti81). Although the point estimates for migration parameters are consistent with our other analyses, we note that some caution is warranted due to the very wide CIs obtained, especially in comparisons including *L. colocolo*.

Discussion

Patterns of hybridization and introgression between L. tigrinus and L. geoffroyi

The four types of molecular markers analyzed here supported the inference of hybridization and introgression between *L. tigrinus* and *L. geoffroyi*. The introgression of foreign alleles was detected in both species for all markers, supporting the interpretation that gene flow between them in their inferred hybrid zone is bidirectional. However, an evaluation of the number of individuals from both species that showed some evidence of hybridization/introgression indicates that the magnitude of introgression between these taxa may be asymmetric. For all of the investigated markers, the number of individuals with an *L. geoffroyi* phenotype showing some evidence of hybridization with *L. tigrinus* was higher than those presenting an *L. tigrinus* phenotype, indicating that rates of genomic introgression into the former population may be higher than in the opposite direction. Estimations of migration rates performed with LAMARC, mainly for the mtDNA, indicate exactly this same pattern. Asymmetric introgression seems to be a common pattern in

carnivore hybrid zones (*e.g.* Roy *et al*. 1994, Vilà & Wayne 1999, Randi & Lucchini 2002), and may be related to several aspects, such as differences in local density between the two hybridizing populations that may lead to the increased pressure of genomic introgression in one direction *versus* the other. Although very little is known about the relative densities of *L. tigrinus* and *L. geoffroyi* in the wild, preliminary field data on these species indicate that both are quite common in the vicinities of their area of geographic contact in southern Brazil (Eizirik *et al*. 2006, F. Mazim, pers. comm., C. B. Kasper, pers. comm.), so that evidence is still lacking for uneven abundances having played a role in this process. Differences in mating system and physiological characteristics of each species, including different estrus periods, parental care and socialization may also favor asymmetric pressures of introgression. In case of hybridization between canids, for example, these differences appear to be important for determining the predominant direction of introgression (*e.g.* Roy *et al*. 1994, Vilà & Wayne 1999). Since very little information is currently available about these aspects of the biology of *L. tigrinus* and *L. geoffroyi*, it is presently difficult to evaluate whether the asymmetric introgression observed has been influenced by such patterns. Different selection pressures against foreign alleles may also be acting in each species (*e.g.* Cianchi *et al*. 2003). In this case, it is possible that *L. tigrinus* represents a more specialized taxon with stronger co-adapted genetic complexes, which may be less permeable to foreign alleles than *L. geoffroyi*. If that was the case, selection might favor hybrids that mate within the latter species, possibly exhibiting a lower reduction in viability and fertility.

In addition to the asymmetric introgression pattern between these two cat species, the estimation of migration rates also revealed different patterns of introgression among markers. For both species, higher migration rates were inferred from mtDNA than from Y-linked and X-linked segments. The lower inferred introgression of Y-linked segments may be due to reduced hybrid male viability and/or fertility related to "Haldane's rule", a well-known hypothesis that predicts that the heterogametic sex (males in mammals) usually suffers more than the homogametic sex with reductions on fitness in cases of hybridization. This would lead to the hypothesis that introgression into both species occurs principally through back-crossing with females. Lower or absent gene exchange in markers exclusive of the heterogametic sex, as may be the case of *L. tigrinus* and *L. geoffroyi*, is commonly found in lepidopteran (*e.g.* Cianchi *et al*. 2003) and bird hybrid zones (*e.g.* Helbig *et al*. 2001, Helbig *et al*. 2005) and has also been observed in other mammal species (*e.g.* Walker *et al*. 1999, Vrana *et al*. 2000, Fickel *et al*. 2007).

Combining the information obtained from all the analyzed molecular markers, we can observe a high number of complex genetic combinations that could not have arisen from a simple pattern of hybridization. The hybrid zone between *L. tigrinus* and *L. geoffroyi* must involve the production of viable and fertile F1 hybrids (*i.e.* presenting at least partial viability and fertility), able to cross with each other and to produce F2 - F*n* hybrid generations, as well as to backcross with both parental species. The occurrence of backcrosses with both parental species is supported by the presence of high values of mean microsatellite-based assignment (≥ 0.9 in some cases) to their respective phenotypic population for individuals of both species that bear some evidence of hybridization in the mtDNA or sex-chromosome markers (see Table 4). This evidence may characterize this hybrid zone as a "hybrid swarm", where an extensive variety of recombinant types may be found (Allendorf *et al*. 2001). Additionally, the occurrence of a variety of marker combinations in both phenotype-based species, including intermediate assignments with the microsatellite data for individuals with introgressed haplotypes (see Table 4), indicates the existence of ongoing hybridization, or at least that it has been quite frequent in the recent past. The inclusion of sex-chromosome nuclear introns as molecular markers was extremely important to identify this complex pattern of hybridization, which was not possible with our previous analyses based only on microsatellites and mtDNA (Trigo *et al*. 2008). The combined analysis of these intronic segments with the mtDNA and microsatellite loci shed light into the history of hybridization and introgression between these cats, and may also be useful in similar studies of other carnivores.

Patterns of hybridization and introgression between L. tigrinus and L. colocolo

In contrast to the pattern observed for the hybridization process between *L. tigrinus* and *L. geoffroyi*, we identified much stronger evidence of hybridization and introgression between *L. tigrinus* and *L. colocolo* with the mtDNA sequences than with any other marker. The mtDNA-based analyses revealed a very high rate of basically unidirectional introgression from *L. colocolo* into *L. tigrinus* individuals, almost all of which were sampled in the central and northeastern regions of Brazil (CNE). On the other hand, the analyses of the X- and Y-chromosome markers for these same individuals indicated the presence of haplotypes that seem to be autochthonous of *L. tigrinus*, or at least very distinct from those sampled in *L. colocolo*. A potential caveat here is that *L. colocolo* has been shown to possess populations that are geographically structured (Johnson *et al*. 1999), and little is still known about Brazilian populations of this species. It is thus still possible

that these "autochthonous" *L. tigrinus* alleles in fact originate from a genetically distinct *L. colocolo* population that has so far not been sampled. Likewise, the microsatellite data also showed very little evidence of hybridization between these species, with the great majority of *L. colocolo* individuals presenting high (> 0.9) probabilities of genomic assignment to their respective phenotypic population, and misplaced *L. tigrinus* samples (according to their mtDNA) presenting values < 0.1 of association to the *L. colocolo* cluster. Therefore, in contrast to the mtDNA data, all nuclear markers indicate basically the presence of an *L. tigrinus* genome in these individuals.

Evidence of hybridization based on the other molecular markers was restricted to only two individuals. The first case was represented by one *L. tigrinus* sample from the central Brazilian region (bLti81) which was strongly associated to the *L. colocolo* population according to the microsatellite data, and also had mtDNA and *PLP1* haplotypes from this same species. As a female, it could not be assessed for the Y segments to confirm a possible hybrid origin with *L. tigrinus*, and this way, the only evidence of hybridization at this sample was related to phenotype *vs.* genetic assignment, implying in the possibility of an erroneous morphological identification (especially since the animal was melanistic). The second evidence of hybridization between these two species was represented by one sample of *L. colocolo* with unknown geographic origin (Lco02) which had partial and simultaneous association to *L. tigrinus* from SSE and *L. colocolo* clusters based on the microsatellite data. Interestingly, this specimen was the only example of mtDNA introgression in the opposite direction (mtDNA from *L. tigrinus* in a *L. colocolo* individual) and of hybridization with *L. tigrinus* from SSE. However as a captive animal, it is difficult to ascertain whether the implied hybridization event (involving a female *L. tigrinus* from SSE and a male *L. colocolo*) happened in the wild or was only a result of a captive mating. These two examples demonstrate the necessity to concentrated efforts in procuring a larger sample of *L. tigrinus* from the central Brazilian region, as well as *L. colocolo,* to better clarify these events.

Overall, our results suggest an asymmetric hybridization that seems to have produced a strong cytonuclear dissociation (*i.e.* the mitochondrial genome was derived from a different lineage relative to the nuclear genes) in the CNE *L. tigrinus* population. These different evolutionary histories for nuclear and mitochondrial genomes was also observed in other hybridization cases between mammals such as coyotes and domestic dogs in the southeastern USA (Adams *et al*. 2003) and forest and savanna African elephants (Roca *et al*. 2004). These patterns were suggested to have been generated by

ancient episodes of hybridization between the implicated species, and to have been lost in the nuclear genome due to several generations of backcrossing, leaving a signature only in the introgressed non recombining mtDNA. In the case of *L. tigrinus* and *L. colocolo*, the probable ancient episodes of hybridization must have involved strong pressures or opportunities favoring primary matings between *L. colocolo* females and *L. tigrinus* males. These matings must have been followed by backcrossings of female hybrids to male *L. tigrinus* for multiple generations, leading to a replacement of the nuclear genome in the population that retained the ancestral maternal mtDNA.

The detected pattern of hybridization between these two cats is even more interesting when we consider the evidence of a historical demographic expansion in the mtDNA haplotypes connected to the 'misplaced' *L. tigrinus* from the CNE regions. Examining Figure 3 and the geographic origin of the individuals (see Supplementary Material), we could verify that the apparent ancestral haplotype of this possible population expansion (H42) was present almost exclusively in individuals from the central region, while the derived ones were represented by central and northeastern individuals, indicating that the former region could have been the origin of the inferred observed expansion. In the case of the canid hybridization in the southeastern USA (Adams *et al*. 2003), the wide distribution of a dog-like haplotype in the coyote population was suggested to be an evidence of a sporadic and old event of hybridization that may have occurred in their ancestors that began to colonize that area. A similar scenario may be inferred in to this case involving *L. tigrinus* and *L. colocolo*, where an ancient event of hybridization between them may have occurred in an ancestral population that began to colonize parts of the central and northeastern regions of Brazil.

Past history, potential evolutionary significance and consequences of hybridization

We have previously suggested that *L. tigrinus* has had in its evolutionary history at least one episode of demographic expansion following a previous phase of smaller population size (Trigo *et al*. 2008). That process may have been the cause for the secondary contact between *L. tigrinus* and its two congeners, with which it had probably not developed complete intrinsic reproductive barriers during isolation and consequently was able to hybridize (Arnold 1992). Considering the age of the *L. tigrinus* mtDNA clade [75.7 Kya (28.7 – 157 Kya)] estimated in our previous work, this demographic expansion may have been initiated in the late Pleistocene, more precisely in the last interglacial period. Many palynologic studies in Brazil suggested that most rainforest environments

was restricted to refuges areas during the dry periods of the Quaternary, and suffered with a substancial growth when the clime suffers with a significative increase in moisture levels (Ledru *et al.* 1996, Behling *et al.* 1998, Behling & Negrelle 2001, Behling 2002). As *L. tigrinus* is considered to be associated to forested habitats (Nowell & Jackson 1996), the inferred demographic expansion of *L. tigrinus* may have been connected to expansion of such biomes during these cycles. However, when exactly the geographic contact between *L. tigrinus* and *L. geoffroyi* may have been established is still unknown and the definition of the age when the hybridization between these species began is still one of the major challenges ahead in the effort to characterize this hybrid zone.

In spite of the unknown age of begin, hybridization between *L. tigrinus* and *L. geoffroyi* seems to be currently ongoing or at least that it was frequent in near past, with high rates of introgression into both parental populations in areas near the contact zone in RS state. This pattern of hybridization may have multiple outcomes with respect of the evolution of the species involved, including (1) the complete admixture of the two species, and (2) the maintenance of a stable hybrid zone affecting only the local populations around the contact area (Allendorf *et al*. 2001). Our results show that, at least with the microsatellite data, the two cat species present an apparent genetic continuum at least in their contact area (see Figure 5) probably due to extensive rates of hybridization and introgression. Therefore, these events may have been reducing the level of genetic differentiation between the two species, principally by homogenizing the populations geographically close to the contact zone. Although the hybridization and introgression detected seems to be extensive, according to our results, it is strongly concentrated in RS state, where their contact zone occurs (Eizirik *et al*. 2006), with very little evidence of hybrid animals more distant from it. Considering that this region comprises only a small area relative to the broad distribution of each species, it is possible that this hybrid zone has been kept stable for many generations, possibly due to the existence of some kind of selection against hybrids (Barton & Hewitt 1985, Harrison 1993, Barton 2001).

In contrast, the hybridization between *L. tigrinus* and. *L. colocolo* has very different patterns and likely evolutionary outcomes. First, we found strong cytonuclear dissociation, which probably is associated to ancient events of hybridization that were imprinted only in the non-recombinant mtDNA. Second, the introgressed mtDNA haplotypes from *L. colocolo* into *L. tigrinus* was almost exclusively recorded in individuals from the CNE regions. Third, these same introgressed haplotypes exhibit a demographic expansion pattern from the central to northeastern regions. And fourth, these misplaced individuals

represent an apparent genetically differentiated subpopulation of *L. tigrinus* (based on microsatellite data set and on X and Y chromosome segments), that presents unique haplotypes for the individuals sampled in these regions. Considering all this information along with the demographic expansion of *L. tigrinus* previously detected, we can hypothesizes a similar initial scenario of hybridization with *L. colocolo* to that proposed with *L. geoffroyi*, but with different consequences. The central and northeastern Brazilin regions are characterized principally by two types of open formation: Caatinga in northeastern Brazil and Cerrado in the central area. In spite of the xeromorphic characteristic of these two kinds of vegetation, forest remnants persist within them. The origin of these remnants may be associated with humid phases of the Pleistocene which permitted the tropical forests to expand, extending their limits into what is now the xeric Caatinga and Cerrado (De Oliveira 1999, Carnaval 2002). It is possible that *L. tigrinus* individuals accompanied these forest expansions and initially achieved demographic contact with *L. colocolo* in the central Brazilian region. As in the expansion events, the males may be the first to disperse beyond the edge of their ranges (Adams *et al*. 2003), male *L. tigrinus* may have had difficulties to encounter potential mates in the new areas, favoring the inter-specific crossbreeding with female *L. colocolo* (which could have been more abundant in the area at the time). The female hybrid offspring may have then backcrossed with *L. tigrinus* males and the descendents of this cross may have expanded their range throughout the northeastern and central Brazilian regions.

In conclusion, we can suggest based on our molecular markers that two different patterns of hybridization may have been caused by events of demographic expansion of one of the species involved. Additional investigations are necessary to further test the proposed scenarios, especially for the *L. tigrinus* and *L. colocolo* hybridization, including a larger sample of this latter species and the inclusion of an even broader array of molecular markers. Nevertheless, our present level of understanding of this process is already sufficient to provide interesting insights into the history of hybridization and introgression among these three cats, as well as its possible connection to past and present biogeographic changes in the Neotropical region.

Figure Legends

Figure 1. A) Geographic distribution of *Leopardus tigrinus* (grey-shaded area), *Leopardus geoffroyi* (area defined by the grey broken line) and *L. colocolo* (area defined by the black

dotted line) in South America (modified from Oliveira 1994, Eisenberg & Redford 1999, Nowell & Jackson 1996). The black star indicates the area where hybridization events between *L. tigrinus* and *L. geoffroyi* were detected, and the grey star indicates the hybridization events between *L. tigrinus* and *L. colocolo*. B) Map showing the distribution of the *Leopardus tigrinus*, *L. geoffroyi* and *L. colocolo* samples utilized in this study with the indication of the four geographic Brazilian regions sampled in this study. The abbreviations indicate the Brazilian states and other South American countries from which samples were included in the study. South American countries: Uruguay (URU), Argentina (ARG), Bolivia (BOL) and Chile (CH). Political Brazilian Regions and their respective states: South region - Rio Grande do Sul (RS), Santa Catarina (SC) and Paraná (PR); Southeast region - São Paulo (SP), Rio de Janeiro (RJ), Espírito Santo (ES) and Minas Gerais (MG); Central region - Mato Grosso do Sul (MS) and Goiás (GO); Northeast region - Sergipe (SE), Pernambuco (PE), Ceará (CE), Piauí (PI) and Maranhão (MA).

Figure 2. Haplotype network based on (A) mitochondrial DNA; (B) an intron of *PLP1* gene, located on the X chromosome; and (C) concatenated Y chromosome sequences from the introns of *ZFY* and *SMCY3* genes. Each unique haplotype is represented by a circle proportional in size to its frequency; colors indicate the frequency of each haplotype in each species (black = *L. geoffroyi*, light grey = *L. tigrinus*, dark grey = *L. colocolo*, white circles = outgroups). Bars placed on connecting branches indicate the number of substitutions between haplotypes.

Figure 3. Mismatch distribution analysis performed with the *Leopardus colocolo* mtDNA haplogroup: A) including all samples belonging to this clade (n = 30); B) including only *L. colocolo* individuals (n = 7); and C) including all *L. tigrinus* individuals bearing *L. colocolo* haplotypes along with a single *L. colocolo* individual (Lco13) which shared one of those haplotypes (n = 23). The main peak in the first and third graphs represents 1 bp difference between sequences.

Figure 4. Bar plotting of the results obtained from STRUCTURE using K = 4. Each individual is represented by a vertical line. Colors indicate the proportion of membership of each individual into the 4 clusters: black = cluster 1 (*L. geoffroyi*), light grey = cluster 2 (*L. tigrinus* from the Southern and Southeastern Brazilian regions), white = cluster 3 (*L.*

tigrinus from the Central and Northeastern Brazilian regions) and dark grey = cluster 4 (*L. colocolo*).

Figure 5. Triangle plot of the results obtained from STRUCTURE showing the genetic differentiation between *Leopardus tigrinus* (light grey circles), *L. geoffroyi* (black circles) and *L. colocolo* (dark grey circles).

Table 1 – Nucleotide diversity, gene diversity and mean number of pairwise differences observed in the *Leopardus tigrinus*, *L. geoffroyi* and *L. colocolo* mitochondrial DNA (mtDNA), X-linked (*PLP1*) intron and Y-linked (*ZFY/SMCY3*) introns data sets.

Species	N*	N° Hap	Polymorphic Sites	Gene diversity ± SE	Nucleotide diversity ± SE	Mean Number of pairwise differences ± SE
mtDNA						
Leopardus tigrinus	98	37	35 (28TS, 5TV, 2I)	0.930 ± 0.016	0.004 ± 0.002	3.180 ± 1.659
Leopardus geoffroyi	70	29	40 (35TS, 5TV)	0.890 ± 0.022	0.006 ± 0.003	4.814 ± 2.379
Leopardus colocolo	30	19	33 (32TS, 1TV)	0.949 ± 0.023	0.006 ± 0.004	3.816 ± 1.975
X-linked intron						
Leopardus tigrinus	90	3	3 (2TS, 1TV)	0.5790 ± 0.0335	0.0012 ± 0.0009	0.9333 ± 0.6485
Leopardus geoffroyi	67	2	1 (1TS)	0.2135 ± 0.0605	0.0002 ± 0.0004	0.2135 ± 0.0261
Leopardus colocolo	6	3	2 (1TS, 1TV)	0.7333 ± 0.1552	0.0011 ± 0.0010	0.8667 ± 0.7008
Y-linked introns						
Leopardus tigrinus	61	3	2 (2TS)	0.2634 ± 0.0689	0.0006 ± 0.0005	0.4623 ± 0.4116
Leopardus geoffroyi	28	1	0	0	0	0
Leopardus colocolo	5	2	1 (1TV)	0.6000 ± 0.1753	0.0007 ± 0.0008	0.6000 ± 0.5622

N* Number of individuals indicated for each clade defined by mtDNA and number of chromosomes for each clade indicated for nuclear introns.

TS, transitions; TV, transversions; I, indels.

Table 2 – Genetic variation in *Leopardus tigrinus*, *L. geoffroyi* and *L. colocolo* samples at eleven microsatellite loci utilized in this study. Deviations from Hardy-Weinberg equilibrium were indicated by an asterisk ($p < 0.05$ after Bonferroni correction for multiple comparisons).

	Leopardus tigrinus			*Leopardus geoffroyi*			*Leopardus colocolo*		
	A	Ho	He	A	Ho	He	A	Ho	He
FCA391	8	0.824	0.790	9	0.718	0.772	6	0.444	0.843*
FCA424	6	0.563	0.661*	7	0.269	0.368	6	0.900	0.795
FCA441	5	0.554	0.675	5	0.577	0.717	5	0.600	0.768
FCA453	7	0.723	0.675	6	0.731	0.718	3	0.600	0.574
FCA723	30	0.781	0.905*	29	0.586	0.956*	6	0.300	0.695
FCA742	9	0.575	0.731*	10	0.828	0.856	7	0.700	0.784
F42	12	0.754	0.866	11	0.808	0.883	7	0.556	0.830
F53	23	0.788	0.923*	19	0.696	0.889*	9	1.00	0.858
F98	7	0.286	0.532*	5	0.513	0.529	7	0.800	0.779
F124	13	0.714	0.761	9	0.636	0.788	11	0.800	0.937
F146	8	0.466	0.588	7	0.705	0.661	6	0.700	0.837
Mean	11.64	0.639	0.737	10.64	0.642	0.740	6.64	0.672	0.791

Abbreviations: A = average number of alleles, Ho = observed heterozygosity and He = expected heterozygosity.

Table 3 – Levels of genetic differentiation among the four clusters defined by STRUCTURE analysis based on Fst and Rst indices.

	L. geoffroyi	SSE *L. tigrinus*	CNE *L. tigrinus*	*L. colocolo*
L. geoffroyi	---	0.105*	0.413*	0.535*
SSE *L. tigrinus*	0.069*	---	0.328*	0.680*
CNE *L. tigrinus*	0.099*	0.176*	---	0.837*
L. colocolo	0.143*	0.119*	0.241*	---

Fst below, Rst above; * $p < 0.001$

Note: SSE – southern and southeastern Brazilian regions; CNE – central and northeastern Brazilian regions.

Table 4 – Summaries of the phenotype-genetic combination found for the four clusters identified with the STRUCTURE analysis. The columns of microsatellite data indicate the mean association (mean *q*) between each class of phenotype-molecular sequence combination and each microsatellite-based cluster. Only individuals with information available for all four types of molecular markers are included.

Phenotype	mtDNA	X-linked intron	Y-linked introns	N	Microsatellites – STRUCTURE clusters *L. geoffroyi* Mean	*L. tigrinus* SSE Mean	*L. tigrinus* CNE Mean	*L. colocolo* Mean
L. geo	*L. geo*	*L. geo*	*L. geo*	16	0.93	0.03	0.03	0.01
L. geo	*L. geo*	*L. geo*	F	16	0.88	0.08	0.02	0.02
L. geo	***L. geo***	***L. tig***	***L. geo***	3	0.93	0.07	0.00	0.00
L. geo	***L. geo***	***L. tig***	***L. tig***	3	0.89	0.07	0.03	0.01
L. geo	***L. geo***	***L. tig***	**F**	1	0.80	0.01	0.19	0.00
L. geo	***L. tig***	***L. geo***	***L. geo***	2	0.83	0.16	0.01	0.00
L. geo	***L. tig***	***L. geo***	***L. tig***	2	0.76	0.23	0.01	0.00
L. geo	***L. tig***	***L. geo***	**F**	2	0.22	0.75	0.02	0.01
L. geo	***L. tig***	***L. tig***	***L. geo***	1	0.89	0.10	0.00	0.01
L. geo	***L. tig***	***L. tig***	***L. tig***	3	0.85	0.10	0.05	0.00
L. tig	*L. tig*	*L. tig*	*L. tig*	32	0.02	0.96	0.01	0.01
L. tig	*L. tig*	*L. tig*	F	12	0.19	0.77	0.01	0.03
L. tig	***L. tig***	***L. geo***	***L. geo***	1	0.02	0.97	0.01	0.00
L. tig	***L. geo***	***L. tig***	***L. tig***	1	0.96	0.02	0.02	0.00
L. tig	***L. geo***	***L. tig***	**F**	3	0.64	0.34	0.02	0.00
L. tig	***L. geo***	***L. geo/L. tig***	**F**	1	0.95	0.05	0.00	0.00
L. tig	***L. col***	***L. tig***	***L. tig***	2	0.18	0.01	0.80	0.01
L. tig	***L. col***	***L. tig***	**F**	5	0.03	0.01	0.95	0.00
L. tig	***L. col***	***L. col***	**F**	1	0.01	0.00	0.00	0.99
L. col	*L. col*	*L. col*	*L. col*	1	0.01	0.01	0.00	0.98
L. col	*L. col*	*L. col*	F	2	0.00	0.01	0.00	0.99

* Note: *L. geo* = *Leopardus geoffroyi*, *L. tig* = *L. tigrinus*, *L. col* = *L. colocolo*, F = female, SSE (southern and southeastern Brazilian regions), CNE (central and northeastern Brazilian regions), N = number of individuals at each class.
** In bold: Classes with evidence of hybridization/introgression according phenotype and molecular sequences.

Table 5 – Demographic parameters inferred for the three hybridizing species (*Leopardus tigrinus*, *L. geoffroyi* and *L. colocolo*) using the coalescent-based approach implemented in the software package LAMARC. Migration rates are expressed as number of migrants per generation (Nm) and indicate here the introgression rate from one species into another. Ninety-five per cent credibility intervals are show in parentheses.

	mtDNA	X-linked segment	Y-linked segments
θ *L. tigrinus*	0.04396 (0.02491 – 0.07689)	0.00029 (0.00007 – 0.00073)	0.00035 (0.00009 – 0.00147)
θ *L. geoffroyi*	0.03387 (0.01711 – 0.05649)	0.00027 (0.00003 – 0.00071)	0.00019 (0.00002 – 0.00090)
θ *L. colocolo*	0.06681 (0.02234 – 0.13657)	0.00030 (0.00004 – 0.00223)	0.00054 (0.00003 – 0.00380)
Nm (*L. tigrinus* into *L. geoffroyi*)	3.656 (0.588 – 12.627)	0.255 (0.008 – 0.732)	0.238 (0.005 – 0.343)
Nm (*L. geoffroyi* into *L. tigrinus*)	1.948 (0.226 – 10.465)	0.230 (0.001 – 0.717)	0.166 (0.001 – 0.910)
Nm (*L. tigrinus* into *L. colocolo*)	0.736 (0.018 – 4.005)	0.047 (0.000 – 0.602)	0.044 (0.000 – 1.033)
Nm (*L. colocolo* into *L. tigrinus*)	2.611 (0.114 – 13.383)	0.119 (0.000 – 2.101)	0.048 (0.000 – 2.275)
Nm (*L. colocolo* into *L. geoffroyi*)	0.005 (0.000 – 2.158)	0.000 (0.000 – 0.993)	0.066 (0.000 – 2.507)
Nm (*L. geoffroyi* into *L. colocolo*)	0.001 (0.000 – 0.744)	0.000 (0.000 – 0.327)	0.043 (0.000 – 0.816)

Supplementary Table 1 - Samples analyzed in the present study.

Samples	**Location (geographic origin)**	**Institution/contact**
Leopardus tigrinus		
bLti01[1,2,4], bLti04[1,2], bLti05[1,2,3,4], bLti09[1,2,4], bLti10[1,2,4], bLti46[1,2,3,4], bLti47[1,2,4], bLti48[1,2], bLti49[1,2,4], bLti51[1,2,3,4], bLti68[1,3,4], bLti69[1,2,3,4], bLti79[1,2,4], bLti80[1,2,3,4], bLti94[1,2,3,4], bLti95[1,2], bLti98[1,2,4], bLti99[1,2], bLti100[1,2,3], bLti102[1,2,4], bLti106[1,2,3,4], bLti108[1,2,4], bLti110[1,2,3], bLti113[1,2,3,4], bLti117[1,2,4], bLti119[1,2,3], bLti120[1,2,3,4], bLti121[1,2,3,4], bLti122[1,2,3,4], bLti124[1,2,3,4], bLti131[1,2,3,4], bLti132[1,2,3,4], bLti133[1,2,3], bLti134[1,2], bLti135[1,2,4], bLti136[1,2,3,4], bLti137[1,2,3,4], bLti138[1,2], bLti139[1], bLti140[1,2,3,4], bLti141[1,2], bLti142[1,2,3], bLti143[1,2], bLti146[1,2], bLti149[1,2]	Rio Grande do Sul/South Region/Brazil	Eizirik, E.; Sapucaia do Sul Zoo; Giacomini, C.; Luchesi, L.; Hohendorff, R.; Cachoeira do Sul Zôo; Salomão, E.; Particular Zoo Maison Forestier; Ott, P.; Indrusiak, C.; Mähler, J.K.; Breier, T.; Brutto, L.F.; Freitas, T.R.O.; Silva J.; Bitencourt, F.; Cechin, S.; Toscan, K.H.; Giasson, L.; Vielmo, P.R.; Andrade, V.; Silva, J.; Particular Zoo Morro Reuter; Correa, M. F.; Kasper, C.B.; Rovedder, C.; Repenning, M.; Silveira, T.; Pedó, E.; Pinto, L.; Marinho, J.; Schmidt, R.; Coelho, I.P.; Passo Fundo Zoo; Green, D.; Rollet, I.; Rosa, J.A.; Behr, E.; Martins, M.; Vinciprova, G.; SMAM Novo Hamburgo; Senra, A.
bLti44[1,2,3,4], bLti114[1,2,3,4], bLti123[1,2,4], bLti125[1,2,3,4], bLti126[1,2,3], bLti127[1,2,3,4], bLti128[1,2], bLti160[1,2,3], bLtiSC[1,2,3]	Santa Catarina/South Region/Brazil	Curitiba Zoo; Santa Catarina Federal University; Castilho, C.; Marins de Sá, L.G.; Boursheid S.A. Engen. e M. Ambiente
bLti31 (Lti39)[1,2], bLti35 (Lti43)[1,2], bLti43[1,2], bLti89[1,2,3,4], bLti90[1,2,3,4], bLti91[1,2], bLti92[1,2,4], bLti93[1,2,3,4], bLti88[1,2,4]	Paraná/South Region/Brazil	CASIB/USA; Cascavel Zoo; Curitiba Zoo; Maringá Zoo
bLti54[1,2], bLti55[1,2], bLti56[1,2,3,4], bLti57[1,4], bLti58[1,2], bLti59[1,2,4], bLti60[1,2,3,4], bLti61[1,2,3,4], bLti62[1,2,3,4], bLti64[1], bLti65[1,2,4], bLti66[1,2], bLti70[1,2], bLti71[1,2,3,4], bLti73[1,2], bLti74[1,2,4], bLti75[1,2,4], bLti76[1,2,3,4], bLti77[1,2], bLti78[1,2], bLti84[1,2], bLti86[1,2], bLti87[1,2,3,4], bLti103[1], bLti104[1], bLti301[1], bLtiSP[1,2]	São Paulo/Southeast Region/Brazil	Mogi Guaçú Zoo; Limeira Zoo; Piracicaba Zoo; São Bernardo do Campo Zoo; Jundiaí Zoo; São José do Rio Pardo Zoo; Sorocaba Zoo; São José do Rio Preto Zoo; Bauru Zoo; Pedreira Zoo; Pedreira Zoo; Leme Zoo; Associação Mata Ciliar/São Paulo; Morato, R.; Oliveira, T.
bLti53[1,2]	Rio de Janeiro/Southeast Region/Brazil	Rio de Janeiro Zoo
bLti97[1,2,3,4]	Espírito Santo/Southeast Region/Brazil	Sana, D.

Supplementary Table 1 (Cont.)

Samples	**Location (geographic origin)**	**Institution/contact**
bLti109[1 2 3 4]	Minas Gerais/Southeast Region/Brazil	Rodrigues, F.
bLti24 (Lti32)[1 2 4], bLti28 (Lti36)[1 2], bLti85 (Lti31)[1 2 3 4], bLti96[1 2 3 4]	Goiás/Central Region/Brazil	Goiânia Zoo; Brasília Zoo; Rodrigues, F.; NCI-USA donation
bLti72[1 2 3 4], bLti81[1 2 4]	Mato Grosso do Sul/Central Region/Brazil	Catanduva Zoo; Sana, D.
bLti107[1 2 4], bLti118[1 2 4], bLti129[1 2 4], bLti130[1 2 3 4], bLti152[1 2 3], bLti156[1 2]	Piauí/Northeast Region/Brazil	Particular Zoo Morro Reuter; Oliveira, T.G.
bLti147[1 2 3], bLti148[1 2 3], bLti154[1 2], bLti155[1 2 3], bLti157[1 2 3], bLti7MA[1 2]	Maranhão/ Northeast Region/Brazil	Tchaicka, L.; Oliveira, T.G.
bLti150[1 2 3], bLti151[1 2 3], bLti153[1 2]	Ceará/ Northeast Region/Brazil	Oliveira, T.G.
bLtiSE1[1 2]	Sergipe/ Northeast Region/Brazil	Aracaju Zoo; Magina, G.C.T.
bLtiPE2[1 2]	Pernambuco/ Northeast Region/Brazil	Recife Zoo
bLti29 (Lti37)[1 2], bLti30 (Lti38)[1 2]	Iguazu, Curuguaty/Paraguai,	Refugio Itaipu Paraguaio (NCI-USA donation)
bLti105(Lti17)[1 2]	Unknown origin	NCI-USA donation

Supplementary Table 1 (Cont.)

Samples	Location (geographic origin)	Institution/contact
Leopardus geoffroyi		
bLge01[1,2,3,4], bLge02[1,2,3,4], bLge03[1,2], bLge04[1,2,3,4], bLge05[1,2,3,4], bLge06[1,2], bLge07[1,2,3], bLge08[1,2,3,4], bLge10[1,2,4], bLge11[1,2,3,4], bLge12[1,2,3,4], bLge13[1,2,3,4], bLge28[1,2,3,4], bLge29[1,2,3,4], bLge31[1,2,3,4], bLge32[1,2,3,4], bLge33[1,2,3,4], bLge35[1,2,3,4], bLge36[1,2,4], bLge37[1,2,4], bLge38[1,2,4], bLge39[1,2], bLge41[1,2,3,4], bLge42[1,2,3,4], bLge43[1,2,3,4], bLge44[1,2,4], bLge46[1,2,3,4], bLge47[1,2,3,4], bLge49[1,2,3,4], bLge59 (Oge49)[1,2], bLge60 (Oge50)[1,2], bLge70[1,2,4], bLge71[1,2,4], bLge72[1,2,3], bLge73[1,2,3], bLge74[1,2,4], bLge75[1,2,4], bLge76[1,2,3,4], bLge77[1,2,3,4], bLge78[1,2,4], bLge79[1,2,3,4], bLge80[1,2,3,4], bLge89[1,2,4], bLge90[1,2,3,4], bLge91[1,2,3,4], bLge92[1,2,3,4], bLge93[1,2,4], bLge94[1,2,4], bLge95[1,2], bLge96[1,2], bLge97[1,2,4], bLge(Nid11)[1,2]	Rio Grande do Sul /South Region/Brazil	Sapucaia do Sul Zoo; Cachoeira do Sul Zoo; Sana, D.; Salomão, E.; Ott, P.; Veronese, L.; Triervaeiler,F.; Indrusiak,C.; Trigo,T.C.; Sapucaia do Sul Zoo; Pontes, G.; Martins, M.; Trigo,T.; Trigo,C.; Andrade,M.; Scherer,A.; Cabral, L.; Zachia, R.; Behr, E.R.; Giasson, L.O.M.; Michalski, F.; Freitas, T.R.O.; Stoltz, J.; Quinta da Estância Grande; Cachoeira do Sul Zoo; Mazim, F.D.; Soares, J.B.G.; Tobaco, M.A.; Marinho, J.; Fundação Zoobotânica Rio Grande do Sul; Jardim, M.M.A.; NCI-USA donation.
bLge09[1,2,4], bLge50 (Oge17)[1,2], bLge51 (Oge21)[1,2], bLge52 (Oge26)[1,2,4], bLge53 (Oge29)[1,2]	Argentina	Eizirik. E.; La Plata Zoological Park; Cordoba Zoological Park; NCI-USA donation
bLge20[1,2,3,4], bLge54 (Oge32)[1,2], bLge55 (Oge37)[1,2,4], bLge56 (Oge38)[1,2,4], bLge57 (Oge39)[1,2,4]	Uruguay	Museo de Cien. Nat., Montev./ D'Elia, G.; Mercedes Zoological Park; Cerro Pan de Azucar Reprodution Center, NCI-USA donation
bLge58 (Oge48)[1,2]	Brazil	Itaipu/Brazil; NCI-USA donation
bLge62 (Oge59)[1,2,4], bLge63 (Oge60)[1,2], bLge64 (Oge61)[1,2,4], bLge65 (Oge62)[1,2,4], bLge66 (Oge63)[1,2,4], bLge67 (Oge64)[1,2,4], bLge68 (Oge65)[1,2,4], bLge81[1,2], bLge82[1,2], bLge83[1,2,3], bLge84[1,2], bLge85[1,2,3,4], bLge86[1,2,3,4], bLge87[1,2,3,4], bLge88[1,2,3]	Bolivia	Santa Cruz Zoo; NCI-USA donation

Supplementary Table 1 (Cont.)

Samples	Location (geographic origin)	Institution/contact
Leopardus colocolo		
bLco04 (Lco06)[1 2 3], Lco26[1 2 3]	Chile	NCI-USA donation, SAG
bLco05 (Lco07)[1 2 3 4]	Argentina	NCI-USA donation
Lco09[1 2 4]	Uruguay	NCI-USA donation; Mercedes Zoological Park
bLco16[1 2], bLco17[1 2 3]	Rio Grande do Sul/South Region/Brazil	Bencke, G.; Marinho, J.; Mähler, J.
Lco02[1 2]	?	NCI-USA donation
Lco13[1 2 4]	Goiás/Central Region/Brazil	São Paulo Zoo (NCI-USA donation)
Lco23[1 2 3]	La Paz Dept., Bolivia	La Paz Zoo (NCI-USA donation)
Lco30[1]	?	NCI-USA donation
Leopardus guigna		
bLgu01(Ogu02)[2 4], bLgu02(Ogu03)[2 4]	Chile	NCI-USA donation
Leopardus wiedii		
bLwi32[2 3 4]	São Paulo/Southeast Region/Brazil	Tapiraí Zoo
Leopardus pardalis		
bLpa72[2 4]	São Paulo/Southeast Region/Brazil	São Bernardo do Campo Zoo

Note: samples typed for each kind of molecular markers: 1) microsatellites; 2) mtDNA; 3) *ZFY/SMCY3* Y-linked chromosome introns and 4) *PLP1* X-linked chromosome intron.

Supplementary Table 2 – List of individuals that bear each mitochondrial DNA haplotype. The geographic distributions of haplotypes are also indicated. The misplaced haplotypes and individuals are indicated in bold.

Haplotypes	Individuals	Geographic Origin
***Leopardus geoffroyi* Clade**		
H19, H20, H21, H22, H23, H24, H29, H30, H31, H32, H33, H34, H35	bLge62, bLge65, bLge63, bLge64, bLge66, bLge67, bLge68, bLge81, bLge82, bLge83, bLge87, bLge84, bLge85, bLge86, bLge88	BOL
H7, H13, H14, H15	bLge09, bLge50, bLge53, bLge51, bLge52	ARG
H16	bLge54	URU
H8, H9, H12, H17, H18, H26, H36,	bLge10, bLge12, bLge37, bLge39, bLge41, bLge43, bLge44, bLge47, bLge70, bLge73, bLge89, bLge91, bLge94, bLge95, bLge96, bLge97, bLge36, bLge58, bLge59, bLge77, bLge92	RS
H3	bLge03, bLge04, bLge05, bLge20, bLge28, bLge29, bLge31, bLge32, bLge33, bLge38, bLge42, bLge56, bLge75, **bLti121**	RS/URU
H11	bLge35, bLge49, bLge55, bLge57, bLge71, bLge76, bLge78, bLge90, **bLti01, bLti09, bLti49, bLti79**	RS/URU
H47	**bLti65**	SP
H51	**bLti77**	SP
***Leopardus tigrinus* Clade**		
H46, H49, H50, H52, H54, H55	bLti54, bLti70, bLti74, bLti78, bLti84, bLti86	SP
H56, H57, H58,	bLti90, bLti91, bLti92	PR
H66, H68, H69, H70	bLti114, bLti127, bLti123, bLti125, bLti128	SC
H60	bLti96	GO
H61	bLti97	ES
H40, H41, H48, H62, H63, H73, H74	bLti04, bLti10, bLti69, bLti99, bLti100, bLti122, bLti132, bLti141	RS
H64	bLti105	?
H43	bLti31, bLti58, bLti62, bLti66, bLti71, bLti72, bLti75	PR/SP/MS
H44	bLti47, bLti61, bLti110, bLti124, bLti131, bLti134, bLti136, bLti142, bLti143, bLtiSC	RS/SP/SC
H45	bLti53, bLti56, bLti73, bLti109	RJ/SP/MG
H59	bLti93, bLti95, bLti160	PR/RS/SC
H1	**bLge01**, bLti48, bLti117, bLti135, bLti149	RS
H2	**bLge02, bLge46, bLge60**, bLti51, bLti94	RS

Supplementary Table 2 (Cont.)

Haplotypes	Individuals	Geographic Origin
H4	**bLge06**	RS
H5	**bLge07**, bLti98, bLti106, bLti119	RS
H6	**bLge08, bLge11, bLge74**, **bLge(Nid11),** bLti29, bLti35, bLti55, bLti59, bLti60, bLti76, bLti80, bLti87, bLti102, bLti108, bLti113, bLti120, bLti137, bLti138, bLti140, bLti146, **Lco02**	RS/PAR/PR/SP
H10	**bLge13**	RS
H25	**bLge72**	RS
H27	**bLge79**, bLti43, bLti44	RS/PR/SC
H28	**bLge80**, bLti30, bLti46, bLti89, bLti126, bLti133	RS/PAR/SC/PR
H37	**bLge93**, bLti05	RS
***Leopardus colocolo* Clade**		
H71, H72, H78	**bLti129, bLti130, bLti152**	PI
H75, H76, H79	**bLti147, bLti148, bLti155**	MA
H84, H85	bLco16, bLco17	RS
H81, H87	bLco04, Lco26	Chile
H80	**bLtiSE**	SE
H82	bLco05	ARG
H83	Lco09	URU
H86	Lco23	BOL
H77	**bLti151**	CE
H42	**bLti24, bLti28, bLti85, bLti88**	GO/PR
H53	**bLti81, bLti150**, Lco13	MS/CE/GO
H65	**bLti107, bLti153, bLti154**	PI/CE/MA
H67	**bLti118, bLti156, bLti157, bLtiPE, bLtiMA**	PI/MA/PE
Leopardus pardalis		
H88		
Leopardus wiedii		
H89		

Supplementary Table 3 – *PLP1* and *ZFY*/*SMCY3* introns haplotypes identified from the six Neotropical cats analyzed [*Leopardus tigrinus* (bLti), *L. geoffroyi* (bLge), *L. colocolo* (bLco/Lco), *L. guigna*, *L. wiedii* and *L. pardalis*). Only variable sites are shown. Site numbers (vertical notation) refer to the aligned position in our 808 and 824 pb data set from *PLP1* and *ZFY*/*SMCY3*, respectivelly. For each haplotype is described the total number of samples where it was identified and the list of individuals with geographic origin information for the misplaced ones in parenthesis. The heterozygotes individuals were underlined. The misplaced individuals are indicated in bold.

Haplotypes identification	Variable sites	Chromosomes
***PLP1* X-linked intron**		
	112334444567 7260683567463 89693317045848	
Hp1	TGGATGGATTGGGC	41 *L. tigrinus*, 9 *L. geoffroyi* (Rio Grande do Sul, Brazil)/ bLti01, bLti01II, <u>bLti09</u>, bLti10, bLti10II, bLti46, bLti49, bLti49II, bLti51, <u>bLti57</u>, <u>bLti59</u>, bLti69, bLti71, bLti72, <u>bLti74</u>, bLti76, bLti80, bLti90, bLti92, bLti92II, bLti94, bLti98, bLti98II, bLti102, bLti102II, bLti106, bLti108, bLti113, <u>bLti117</u>, bLti120, bLti122, bLti123, bLti123II, bLti124, bLti125, bLti127, bLti131, bLti132, bLti136, bLti137, bLti140, **bLge02, bLge04, bLge05, bLge08, bLge13, bLge32, bLge42, bLge49, bLge79**
Hp2	C.............	26 *L. tigrinus*, 3 *L. geoffroyi* (Rio Grande do Sul, Brazil)/ <u>bLti09II</u>, bLti44, bLti47, bLti47II, bLti56, <u>bLti57II</u>, <u>bLti59II</u>, bLti60, bLti61, bLti62, bLti65, <u>bLti74II</u>, bLti75, bLti75II, <u>bLti79</u>, bLti87, bLti89, bLti93, bLti96, bLti97, bLti109, bLti114, <u>bLti117II</u>, bLti121, bLti135, bLti135II, **bLge10, bLge10II, bLge12**
Hp3	C..G..........	56 *L. geoffroyi*, 3 *L. tigrinus* (Rio Grande do Sul, Brazil)/ bLge01, bLge09, bLge09II, bLge11, bLge20, bLge29, bLge31, bLge33, bLge35, bLge36, bLge36II, bLge37, bLge37II, bLge38, bLge38II, bLge41, bLge43, bLge44, bLge44II, bLge47, bLge52, bLge55, bLge55II, bLge56, bLge57, bLge57II, <u>bLge62</u>, bLge64, bLge65, bLge65II, bLge66, bLge70, bLge70II, bLge71, bLge71II, bLge74, bLge74II, bLge75, bLge75II, bLge76, bLge77, bLge78, bLge78II, bLge85, bLge86, bLge87, bLge89, bLge90, bLge91, bLge92, bLge93, bLge93II, bLge94, bLge94II, bLge97, bLge97II, **bLti05, bLti68, <u>bLti79</u>**
Hp4	C..GC.........	8 *L. geoffroyi*/ bLge46, bLge28, bLge67, bLge67II, bLge68, bLge68II, bLge80, <u>bLge62II</u>
Hp5	C..G........A.	*Leopardus guigna*
Hp6	C..G......T...	11 *L. tigrinus* (Central and Northeastern Brazilian Regions)/ bLti24, bLti24II, bLti85, bLti88, bLti107, bLti107II, bLti118, bLti118II, bLti129, bLti129II, bLti130

Supplementary Table 3 (Cont.)

Haplotypes identification	Variable sites	Chromosomes
Hp7	C..G.AAC.C.T..	*L. wiedii* and *L. pardalis*
Hp8	CAAG...C.....T	**bLti81, bLti81II** (Mato Grosso do Sul, Brazil), Lco13
Hp9	C.AG...C.....T	Lco07
Hp10	CAAG...CG....T	Lco09, Lco09II
***ZFY/SMCY3* Y-linked introns**		
	112234567 4377988270 8439608022	
Hy1	TCGCCATAAC	26 *L. geoffroyi*, 2 *L. tigrinus* (Rio Grande do Sul, Brazil)/ bLge01, bLge05, bLge07, bLge08, bLge11, bLge20, bLge28, bLge29, bLge31, bLge32, bLge33, bLge35, bLge41, bLge43, bLge47, bLge49, bLge73, bLge76, bLge77, bLge83, bLge85, bLge86, bLge87, bLge88, bLge91, bLge92, **bLti05**, **bLti119**
Hy2	C.......G.	42 *L. tigrinus*, 10 *L. geoffroyi* (Rio Grande do Sul, Brazil)/ bLti44, bLti46, bLti51, bLti56, bLti60, bLti61, bLti62, bLti68, bLti69, bLti71, bLti72, bLti76, bLti80, bLti87, bLti89, bLti90, bLti93, bLti94, bLti96, bLti97, bLti100, bLti106, bLti109, bLti110, bLti113, bLti114, bLti120, bLti121, bLti122, bLti124, bLti125, bLti126, bLti127, bLti131, bLti132, bLti133, bLti136, bLti137, bLti140, bLti142, bLti160, bLtiSC, **bLge02**, **bLge04**, **bLge12**, **bLge13**, **bLge42**, **bLge46**, **bLge72**, **bLge79**, **bLge80**, **bLge90**
Hy3	........G.	2 *L. tigrinus*: Central and Northeastern Brazilian Regions/ bLti85, bLti130
Hy4	......C.G.	7 *L. tigrinus*: Central and Northeastern Brazilian Regions/ bLti147, bLti148, bLti150, bLti151, bLti152, bLti155, bLti157
Hy5	.T..AG.T.G	3 *L. colocolo*/ bLco06, bLco07, Lco17
Hy6	.T..AG...G	2 *L. colocolo*/ Lco23, Lco26
Hy7	.TAAA.????	*L. wiedii*

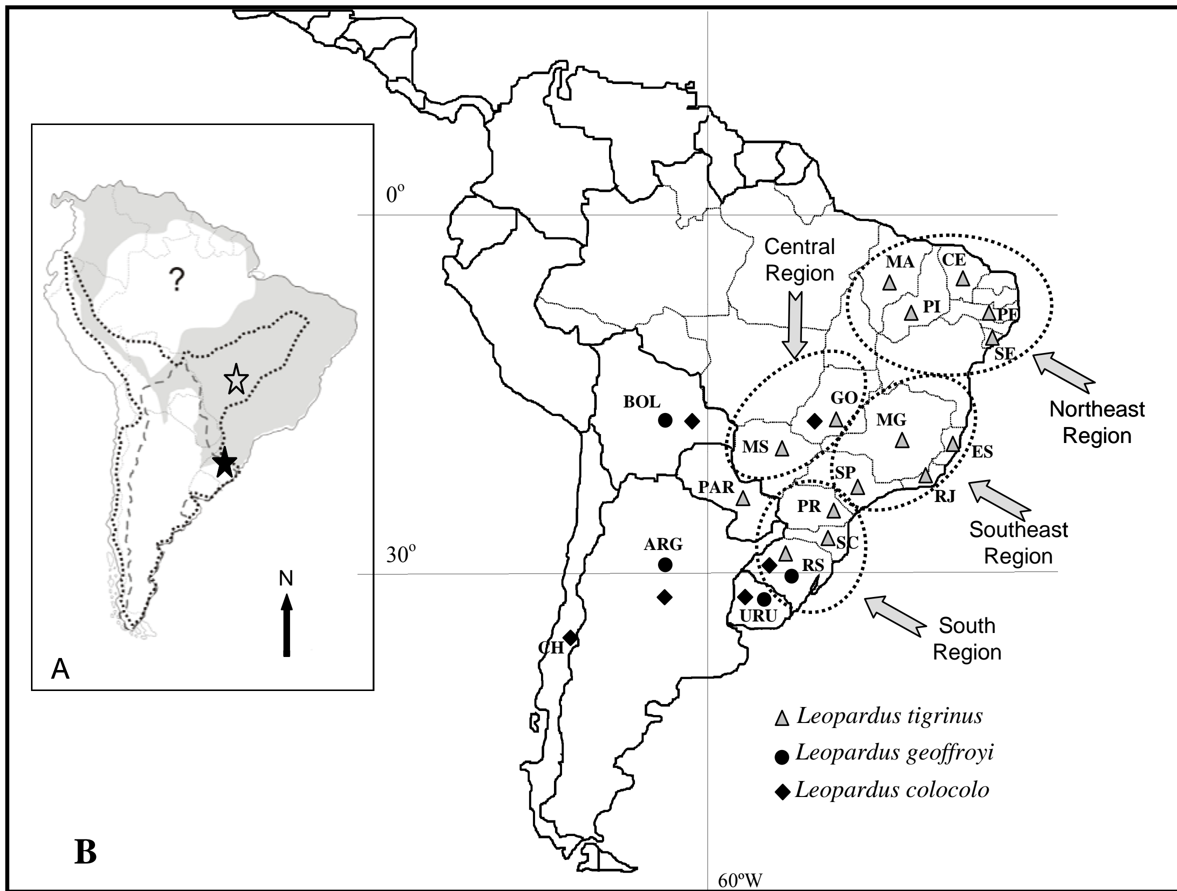

Figure 1 – Capítulo II

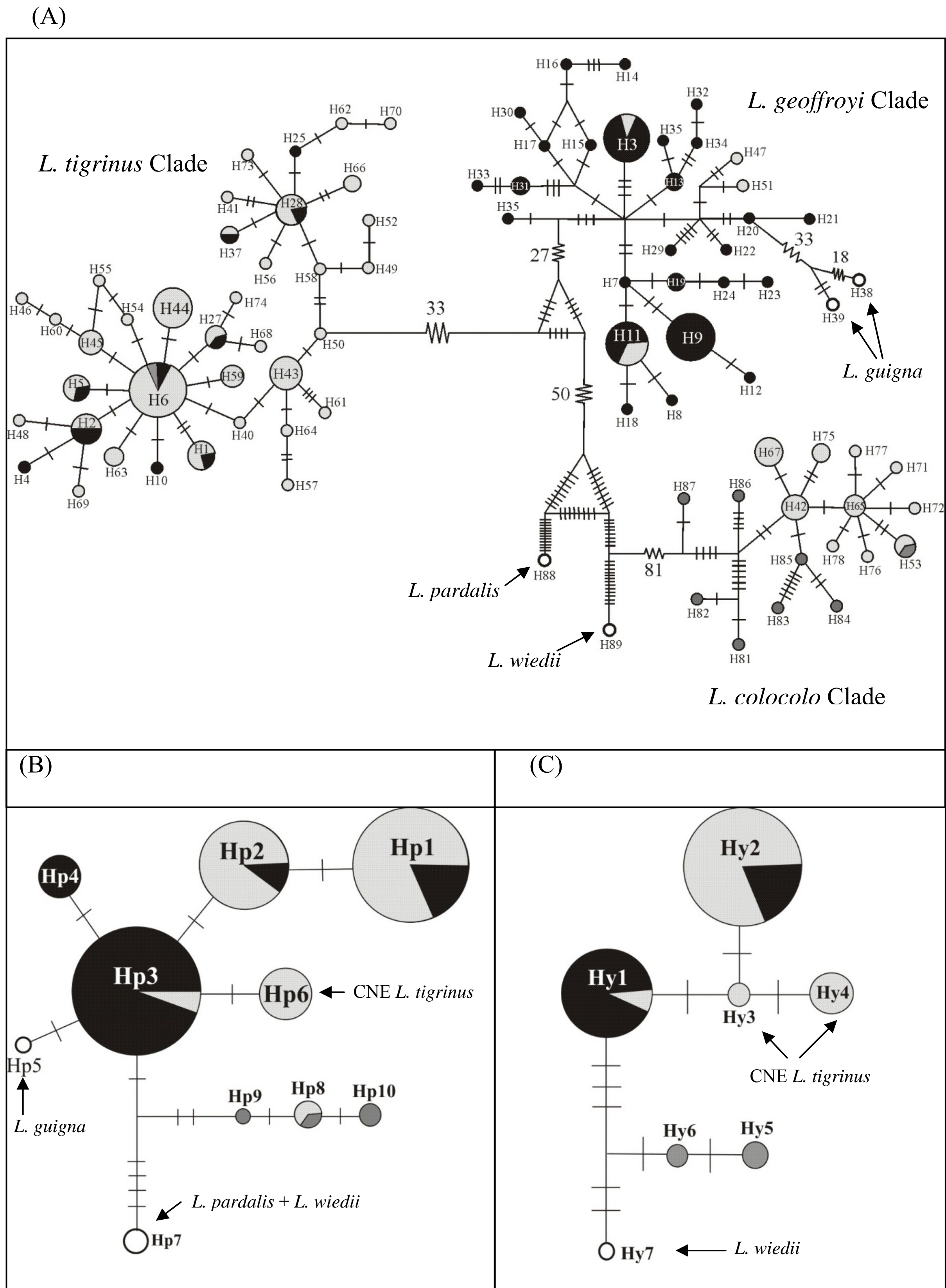

Figure 2 – Capítulo II

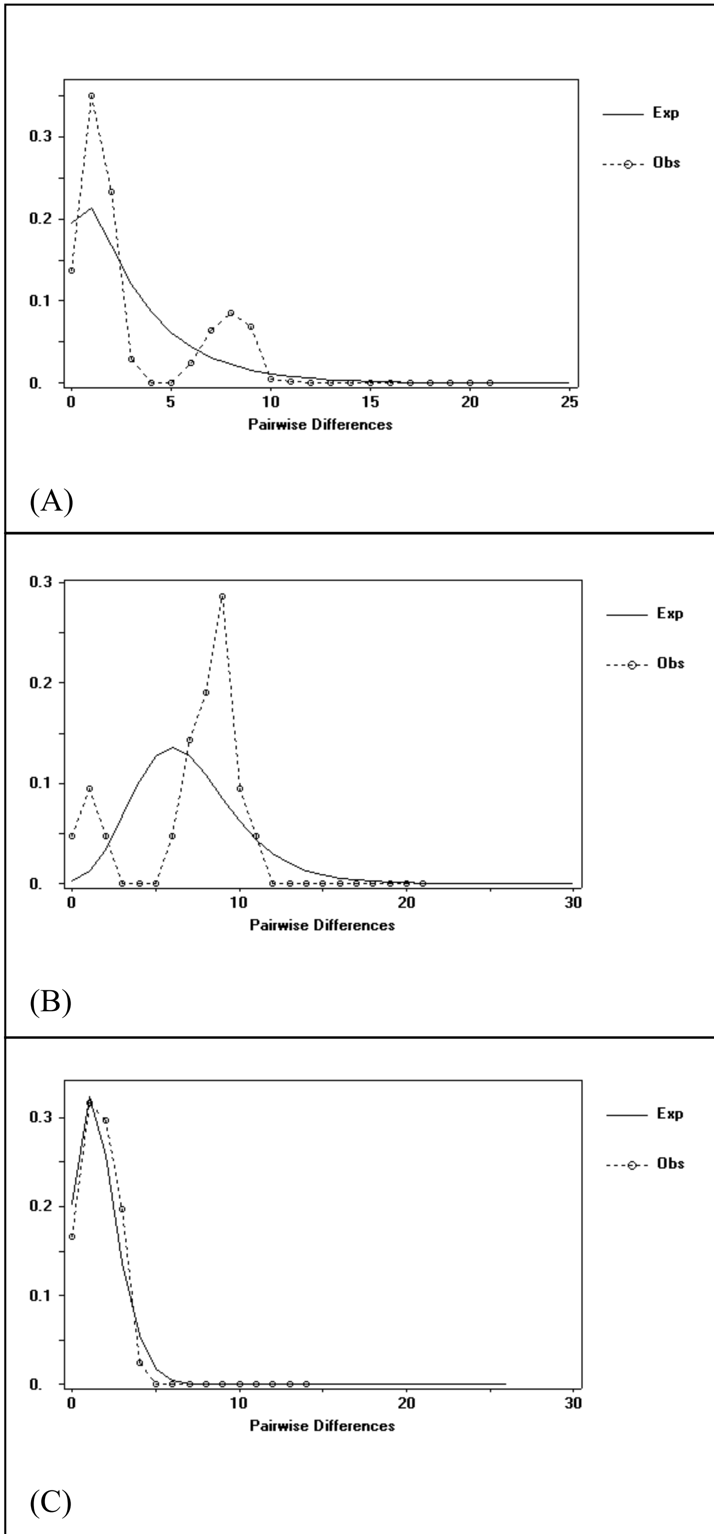

Figure 3 – Capítulo II

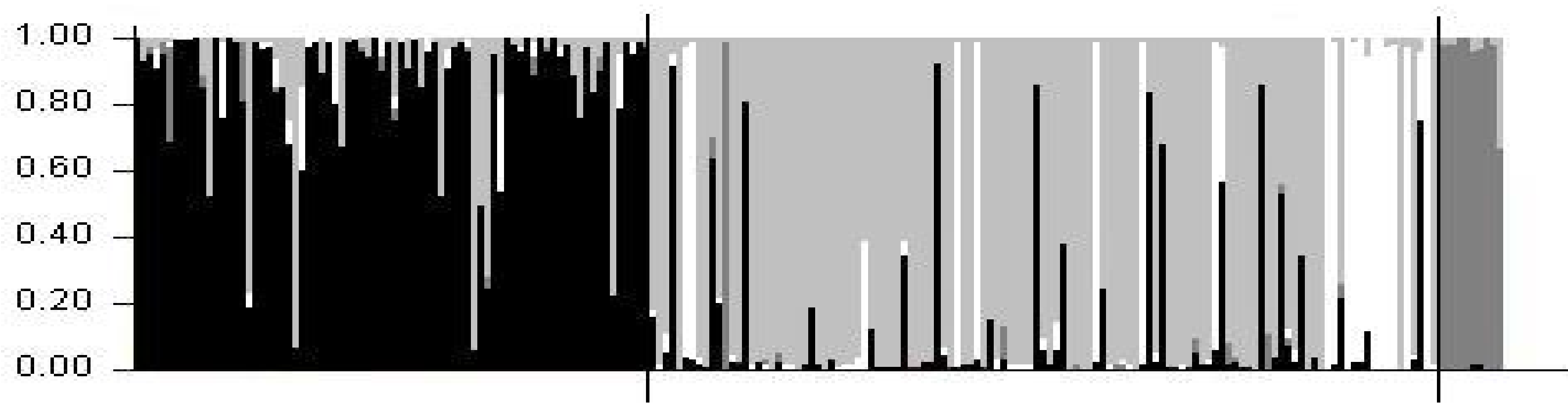

Figure 4 – Capítulo II

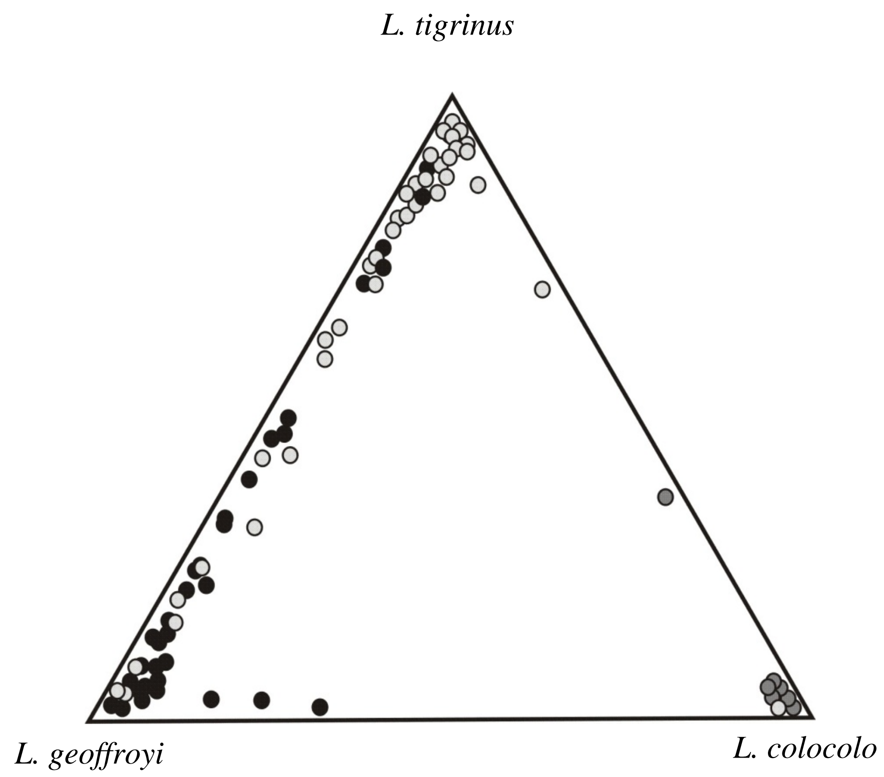

Figure 5 – Capítulo II

CAPÍTULO III

MANUSCRITO EM PREPARAÇÃO

Molecular and morphological characterization of an extensive hybrid zone between *Leopardus tigrinus* and *L. geoffroyi* (Mammalia, Carnivora, Felidae) in southern Brazil

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Keywords: hybridization, introgression, morphology, molecular markers, *Leopardus tigrinus*, *Leopardus geoffroyi*
Running title: Genetic and morphology of hybridizing Neotropical cats

Abstract

Increased attention towards the Neotropical cats *Leopardus tigrinus* and *L. geoffroyi* was prompted after genetic studies identified the occurrence of hybridization between them at their contact geographic zone in southern Brazil. In the present study we analyzed several types of molecular markers described by a previous work, aiming to characterize the extension and structure of the hybrid zone between these two cats at a local level. A morphological evaluation of the species was also conducted with the objective to test the level of morphological differentiation between the two species in their contact zone and the correlation between intermediate morphologies and genetic identification of parental and hybrid individuals. We found an extensive and complex hybridization pattern, with *ca.* 60% of the total population identified as hybrids resulting from post-F1 generations. Despite the strong level of hybridization/introgression detected, the two species seem to maintain their differentiated basic phenotypes in the vicinities of the contact zone, indicating that selective pressures may act against introgression of some morphology determinant genes.

Introduction

Hybridization between species is currently considered to be a natural evolutionary process playing an important role in the evolution/speciation of the organisms (Arnold 1992, Harrison 1993, Barton 2001). However, some anthropogenic disturbances such as the introduction of exotic species or habitat alteration may promote or increase hybridization, and thus compromise the genetic integrity of the implicated species (Huxel 1999, Rhymer & Simberloff 1996, Allendorf *et al*. 2001). Inter-specific hybridization processes arising from natural or human-induced causes may have multiple evolutionary consequences. In one extreme, hybridization may produce only sterile F1s, where the major effect for the involved species will be basically the waste of reproductive efforts rather than genetic admixing. At the other extreme, however, hybridization may produce fertile F1s able to cross with each other and also with the parental species, leading to widespread introgression that may generate a complete admixture between the two original organisms (Arnold 1992, Harrison 1993). The characterization of the nature of each particular hybrid zone, with the identification of its history and the main forces promoting its formation and maintenance, is crucial because these aspects may lead to relevant considerations on management and conservation of the species involved (Allendorf *et al.* 2001).

An initial and important issue in the investigation of a hybrid zone is the accurate identification of hybrids and parental types. The detection of hybrid individuals relied upon morphological characteristics until the mid-1960s. However, the utilization of morphological characters alone to distinguish between pure and admixed individuals is generally inappropriate, because not all morphological variation has a genetic basis and most of the phenotypic characters have multifactorial determinants (Allendorf *et al*. 2001). Additionally, morphological characters do not allow one to determine whether an individual is a first or later generation hybrid, which is crucial to the accurate characterization of hybrid zones. The development of new molecular techniques and powerful statistical tools for individual-based analysis (*e.g*. Pritchard *et al*. 2000, Anderson & Thompson 2002) allows the more precise identification of hybrids, as well as the proportion of admixture at the individual or population level. These pieces of information greatly contribute to shed light on important aspects of hybrid zone formation and evolution, including the magnitude, symmetry and consequences of genetic introgression.

Since the discovery of the occurrence of hybridization between two endangered Neotropical small cats, *Leopardus tigrinus* and *L. geoffroyi*, concentrated in their area of geographic contact in southern Brazil (Trigo *et al*. 2008, Trigo *et al.* in prep. Capítulo II), increased attention has been focused on their ecological, morphological and genetic characterization. The two species are closely related according to both molecular and morphological data (Salles 1992, Johnson *et al*. 2006), and are currently included in several lists of endangered fauna (Marques *et al*. 2002, Wozencraft 2005). They present basically parapatric geographic distributions at the Neotropical Region, with *L. tigrinus* occurring from southern Costa Rica to southern Brazil and *L. geoffroyi* from Bolivia, Paraguay and southern Brazil to the southern tip of South America (Oliveira 1994, Nowell & Jackson 1996, Eisenberg & Redford 1999). Overlap between their distributions seems to be quite limited, with an extremely restricted contact zone having been documented in the southernmost Brazilian state, Rio Grande do Sul (RS) (Eizirik *et al*. 2006), where subsequent genetic studies indicated the occurrence of hybridization between them (Trigo *et al*. 2008, Trigo *et al*. in prep. Capítulo II).

Hybridization between *L. tigrinus* and *L. geoffroyi* was first documented with the analysis of microsatellites and mitochondrial DNA (mtDNA) sequences by Trigo *et al*. (2008). In that study, the microsatellite loci employed presented weak statistical support to precisely identify hybrid individuals, which was accomplished more reliably by comparing phenotype and mtDNA-based data. Subsequently, this inter-specific hybridization was characterized in a more detailed study (Trigo *et al*. in prep. Capítulo II) which analyzed a larger number of molecular makers, including microsatellite loci, mtDNA and nuclear introns, leading to a better understanding about the main introgressive patterns acting between these species. Extensive rates of hybridization were detected in the vicinities of the contact zone, with a bidirectional introgression pattern recorded in all types of molecular markers, leading to a complex and varied array of genetic combinations among the admixed descendents. Despite these contributions to the understanding of the *L. tigrinus vs. L. geoffroyi* hybrid zone, no statistical treatment has so far been applied to verify the predominant genetic categories of hybrids (*e.g.* F1, F2 or backcrosses) in that admixed population, and the real magnitude of hybridization at a local level.

Although these two previous studies mentioned the existence of some individuals with ambiguous phenotypic characteristics, the great majority of the analyzed animals could be easily identified at species level on the basis of their morphology. The distinction between the two cats was normally based on the body size and pelage patterns (Ximenez

1975, Oliveira 1994, Sunquist & Sunquist 2002). In general, *L. tigrinus* has a more gracile appearance, with the total length varying between 71 to 93.6 cm and weight between 1.75 to 3.5 kg, while *L. geoffroyi* is larger and more robust, with a total length and weight varying between 69 to 125 cm and 2.2 to 7.8 kg, respectively. The *L. tigrinus* pelage color has a yellowish/ochre background with mostly open rosettes, while *L. geoffroyi* presents a gray/yellowish background pelage color with solid black spots instead of rosettes. In spite of these usual standards for identification, some animals with atypical morphology, which appeared to be intermediate between the two species, have been documented to occur in RS state since the early 1990s (Mazim *et al*. 2004, Eizirik *et al*. 2006). These atypical forms were predominantly characterized by an intermediate pelage pattern, which was hypothesized to be associated with hybridization events between these species in that area (Eizirik *et al*. 2006). Although the occurrence of hybridization in that region has now been documented with genetic data, so far no analysis has been performed testing the possible correlation between these intermediate phenotypes and their genetic status as hybrids.

To better understand the role that hybridization plays in the *L. tigrinus* and *L. geoffroyi* populations, as well as to help guide future management strategies for these species, it is critical to determine the extent and nature of the admixture events occurring between these cats. The genetic study conducted by Trigo *et al*. (in prep. Capítulo II) defined several molecular markers that are informative for the investigation of hybridization and introgression between these two cat species, allowing the extent and character of the admixture to be explored. In the present study, the previous genetic data generated for these two cats were used with the aim of evaluating the magnitude of admixture (at both population and individual levels) in the geographic area of Rio Grande do Sul state, southern Brazil, including a detailed attempt to identify pure and different hybrid categories. We specifically tested, with simulation analyses, the effectiveness of microsatellite loci in the distinction among these genetic categories. We also performed morphological analyses of a subset of the included specimens, and evaluated the level of differentiation between the two species and the correlation between genetic and morphological variation in this region. The results obtained here demonstrate that, although the two cats species can in most cases be morphologically differentiated in this contact zone, a small proportion of the individuals are completely pure in these populations, and many are the result of complex admixed combinations derived from hybridization events.

Materials and Methods

Sample collection and morphological identification

The focal genetic samples included in this study comprised 44 *L. tigrinus* and 49 *L. geoffroyi* individuals from Rio Grande do Sul state (RS), Brazil, analyzed by Trigo *et al*. (in prep. Capítulo II) (Table 1, Figure 1). These animals were subjected to in-depth analyses aiming to dissect the genetic composition of these populations sampled in the region containing the hybrid zone between these species. To assist in the genetic assignment of these RS individuals, additional samples also analyzed at this same genetic study and representing allopatric areas (*i.e.* locations where only one of the two species occurred) farther from RS state were included as control "pure" populations. This sample comprised 52 *L. tigrinus* (excluding individuals with evidence of hybridization with *L. colocolo*; see cited work for more details) and 26 *L. geoffroyi*. The entire sample included in this study was analyzed for two different mtDNA fragments [5' portion of the mtDNA control region (Tchaicka *et al*. 2007) and a segment of the *ND5* gene (Trigo *et al*. 2008)]; the second intron of the X-linked *Proteolipid Protein 1* (*PLP1*) gene (Murphy *et al*. 1999); one intron each of the Y-linked genes *ZFY* and *SMCY3* (Pecon-Slattery & O´Brien 1998, Hellborg & Ellegren 2003); and eleven microsatellite markers developed originally for the domestic cat (FCA391, FCA424, FCA441, FCA453, FCA723, FCA742, F42, F53, F98, F124, F146; Menotti-Raymond *et al*. 1999, 2005).

The RS samples were obtained from captive animals or individuals that had been road-killed or killed/caught by farmers. The identification of each individual was based on external morphology including pelage pattern and body size. Individuals with ambiguous characteristics such as intermediate pelage pattern were identified at species level based on body proportions, with more robust individuals identified as *L. geoffroyi* and more gracile ones as *L. tigrinus*. A sub-sample of these animals was assessed with morphological analysis (see below), including only individuals that could be measured for all the predefined measurements. For this set of samples were collected gender information, photographs of diverse body angles, observations on pelage details and body measurements.

Analysis of Genetic Data

Hybridization between *L. tigrinus* and *L. geoffroyi* was essentially recorded in RS state in the two previous studies focusing on these species (Trigo *et al*. 2008 , Trigo *et al.*

in prep. Capítulo II). However, those studies indicated that some individuals from locations outside this state had relatively low values of association to their phenotype-based population on the basis of microsatellite data. Due to this fact, the first step of our analysis involved the definition of the purest individuals among the 52 *L. tigrinus* and 26 *L. geoffroyi* sampled from areas out of RS state. For this purpose, the microsatellite data set and the Bayesian method implemented in STRUCTURE 2.2 (Pritchard *et al*. 2000) were used to assign individuals to their source population. The analysis was performed under a model allowing admixture, assuming correlated allele frequencies between groups (Falush *et al*. 2003) and using no prior information of phenotypic classification in 500,000 Markov Chain Monte Carlo (MCMC) iterations after a burn-in period of 200,000 replicates. The inferred individual coefficient of membership *q* was then evaluated to identify the purest allopatric individuals of each species. Subsequently, the individuals were investigated for the presence of any introgressed haplotype for the sequence markers analyzed (mtDNA, X and Y chromosome introns), according to the species-specific haplotypes described by Trigo *et al*. (in prep. Capítulo II). The purest individuals thus defined were then assumed to be a representative sample of each original species population, without the influence of the hybridization events, and are referred to here as "allopatric populations".

For microsatellite data, deviations from Hardy-Weinberg Equilibrium (HWE) and linkage equilibrium (LE) (for the following four predefined populations: allopatric *L. geoffroyi*, allopatric *L. tigrinus*, RS *L. geoffroyi* and RS *L. tigrinus*) were tested using the software packages ARLEQUIN 3.11 (Excoffier *et al*. 2005) and GENEPOP 3.0 (Raymond & Rousset 1995). The genetic differentiation among these populations according to both microsatellite and sequence data was assessed with an Analysis of Molecular Variance (AMOVA; Excoffier *et al*. 1992) implemented in ARLEQUIN, using 10,000 permutations to test the statistical significance of the estimated values.

To estimate the genetic contribution of the two parental species to the genotypes of each RS individual (*i.e.* to estimate individual admixture proportions), the microsatellite data set was analyzed using two different Bayesian clustering methods. First, STRUCTURE was used to assign individuals to populations according the individual coefficient of membership *q* and its associated 90% credibility interval (CI), following the same conditions described above. This method basically distinguishes between pure and hybrid individuals, where the former ones are highly associated with only one of the two parental clusters and the latter ones present intermediate and simultaneous association to both parental clusters. The second Bayesian method is implemented in the program

NEWHYBRIDS (Anderson & Thompson 2002), which rather than assigning individuals to a single hybrid category, computes the posterior probability (Q) that an individual in the sample belongs to each of six genotypic classes: pure I, pure II, F1, F2 (*i.e*. F1 x F1), backcrosses I and backcrosses II. This analysis was performed using the genotypic classes and the allele frequencies assumptions as described in Anderson & Thompson (2002) in runs of 500,000 sweeps after a burn-in period of 200,000 sweeps.

To determine the range of q values expected for each genetic category, and to assess the power of our admixture analyses (using STRUCTURE and NEW HYBRIDS) to distinguish between parental and different hybrid categories, the program HYBRIDLAB 0.9 (Nielsen *et al*. 2006) was used to simulate parental and hybrid genotypes based on our original data. The purest individuals from allopatric populations of each species, as defined by the first STRUCTURE analysis, were used as the source parental populations for genotype simulations. First, these populations were used to simulate five hundred individuals of each parental species, which were used to generate the same number of F1, F2 and three generations of backcrosses to each parental species. All simulated genotypes were then run in STRUCTURE and NEWHYBRIDS with the same conditions described above. The same simulated parental populations were also employed with the two Bayesian approaches to estimate the RS individuals' admixture proportion.

After the microsatellite-based independent analysis, each of the RS specimens was examined for its DNA sequence markers (mtDNA, X and Y chromosome introns). The presence of any incongruence between phenotypic identification and at least one of the three segments was considered as an evidence of a hybrid origin. This hybrid identification based on DNA sequences was then compared and added to the microsatellite-based hybrid identification in order to assess the number and proportion of hybrids existing in each phenotypic population and, consequently, the extent of hybridization in the RS population.

Finally, we evaluated the population admixture proportions through the estimation of the genetic contribution of each parental species to the hybridizing population, using the program ADMIX 2.0 (Dupanloup & Bertorelle 2001). In this analysis, we used the admixture coefficient (mY) introduced by Bertorelle & Excoffier (1998), which takes into account information regarding the degree of molecular divergence between alleles in addition to allele frequency differences. Under this model, molecular divergence was estimated from the average squared difference in allele sizes for microsatellite data and number of substitutions for molecular sequences (Bertorelle & Excoffier 1998). The analysis was performed for the entire set of markers (microsatellites, mtDNA, X and Y

chromosome introns), using the individuals from allopatric populations defined by our first analyses as representatives of each purebred species, and the hybrids identified by microsatellite and sequences data pooled together as representatives of the hybridizing population.

Analysis of morphological data

We obtained a sample of 23 *L. geoffroyi* and 19 *L. tigrinus* individuals that were suitable for the morphological analysis (Table 1). All the analyzed specimens were from RS state, with the exception of two *L. tigrinus* individuals from Santa Catarina state, adjacent to RS at its northern boundary.

Two different approaches were employed to morphologically characterize the two focal cats in the RS state. The first one involved the classification of all 42 individuals according to a pelage pattern scheme, taking into account the previous observation of atypical forms in the state predominantly characterized by an intermediate pelage pattern. For this purpose, we used photographs from the lateral-anterior portion of each individual's body in order to classify it into one of three arbitrarily defined categories: 1) PT: pelage typical of *L. tigrinus*, with orange background and presence of rosettes; 2) PG: pelage typical of *L. geoffroyi*, with grey/yellowish background and solid spots; 3) PI: defined as intermediate pelage including individuals that presented characteristics incongruent with the two previous categories, such as incomplete rosettes formed by the joining of adjacent spots.

The second approach involved the assessment of the level of morphological differentiation between the two species through a morphometric analysis. Twenty six measurements were taken from each specimen, including 23 body dimensions and three tooth measures (Table 4). Some individuals of our sample (*L. tigrinus*, n = 13 and *L. geoffroyi* n = 12) had a pair of measurements collected by two independent researchers. The Student test (*t*-test) for paired samples were used to assess the existence of significant differences between these paired measures for each species, independently. Only one measurement (ear length) differed significantly between the two independent researchers for both species ($p < 0.05$) and was then excluded from further analysis, since this data set was then considered less reliable. After this preliminary test, only the measurements taken by one of the researchers were maintained for the subsequent analyses. We employed univariate analysis (*t*-test) to verify the existence of significant differences between species and sexes for each individual measurement. The Analysis of Variance (ANOVA) with a

subsequent Tukey-Kramer Multiple Comparison Test was employed to verify significant differences between the four sex-species groups (*L. geoffroyi* males, *L. geoffroyi* females, *L. tigrinus* males and *L. tigrinus* females). To explore multivariate differences between species and sexes we performed a Principal Component Analysis (PCA) over the variance-covariance matrix of the logarithms of all measurements and a Discriminant Analysis to classify individuals and to evaluate the exact association between the individuals and their original morphological population. All statistical analyses were performed using the software package NCSS (Hintze 2006).

Correlation between genetic and morphological data

To test the relationship between the morphological identification based on measurements and pelage pattern with the genetic identification of pure and hybrid origin, we conducted a Correspondence Analysis using the software MVSP 3.13 (Kovach 1998). For that purpose, we constructed a contingency table with the number of individuals associated to each of the morphological and genetic classes. The morphological categories were defined as follows: 1) GPG: individual associated with the *L. geoffroyi* morphological cluster with pelage pattern typical of this same species; 2) GPI: individual associated with the *L. geoffroyi* morphological cluster but with an intermediate pelage pattern; 3) TPT: individual associated with the *L. tigrinus* morphological cluster and pelage pattern typical of this species; and 4) TPI: *L. tigrinus* morphological cluster but presenting an intermediate pelage pattern. The subdivision of the sample into genetic categories included: 1) pure *L. geoffroyi* (LGE); 2) pure *L. tigrinus* (LTI) and 3) individuals with a hybrid origin (HYB).

Results

Genetic Analysis

The majority of the individuals from the allopatric areas had very high probabilities of belonging to their respective species [84.6% (66/78) with $q \geq 0.90$] according to the STRUCTURE analysis. However, some of these individuals had a very broad associated CI for their q value. Therefore, to be more conservative, we selected only the individuals with q-values ≥ 0.90 and CI intervals with the maximum breadth of 0.8 - 1.0. Adhering to this criterion, we identified 31 *L. tigrinus* and 19 *L. geoffroyi* that represented the purest possible set of each species, and used these animals to constitute the allopatric populations.

Corroborating this microsatellite-based assessment, none of these individuals had any evidence of introgression for any of the available molecular sequences.

Considering the four populations evaluated here, significant departures from Hardy-Weinberg expectations were observed in three of them: two loci for the RS *L. tigrinus* (FCA742 and FCA723) after a Bonferroni correction (α = 0.05), one locus for the *L. geoffroyi* allopatric population (FCA723) and two loci for RS *L. geoffroyi* (FCA723, F53). As FCA723 was the only locus that presented HWE departures in more than one population, and taking into account the evidence of null alleles detected for it by Trigo *et al*. (2008), it is possible that these deviations may be in part caused by genotyping errors. In contrast, the departures detected at the other two loci were apparently consequences of the hybridization events. On the other hand, all pairwise locus combinations were in Linkage Equilibrium for the four populations (α = 0.05, after Bonferroni correction for 55 comparisons). The AMOVA results clearly demonstrated the effects of hybridization between the two species at the population level, where the four types of molecular markers demonstrated higher genetic similarity between *L. tigrinus* and *L. geoffroyi* individuals from RS state than between the allopatric populations of each species (Table 2).

The results of the simulated genotypes showed that different genetic categories had different detectabilities according to our microsatellite data set. The parental genotypes from both species were clearly segregated, with 94.8% of the *L. geoffroyi* individuals with $q \geq 0.9$ and 91% of the *L. tigrinus* with $q \leq 0.1$ (q in this case arbitrarily refers to assignment to the *L. tigrinus* population) (Figure 2a). Simulated F1s and F2s had very similar q distributions, which were quite different from those of the parentals, with 100% of F1 hybrids and 98.8% of F2s presenting $0.9 > q > 0.1$, and a mean of 0.5 (Figure 2b, c). From the simulations, only 0.6% of the F1 hybrids and 4.2% of the F2s overlapped with the simulated *L. geoffroyi* parental population, and 2% of the F1s and 7.8% of the F2s with the simulated *L. tigrinus* parental population. The CIs were also an important measure of statistical support to differentiate parentals from F1 and F2 hybrid categories. While more than 97% of the simulated parental genotypes had CIs including 1 or 0, respectively (*i.e.* accepting the hypothesis that their ancestry lay exclusively in one of the two populations), 100% of F1 and 97.4% of F2 simulated genotypes presented CIs excluding 1 and 0 (*i.e.* rejecting the hypothesis that their ancestry lay exclusively in one of the two sampled populations). The backcrosses were not as easily distinguished from parentals as was expected. The q-values generated from first-generation backcrosses into *L. geoffroyi* (BG1) ranged from 0.42 to 0.98, with a mean of 0.76 and a small percentage (10.8%) higher than

0.9 (Figure 2d). Similarly, q-values of first-generation backcrosses into *L. tigrinus* (BT1) ranged from 0.04 - 0.74 (with a mean of 0.26), and 11.4% of these simulated individuals had $q < 0.10$ (Figure 2g). These two hybrid categories showed CIs that mostly excluded 1 and 0 (85.4% of the BG1 and 82.4% of the BT1), as had been observed in the cases of F1s and F2s. Second- and third-generation backcrosses into both *L. geoffroyi* and *L. tigrinus* (BG2, BG3, BT2, BT3) had a higher proportion of overlap with the simulated parental genotypes. In this simulated scenario, 41.4% and 63.4% of the BG2 and BG3, respectively, had $q \geq 0.9$ (Figure 2e, f), with CIs predominantly including 1 (52% for BG2 and 70.4% for BG3). Likewise, 47.6% and 67.4% of the BT2 and BT3 categories had $q \leq 0.10$ (Figure 2h, i) and CIs predominantly including 0 (53.4% for BT2 and 71.8% for BT3).

On the basis of the simulated genotypes, the STRUCTURE analysis using the eleven microsatellite loci could distinguish essentially the F1, F2 and first-generation backcrosses from the parental individuals of both species under the following criterion: pure *L. geoffroyi* individuals are assumed to present q-values ≥ 0.90 with associated CIs including 1, pure *L. tigrinus* with q-values ≤ 0.10 with CIs including 0, and hybrids will have $0.90 > q > 0.10$ with CIs excluding both 1 and 0 values. In such a case, backcrosses beyond the first generation would not be reliably detected by the microsatellite data alone, and would only be identified by introgressed sequences into an apparently pure microsatellite background. Despite the detectability of F1, F2 and first-generation backcrosses, the distinction among these three hybrid categories was ineffective due to the high overlap in their q distribution (see Figure 2).

Based on the criterion established with the simulated genotypes, the STRUCTURE analysis of the RS individuals revealed a total of 15 pure *L. geoffroyi*, 17 pure *L. tigrinus* and 46 hybrids (27 phenotypic *L. geoffroyi* and 19 *L. tigrinus*), indicating that the RS region was largely represented by individuals with an admixed origin, totaling 49.5% of the total sample from this region (Figure 3). An additional 15 individuals representing both species could not be accurately classified because they presented intermediate q-values but CIs that included 1 or 0, thus being excluded from the criteria used to define both pure and hybrid animals.

The analysis of the simulated data with NEWHYBRIDS yielded less stringent conclusions with respect to the definition of purebreds *vs.* hybrids based on the eleven microsatellite loci used in this study. Only 82% of the simulated *L. geoffroyi* parental genotypes and 81.2% of the *L. tigrinus* had probabilities ≥ 0.90 of assignment to their respective genetic category. However, if we considered a threshold of 0.80 to distinguish

purebred individuals from hybrids in the NEWHYBRIDS analysis, we observed a higher proportion of correct assignment: 93.80% for *L. geoffroyi* and 93% of *L. tigrinus*. The simulated hybrid genotypes for the F1, F2, BG1 and BT1 categories were predominantly assigned to one of the hybrid categories defined by NEWHYBRIDS, but the distinction among them was much less accurate. The great majority of these individuals (90.3%) presented assignment proportions to their respective genetic category that was lower than 0.80, and several of them exhibited simultaneous and similar assignment probabilities to two or more of the four hybrid categories. This analysis also resulted in a high proportion of misplaced individuals from subsequent backcross generations, where 42.8% of BG2, 66.40% of BG3, 50.80% of BT2 and 72.2% of BT3 were identified as being pure applying a threshold of 0.80.

Following up on the investigation of individual admixture proportions based on the microsatellite data, we examined the available DNA sequences (mtDNA, X and Y chromosome introns) for each of the RS specimens. Evaluating all molecular markers together, we could observe several complex genetic combinations. Concordant evidence for a hybrid origin with microsatellite and DNA sequences was obtained for nineteen samples (Table 3). In addition, six samples considered to be pure individuals based on the microsatellite markers showed some evidence of hybridization with the DNA segments, while 27 others showed only evidence of hybridization with the microsatellites. In addition, four individuals among the 15 whose definition as pure or hybrid had been ambiguous with the microsatellite data (bLge02, bLge10, bLge49, bLge72) presented evidence of hybridization with the DNA sequences. There was only one case compatible with an F1 hybrid in the *L. tigrinus* phenotypic population (bLti79), and none in the *L. geoffroyi* population. The majority of the identified hybrids were compatible with F2 hybrids and/or backcrosses with both parental species (see Table 3).

Finally, based on the complete data set, and considering that an individual was only considered to be pure if it presented no evidence of hybridization in any of the molecular markers, 35 individuals of the *L. geoffroyi* phenotypic population (71.43%) and 21 of the *L. tigrinus* phenotypic population (47.73%) were identified as hybrids (see Table 3). This remarkable level of admixture implies an extensive process of population mixing, where at least 60% of our total sample constitutes a genetic population originated from hybridization events, which according to the ADMIX analysis contains a higher genetic contribution of *L. tigrinus* ($mY = 0.678 \pm 0.079$) than *L. geoffroyi* ($mY = 0.322 \pm 0.078$).

Morphological Analyses

The first morphological approach, wich includes the evaluation of the pelage pattern of the individuals sampled, placed eight of the 23 *L. geoffroyi* in the PG category, and ten in the PI group. Five individuals were melanistic and were thus excluded from the analysis due to the difficulty in reliably typing their coat patterns. Eleven *L. tigrinus* individuals were included in the PT category, and nine in the PI group. The pelt of one *L. tigrinus* individual was extremely damaged in the analyzed body region, and thus also had to be excluded from the analysis.

For the morphometric analysis, all the 25 measurements evaluated independently showed significant differences between species, with *L. geoffroyi* being always larger than *L. tigrinus* (Table 4). Statistically significant differences ($p < 0.05$) were also found between males and females of *L. geoffroyi* for almost all measures evaluated independently (exceptions: NC, ATW, PTW and UFP; see Table 4 for measurements abbreviations), indicating the presence of sexual dimorphism for this species with males being larger than females. On the other hand, the sexual dimorphism was not so clear for *L. tigrinus* group where only ten variables showed significant differences between males and females (TBL, HL, SH, NC, HC, PLF, APW, PPL, AFW and PFL; $p < 0.05$).

For the multivariate analysis, the two first principal axis of the PCA explained 77.59% of the total observed variability (individual percent of PCI = 72.34% and PCII = 5.25%), with PCI including all the 25 measures taken and PCII including upper canine width, lower canine width and head length. The measurements with highest loadings on PCI were weight, head circumference, total body length and posterior footpad width. Statistically significant differences were found between species for PC1 (t-value = 5.334, $p < 0.001$) but not for PCII (t-value = 1.27, $p > 0.05$), with the PCA diagram showing the predominance of *L. tigrinus* individuals in the lower area of the plot, and *L. geoffroyi* to the upper area (Figure 4A).

The Discriminant Analysis yielded 95.2% correct classifications of the individuals to their respective species category, with only one *L. geoffroyi* specimen (bLge37) associated to the *L. tigrinus* sample with a probability of 61.8%, and no equivalent case in the opposite direction. As it corresponds to a female *L. geoffroyi*, we performed an ANOVA to evaluate the differences between sexes and between species to ascertain that this erroneous classification is not correlated with an overlap betweeen female *L. geoffroyi* and *L. tigrinus* individuals. The ANOVA showed statistically significant differences among the four different groups (GM: *L. geoffroyi* males, GF: *L. geoffroyi* females, TM: *L.*

tigrinus males and TF: *L. tigrinus* females) for PCI (F = 27.090, p < 0.001) but nor for PCII. The Tukey-Kramer Multiple Comparison test indicated statistical significance for most comparisons, with the exceptions being between males and females of *L. tigrinus*, and between female *L. geoffroyi* and male *L. tigrinus.* These results indicated again a clear sexual dimorphism for *L. geoffroyi* but not for *L. tigrinus,* and demonstrate the existence of a larger overlap in size between male *L. tigrinus* and female *L. geoffroyi* that may account for the erroneous classification detected in the Discriminant Analysis (Figure 4B).

Correlation between the genetic and morphological data

The Correspondence Analysis (CA) plot (Figure 5) showed an association of the genetic categories related to pure individuals with those of typical morphology of each species, while the genetic classes represented by the hybrid individuals seemed to be more associated to the morphologically intermediate categories (GPI and TPI). Axis 1 explained 85.93% of the total variance of the data, and seemed to separate the two genetic variables representing the pure specimens (LGE *vs.* LTI) and the different categories of morphological identification included in the two species (TPT/TPI *vs.* GPG/GPI); an intermediate position was observed for the morphologically intermediate categories and for the hybrid genetic category (HYB). Axis 2, on the other hand, seemed to separate the genetic and morphological categories associated with pure individuals *versus* those containing hybrids. These results suggested that the atypical morphology characterized by an intermediate pelage pattern may be in part related to a hybrid origin.

Discussion

Genetic identification of hybrids and extent of hybridization

The genetic analyses performed here allowed an in-depth assessment of the power and limitations of the molecular markers employed by Trigo *et al*. (in prep. Capítulo II) in their study of the hybrid zone between *L. tigrinus* and *L. geoffroyi* in southern Brazil. Simulations and assignment tests conducted with the microsatellite data set, along with the DNA sequence segments, have permitted the identification of an extensive rate of hybridization, with an important impact on the genetic composition of both species around this contact zone.

Many studies conducted on hybridization have used programs such as STRUCTURE and/or NEWHYBRIDS to identify hybrid *vs.* purebred individuals (*e.g.*

Beaumont *et al*. 2001, Pierpaoli *et al*. 2003, Trigo *et al*. 2008). In these studies, arbitrarily selected threshold *q*-values have been used to distinguish between hybrid and purebred individuals, leading to frequent controversies about the applicability and credibility of these distinctions. Recently, genotype simulations performed with HYBRIDLAB have been used by several studies on hybridization (*e.g.* Lancaster *et al.* 2006, Oliveira *et al*. 2008, Schwartz & Beheregaray 2008) to determine the best criterion to distinguish between pure and hybrid categories, and to predict the power to detect hybrids and different classes of admixed individuals. In the present study, the use of simulated genotypes was extremely useful to infer the power (*i.e.* the proportion of individuals in a group that were correctly assigned) of the eleven microsatellite loci to distinguish between these genetic categories. Through the generation of simulated genotypes, we could conclude that the Bayesian analysis performed using STRUCTURE with the eleven microsatellite loci and the defined allopatric populations as surrogate parental groups was highly efficient to distinguish purebred from F1 and F2 individuals. However, the power to distinguish purebred from backcrosses (especially beyond the first generation) was reduced. The analysis using NEWHYBRIDS was less conclusive, but yielded a similar pattern. Such reduction of efficiency has also been reported in studies that used simulated genotypes and may be mainly related to the presence of few diagnostic alleles for the species involved and to the small number of loci employed (*e.g.* Lancaster *et al.* 2006, Schwartz & Beheregaray 2008, Vähä & Primmer 2006). In the cases of highly hybridizing species, in which most of the individuals are admixed, the distinction between backcrosses and purebred individuals is even more difficult and often requires a relatively high number of diagnostic alleles for the species involved and a large number of microsatellite loci (Pritchard *et al*. 2000, Allendorf *et al.* 2001, Vähä & Primmer 2006). On the other hand, the distinction among the different hybrid categories was poorly accomplished with either software, and may again be related to the small number of microsatellite loci used. According to Vähä & Primer (2006), only when 48 loci were used and the genetic divergence between the hybridizing parental populations was Fst = 0.21, did they obtain over 95% of backcross, F1, F2 and parental individuals assigned correctly to each specific hybrid or parental group. Considering all of these observations, the present data set, which shows an Fst value of 0.168 between the pure parental populations defined in this study, using eleven microsatellite loci, presented considerable power to distinguish between purebred and hybrid individuals, but was not able to discriminate among different hybrid categories.

Analysis of the microsatellite data from the RS population has permitted the identification of a large number of hybrids, accounting for *ca.* 60% of our total sample when the sequence data is included. These results indicate that this system consists of the most extensive hybridization process documented for carnivores until now, even taking into account the highly known hybridizing populations of wildcats and domestic cats in Hungary (Lecis *et al*. 2006). Considering the results of the simulated data, that indicated purebred individuals from both species with very similar q distributions to backcrosses beyond the second generations, it is possible that the number of hybrids detected is still underestimated. This extensive rate of hybridization and introgression seems to be the main cause of the higher genetic similarity detected between phenotypic populations of *L. tigrinus* and *L. geoffroyi* in the RS contact zone than between the two allopatric populations of both species.

In addition to testing and supporting the hybrid identification process, the sequence segments used in combination with the microsatellite assignments contributed to generate important pieces of information regarding the different hybrid categories present in the sampled area, which was not fully possible using the microsatellite data alone. If only the individuals analyzed for the full suite of genetic markers were considered, only one possible F1 hybrid (bLti79) was identified. Interestingly, this road-killed individual was a pregnant female morphologically identified as *L. tigrinus*, supporting our previous inference that F1 hybrid females are fertile (Trigo *et al*. 2008, Trigo *et al*. in prep Capítulo II). Furthermore, those previous studies suggested that mating between hybrids and backcrosses to the two parental populations were also occurring. Our present analyses corroborate this hypothesis, and demonstrate that the majority of the hybrids were compatible with these admixture categories. Our analyses also allowed the detection of more complex hybrid classes, such as in the case of bLge80 (see Table 3), which was a putatively pure individual based on the microsatellite data, but presented sequence segment combinations that implied a F2 hybrid ancestry. It is relevant to point out that we have only simulated microsatellite genotypes for the simplest hybrid scenarios. A more complex pattern, with extensive admixture among various types of hybrid descendants (*e.g.* crosses among F2s or F*n* with backcrosses of different generations) was not tested, and perhaps some individuals in this hybrid zone cannot be confidently assigned to the any of these simple hybrid categories.

Despite the diverse array of molecular tools used in this study, it was still not enough to precisely assign individuals to some specific hybrid classes. Several of the

inferred hybrids was simultaneously compatible with an F2 or backcross status; consequently, it was not possible to determinate with accuracy whether the genetic introgression found in the two morphological populations was predominantly deriving from hybrid backcrosses to the parental species, or from mating among F1 hybrids. This difficulty may be associated with the high rate of hybridization detected here, with a substantial proportion of the RS individuals being identified as admixed due to inter-specific cross-breeding. This finding suggests that fertile hybrids are able to mate among themselves and/or with parental individuals. In this type of hybrid zone, the number of possible genotypic classes to which an individual may belong increases exponentially with the number of generations over which introgression has been occurring, and therefore to distinguish among them becomes increasingly difficult, requiring an extensive number of loci for efficient classification (Boecklen & Howard 1997, Anderson & Thompson 2002).

Despite the difficulty in determining the predominant type of mating occurring in the hybrid zone investigated in this study, some evidence was in agreement with those presented by Trigo *et al*. (in prep Capítulo II) suggesting a higher level of genetic introgression into the morphological *L. geoffroyi* population. First, all molecular markers analyzed independently and also combined identified a higher proportion of hybrids in the phenotypic population of *L. geoffroyi* than in *L. tigrinus* (see Table 3). Second, the ADMIX analysis indicates that the hybridizing population in RS state had approximately 68% of its genomic composition derived from *L. tigrinus*. Such asymmetric introgression in hybrid zones is very common and may suggest selective pressures that favor the hybrid combinations that, when crossed with the parental species, lead to a smaller reduction in viability and fertility, allowing more genes to pass in one direction than in the other (Barton & Hewitt 1985, Harrison 1993).

The extensive rate of hybridization detected between *L. tigrinus* and *L. geoffroyi* in RS state may also indicate the absence of selection and post-zygotic barriers against the hybrids, or at least that they are limited. However, Barton & Hewitt (1985) argue that the great majority of known hybrid zones are maintained at a stable balance between dispersal and such kind of selection. Two different general types of selection are possible to operate in hybrid zones. The first one includes some sort of endogenous selection independent of the environment, represented only by intrinsic reductions on fertility or viability of the hybrid forms. The second one comprises exogenous selection, where different genetic combinations are favored in different environments (Arnold 1993, Barton 2001). Because we currently have no information about reduction in fertility or viability of the various

hybrid categories inferred to exist between these two cat species, and knowledge about their specific habitat associations and requirements is also very scarce, a thorough evaluation of these possible selective constraints will only be possible through the conduction of in-depth physiological and ecological studies focusing on these felids in these and other regions.

Morphological characterization and correlation with the genetic identification

The present study is the first to include the characterization of *L. tigrinus* and *L. geoffroyi* individuals using multiple morphological body measurements. Until now, only studies based on cranial morphology (Salles 1992) and a few body dimensions (such as length and weight) have been published on either species (see Kitchener 1991 and references therein, Johnson & Franklin 1991, Lucherini *et al*. 2006).

The set of measurements employed here seems to be effective for species identification, since it allowed us to detect morphologically distinct groups in RS state that correspond to the two focal cat species. This differentiation was related basically to a smaller size of *L. tigrinus* in comparison to *L. geoffroyi*, which presented higher means for all measurements and higher scores in the multivariate analysis. Differences in body size were also detected between males and females of *L. geoffroyi*, indicating that there is morphological sexual dimorphism for this species, represented by larger size in males, being in agreement with the analyses published by Lucherini *et al*. (2006). The sexual dimorphism for *L. tigrinus* was not clearly defined by our analysis, what may be related in part to the small sample size obtained especially for this species. Our results also enabled the detection of a large overlap in size between females of *L. geoffroyi* and males of *L. tigrinus* hampering the distinction between these morphological categories.

The Discriminant Analysis performed with samples subdivided *a priori* into two groups (*L. tigrinus* and *L. geoffroyi*) led to 95% of correct assignments. This high proportion of correct species-level assignments based on the measurements indicates that there is a detectable morphological segregation between the two cats in RS state, owing mostly to differences in general body size, which does not seem to be strongly influenced by the hybridization events documented here. On the other hand, several specimens presented intermediate patterns of pelage coloration, which appear to be at least partially influenced by hybridization given the results of the Correspondence Analysis. However, the analyses showed statistical limitations due to the small sample size available, which is due to the difficulty in finding specimens (*e.g.* road-killed) that are sufficiently intact to

allow the reliable collection of all body measurements and simultaneously a detailed assessment of pelage coloration. A larger sample size and the introduction of additional body measures and analytical approaches should contribute to shed more light on this observation.

The morphological characterization of *L. tigrinus* and *L. geoffroyi* individuals from RS state performed in this study with two different approaches (body size dimensions and pelage characteristics) indicated that the body proportions are the best criterion to identify these two cat species, especially in the presence of an intermediate pelage. However, in spite of the apparent association between intermediate pelage pattern and genetic identification of hybrids, these morphological characteristics are not sufficient to identify hybrid individuals, but a possible indicative of admixed ancestry in many cases.

Despite the incomplete reproductive isolation detected between the two cat species (reflected by the high rates of hybridization/introgression), the combination of morphology and genetic identification demonstrates that there are some mechanisms that maintain the phenotypic differentiation between them. This incongruence between morphological and genetic identification in highly hybridizing populations was also reported in several other studies (*e.g.* Gaubert *et al*. 2005, Chan *et al*. 2006, Lecis *et al*. 2006), demonstrating the serious limitation of attempting to identify hybrids *vs.* pure individuals on the basis of morphology alone. Identification of hybrids in such extensive hybrid zones using only morphological features can often be challenging because introgression is often not reflected on morphology, and, in particular, after several generations of backcrossing the hybrids may not differ from the parental species, and are then considered to be cryptic hybrids (Rhymer & Simberloff 1996, Allendorf *et al*. 2001). Generally, morphological introgression may follow distributions which differ from those of neutral markers, because the morphological traits may be controlled by genes that can are under selection, sometimes related to the fitness of parental types (Fernandez-Manjarres *et al*. 2006). In this case, the genes underlying the determination of body proportions and coat patterns in *L. tigrinus* and *L. geoffroyi* may be under different selective pressures, leading to different introgression rates among loci. We can hypothesize that body proportions may be coded by larger complexes of co-adapted genes than those determining pelage characteristics, so that the integrated regulation of the former may result in stronger selection restricting phenotypes to distinct adaptive peaks.

In conclusion, this study demonstrated and characterized an extensive rate of hybridization and introgression between *L. tigrinus* and *L. geoffroyi* in RS state, including

mostly non-F1 hybrids. In spite of the inferred high rates of introgression that would lead to rapid homogenization between the two cat species around their geographic contact zone, our morphological analyses suggest that different selective pressures may play an important role in maintaining phenotypic (and to some extent genetic) integrity of the parental species. An asymmetry in the magnitude of introgression seems to occur (favoring backcrosses with *L. geoffroyi*), and the rate of introgression seems to be different in neutral markers relative to phenotype-determining genes, especially those related with body proportions. Overall, this study illustrates the complexity of evolutionary processes acting on hybrid zones, where the maintenance of species distinctness hinges on a balance between different evolutionary forces.

Figure Legends

Figure 1 – A) Map of the *Leopardus tigrinus* (in grey) and *L. geoffroyi* (in black) distributions in South America, with an indication of their potential contact zone (area in dark grey) and the location of Rio Grande do Sul state in southern Brazil (white circle). The question mark indicates an area of uncertain occurrence of *L. tigrinus*. B) Map of Rio Grande do Sul state showing the distribution of the *L. tigrinus* (grey circles) and *L. geoffroyi* (black circles) samples utilized in this study.

Figure 2 – Frequency distributions of q for all hybrid types according to the STRUCTURE analysis of eleven microsatellite loci: a) simulated parental *Leopardus tigrinus* and *L. geoffroyi*; b) simulated F1 hybrids; c) simulated F2 hybrids; d) simulated first generation of backcrosses with *L. geoffroyi*; e) simulated second generation of backcrosses with *L. geoffroyi;* f) simulated third generation of backcrosses with *L. geoffroyi*; g) simulated first generation of backcrosses with *L. tigrinus*; h) simulated second generation of backcrosses with *L. tigrinus*; i) simulated third generation of backcrosses with *L. tigrinus*; j) observed frequency distribution of q values for the *L. tigrinus* and *L. geoffroyi* samples from Rio Grande do Sul state.

Figure 3 - Distribution of q in *Leopardus tigrinus* and *L. geoffroyi* individuals from Rio Grande do Sul (RS) state according to the STRUCTURE analysis. Black central squares

indicate point estimates, and vertical lines represent the 90% posterior credibility intervals associated with each individual.

Figure 4 – Morphological differentiation of *Leopardus tigrinus* and *L. geoffroyi* based on the 25 measures used in the final analyses. A) Morphological differentiation at the species level based on a Principal Component Analysis (*Leopardus geoffroyi*: Lge [n = 23] and *L. tigrinus:* Lti [n = 19]). B) Box plot showing the morphological differentiation of the males and females of the both species based on Analysis of Variance (GM: *L. geoffroyi* males [n = 14]; GF: *L. geoffroyi* females [n = 9]; TM: *L. tigrinus* males [n = 13]; TF: *L. tigrinus* females [n = 6]).

Figure 5 – Correspondence Analysis showing the association between the genetic and morphological categories. The genetic categories are represented by grey triangles and are defined as LTI: pure *L. tigrinus*, LGE: pure *L. geoffroyi* and HYB: individuals carrying a hybrid origin based on the full set of molecular analyses conduced here. The morphological categories are represented by black circles and are defined as TPT: individuals identified as *L. tigrinus* based on body measurements with pelage typical of *L. tigrinus*; TPI: *L. tigrinus* based on body measurements with a pelage pattern seemingly intermediate between the two species; GPG: *L. geoffroyi* based on the body measurements with coat typical of *L. geoffroyi*; and GPI: *L. geoffroyi* based on the body measurements with an intermediate pelage pattern.

Table1. Characterization of entire Rio Grande do Sul (RS) state sample collected for *Leopardus tigrinus* and *L. geoffroyi*.

Leopardus geoffroyi				*Leopardus tigrinus*			
ID.	Sex	Municipality of origin	Collection Year	ID.	Sex	Municipality of origin	Collection Year
bLge01	M	Santa Cruz do Sul	1993	bLti01	F	Triunfo	1993
bLge02	M	Cachoeira do Sul	1994	bLti04	M	Rio Grande do Sul	1993
bLge03	M	Cachoeira do Sul	1994	bLti05	M	Camaquã	1993
bLge04	M	Cachoeira do Sul	1994	bLti09	F	Cachoeira do Sul	1994
bLge05	M	Cachoeira do Sul	1994	bLti10	F	Guaporé	1994
bLge07	M	Cachoeira do Sul	1994	bLti46	M	Garibaldi	1995
bLge08	M	Cachoeira do Sul	1994	bLti47	F	Garibaldi	1995
bLge10	F	Cachoeira do Sul	1994	bLti48	F	Estrela	1995
bLge11	M	Pantano Grande	1994	bLti49	F	Guaíba	1995
bLge12	M	Cachoeira do Sul	1994	bLti51	F	Santa Cruz do Sul	1996
bLge13	M	Eldorado do Sul	1994	bLti68	M	Montenegro	1997
bLge28*	M	Camaquã	1997	bLti69*	M	Santa Cruz do Sul	1997
bLge29*	M	Quaraí	1998	bLti79*	F	Eldorado do Sul	1997
bLge31*	M	Quaraí	1998	bLti80*	M	Glorinha	1998
bLge32*	M	Pântano Grande	1998	bLti94	M	Ibarama	1999
bLge33*	M	Alegrete	1999	bLti95	F	Sarandi	2001
bLge35	M	Rio Grande	2000	bLti98	F	Restinga Seca	2002
bLge36	F	Rio Grande	2000	bLti99	M	Nova Esperança do Sul	1999
bLge37*	F	São Lourenço do Sul	2000	bLti100	M	Santo Antônio da Patrulha	2002
bLge38	F	Santa Maria	2000	bLti102*	F	Erechim	2004
bLge39	M	Jaguari	2000	bLti106*	M	Santa Cruz do Sul	2004
bLge41	M	Itaqui	2000	bLti108	M	Santa Maria	2004
bLge42	M	Barra do Ribeiro	2002	bLti110	M	Itapuã	2003
bLge43	M	São Borja	2002	bLti113	M	Getúlio Vargas	2004
bLge44	F	São Gabriel	2002	bLti117	F	Arroio do Meio	2004
bLge46*	M	Canela	2004	bLti119*	M	Cachoeira do Sul	2005

Table 1 (Cont.)

Leopardus geoffroyi				*Leopardus tigrinus*			
ID.	Sex	Municipality of origin	Collection Year	ID.	Sex	Municipality of origin	Collection Year
bLge47*	M	São Leopoldo	2004	bLti120*	M	Cachoeira do Sul	2005
bLge49*	M	Rio Grande do Sul	2004	bLti121*	M	Triunfo	2005
bLge70	F	Arroio Grande	2003	bLti122*	M	Arroio do Sal	2005
bLge71*	F	Rio Grande	2004	bLti124*	M	Arroio do Meio	2003
bLge72*	M	Encruzilhada do Sul	2005	bLti131	M	Guaporé	1998
bLge73*	M	Cachoeira do Sul	2005	bLti132	M	Rolante	2001
bLge74*	F	Piratini	2004	bLti133	M	Lagoa Vermelha	2002
bLge75*	F	Arroio Grande	2003	bLti134	F	São Pedro de Alcântara	2003
bLge76*	M	Arroio Grande	2004	bLti135*	F	Estância Velha	2004
bLge77*	M	Dom Pedrito	2004	bLti136*	M	Carazinho	2005
bLge78*	F	Rio Grande	2004	bLti137*	M	Morro Reuter	2006
bLge79	M	São Lourenço do Sul	2004	bLti138*	F	Machadinho	2006
bLge80	M	São Lourenço do Sul	2004	bLti140*	M	Ibarama	2006
bLge89	M	Alegrete	2001	bLti141	M	Arroio do Sal	2006
bLge90	M	São Gabriel	2002	bLti142	M	Sarandi	2006
bLge91*	M	Itaqui	2000	bLti143*	F	Novo Hamburgo	2006
bLge92*	M	Alegrete	2006	bLti146	F	Cachoeira do Sul	2002
bLge93*	F	Arroio Grande	2002	bLti149*	M	Forquetinha	2006
bLge94*	F	Jaguarão	2006				
bLge95*	F	Cristal	2004				
bLge96*	M	Pelotas	2006				
bLge97	F	Guaíba	2006				
bLge(Nid11)	M	Rio Pardo	2000				

* Individuals included on the morphological analysis.

Abbreviations: F – female, M – male.

Table 2. Levels of differentiation between the *Leopardus geoffroyi* and *L. tigrinus* populations from allopatric and contact (RS state) areas, based on microsatellite, mtDNA and X and Y chromosome introns.

A	Microsatellite		mtDNA	X chr.	Y chr.
	Fst	Rst			
*Lti*Allop. *vs. Lti*RS	0.039*	0.006	0.062	0.145**	0.026
*Lti*Allop. *vs. Lge*RS.	0.110**	0.092**	0.650	0.480**	0.613**
*Lti*Allop. *vs. Lge*Allop.	0.168**	0.335**	0.938**	0.739**	1.000**
*Lti*RS *vs. Lge*Allop.	0.089**	0.287**	0.794	0.739**	0.868**
*Lti*RS *vs. Lge*RS	0.049**	0.073**	0.540**	0.540**	0.520**
*Lge*Allop. *vs. Lge*RS	0.010*	0.106**	0.137	0.095*	0.145

Abbreviations: *Lti*Allop.: *L. tigrinus* allopatric population; *Lge*Allop.: *Leopardus geoffroyi* allopatric population; *Lti*RS: Rio Grande do Sul *L. tigrinus* population; *Lge*RS: Rio Grande do Sul *L. geoffroyi* population.

Significance level: * $p < 0.05$ ** $p < 0.01$

Table 3. Individuals inferred to have a hybrid (*L. tigrinus vs. L. geoffroyi*) origin based on the complete set of molecular markers used in this study. I) Proportion of membership *q* of each individual inferred by microsatellite analyses using STRUCTURE. The values in parentheses are the posterior credibility intervals. II) Species-specific haplotypes from molecular segments of the mitochondrial DNA (mtDNA), X and Y chromosome introns. Introgressed haplotypes are shown in bold.

Leopardus geoffroyi					*Leopardus tigrinus*				
	I	II				I	II		
ID	Microsatellites	mtDNA	X chr	Y chr	ID	Microsatellites	mtDNA	X chr	Y chr
bLge01ϕ	0.973 (0.839, 1.000)	***Lti***	*Lge*	*Lge*	bLti01*ϕ	0.316 (0.098,0.567)	***Lge***	*Lti*	**F**
bLge02ϕ	0.811 (0.494, 1.000)	***Lti***	***Lti***	***Lti***	bLti05ϕ	0.026 (0.000,0.193)	*Lti*	***Lge***	***Lge***
bLge04*ϕ	0.475 (0.208, 0.737)	*Lge*	***Lti***	***Lti***	bLti09*ϕ	0.546 (0.303,0.782)	***Lge***	*Lti*	**F**
bLge05*ϕ	0.532 (0.293, 0.769)	*Lge*	***Lti***	*Lge*	bLti47*	0.391 (0.097,0.696)	*Lti*	*Lti*	F
bLge07*ϕ	0.432 (0.120, 0.736)	***Lti***	**---**	*Lge*	bLti49*ϕ	0.636 (0.388,0.855)	***Lge***	*Lti*	**F**
bLge08*ϕ	0.554 (0.302, 0.800)	***Lti***	***Lti***	*Lge*	bLti51*	0.317 (0.098,0.575)	*Lti*	*Lti*	F
bLge10ϕ	0.883 (0.635, 1.000)	*Lge*	***Lti***	**F**	bLti68ϕ	0.035 (0.000,0.253)	*Lti*	***Lge***	*Lti*
bLge11*ϕ	0.527 (0.275, 0.777)	***Lti***	*Lge*	*Lge*	bLti79*ϕ	0.561 (0.322,0.795)	***Lge***	***Lge*** / *Lti*	**F**
bLge12ϕ	0.992 (0.956, 1.000)	*Lge*	***Lti***	***Lti***	bLti98*	0.640 (0.379,0.878)	*Lti*	*Lti*	F
bLge13*ϕ	0.483 (0.247, 0.720)	***Lti***	***Lti***	***Lti***	bLti100*	0.285 (0.017,0.558)	*Lti*	---	*Lti*
bLge31*	0.738 (0.486, 0.944)	*Lge*	*Lge*	*Lge*	bLti102*	0.344 (0.055,0.659)	*Lti*	*Lti*	F
bLge32*ϕ	0.567 (0.329, 0.796)	*Lge*	***Lti***	*Lge*	bLti108*	0.313 (0.085,0.586)	*Lti*	*Lti*	---
bLge33*	0.820 (0.600, 0.994)	*Lge*	*Lge*	*Lge*	bLti119*ϕ	0.504 (0.245,0.763)	*Lti*	**---**	***Lge***
bLge35*	0.784 (0.550, 0.957)	*Lge*	*Lge*	*Lge*	bLti120*	0.295 (0.072,0.564)	*Lti*	*Lti*	*Lti*
bLge38*	0.535 (0.272, 0.789)	*Lge*	*Lge*	F	bLti121*ϕ	0.787 (0.543,0.962)	***Lge***	*Lti*	*Lti*
bLge39*	0.645 (0.361, 0.896)	*Lge*	---	---	bLti135*	0.524 (0.276,0.771)	*Lti*	*Lti*	F
bLge42*ϕ	0.494 (0.257, 0.732)	*Lge*	***Lti***	***Lti***	bLti137*	0.244 (0.002,0.514)	*Lti*	*Lti*	*Lti*
bLge46*ϕ	0.327 (0.052, 0.626)	***Lti***	*Lge*	***Lti***	bLti138*	0.361 (0.017,0.664)	*Lti*	---	F
bLge47*	0.703 (0.447, 0.917)	*Lge*	*Lge*	F	bLti140*	0.296 (0.081,0.549)	*Lti*	*Lti*	*Lti*

Table 3 (Cont.)

Leopardus geoffroyi					*Leopardus tigrinus*				
	I	II				I	II		
ID	Microsatellites	mtDNA	X chr	Y chr	ID	Microsatellites	mtDNA	X chr	Y chr
bLge49ϕ	0.773 (0.403, 1.000)	*Lge*	*Lti*	*Lge*	bLti141*	0.408 (0.177,0.661)	*Lti*	---	---
bLge72*ϕ	0.166 (0.000, 0.462)	***Lti***	---	***Lti***	bLti149*	0.458 (0.157,0.742)	*Lti*	---	---
bLge73*	0.404 (0.166, 0.663)	*Lge*	---	*Lge*					
bLge74*ϕ	0.365 (0.121, 0.635)	***Lti***	*Lge*	**F**					
bLge75*	0.606 (0.308, 0.891)	*Lge*	*Lge*	F					
bLge76*	0.526 (0.244, 0.799)	*Lge*	*Lge*	*Lge*					
bLge78*	0.765 (0.536, 0.945)	*Lge*	*Lge*	F					
bLge79*ϕ	0.653 (0.366, 0.900)	***Lti***	***Lti***	***Lti***					
bLge80ϕ	0.942 (0.781, 1.000)	***Lti***	*Lge*	***Lti***					
bLge89*	0.752 (0.507, 0.947)	*Lge*	*Lge*	---					
bLge90*ϕ	0.560 (0.288, 0.824)	*Lge*	*Lge*	***Lti***					
bLge91*	0.645 (0.390, 0.865)	*Lge*	*Lge*	*Lge*					
bLge93*ϕ	0.390 (0.117, 0.692)	***Lti***	*Lge*	**F**					
bLge94*	0.535 (0.282, 0.774)	*Lge*	*Lge*	F					
bLge96*	0.549 (0.285, 0.795)	*Lge*	---	---					
Lge(Nid11)ϕ	0.979 (0.844, 1.000)	***Lti***	**---**	---					

* Evidence of hybridization based on microsatellite data; ϕ Evidence of hybridization based on molecular sequences.

Note: F = Female; *Lge* = *L. geoffroyi* specific haplotype; *Lti* = *L. tigrinus* specific haplotype.

Table 4. Measurements used for the morphological classification of *Leopardus tigrinus* and *L. geoffroyi* individuals from Rio Grande do Sul state, southern Brazil [mean (standard deviation), minimum – maximum range in cm].

Variable	*L. geoffroyi*	*L. tigrinus*	P
Body			
1 – Total Body Length (TBL)	94.65 (6.84), 83 - 104	84.24 (4.74), 78 - 96	< 0.001
2 – Body Length (BL)	50.30 (3.90), 42 - 56	44.21 (3.08), 39 - 50	< 0.001
3 – Tail Length (TL)	31.59 (3.18), 26 - 36	28.45 (2.20), 25 - 34	< 0.001
4 – Head Length (HL)	12.76 (0.86), 11 - 14	11.58 (0.82), 10 - 13	< 0.001
5 – Weight (W)	3.84 (0.84), 2.5 - 5.1	2.81 (0.44), 2.00 - 3.50	< 0.001
6 – Shoulder High (SH)	27.74 (2.36), 23 - 32	24.66 (1.76), 22 - 28	< 0.001
7 – Neck Circumference (NC)	18.93 (2.08), 15 - 24	16.79 (1.46), 13.5 - 19	< 0.001
8 – Breast Circumference (BC)	28.50 (3.24), 24 - 36	25.08 (2.31), 21 - 29	< 0.001
9 – Head Circumference (HC)	22.34 (1.64), 19 - 26	20.26 (1.28), 18 - 23	< 0.001
10 – Posterior Length Foot (PLF)	11.88 (0.89), 10 - 13.5	10.96 (0.72), 10 - 12.5	< 0.001
11 – Ear Length (EL)	4.04 (0.65), 3.19 – 5.45	3.88 (0.44), 3.21 – 4.82	0.363
Anterior and Posterior Limbs			
12 – Anterior Paw Length (APL)	3.18 (0.25), 2.61 - 3.51	2.98 (0.21), 2.57 - 3.30	0.007
13 – Anterior Paw Width (APW)	2.84 (0.28), 2.30 - 3.43	2.60 (0.17), 2.30 - 2.90	0.002
14 – Posterior Paw Length (PPL)	3.33 (0.28), 2.78 - 3.79	3.03 (0.24), 2.63 - 3.50	< 0.001
15 – Posterior Paw Width (PPW)	2.60 (0.27), 2.20 - 3.20	2.38 (0.20), 2.05 - 2.80	0.003
16 – Anterior Footpad Length (AFL)	1.55 (0.17), 1.21 - 1.78	1.40 (0.13), 1.19 - 1.59	0.002
17 – Anterior Footpad Width (AFW)	1.91 (0.23), 1.50 - 2.30	1.66 (0.18), 1.27 – 1.97	< 0.001
18 – Posterior Footpad Length (PFL)	1.47 (0.13), 1.19 - 1.66	1.34 (0.09), 1.15 - 1.50	< 0.001
19 - Posterior Footpad Width (PFW)	1.76 (0.21), 1.34 - 2.05	1.57 (0.08), 1.37 - 1.72	< 0.001
20 – Anterior Toe Length (ATL)	1.00 (0.09), 0.80 - 1.13	0.93 (0.08), 0.80 - 1.05	0.018
21 – Anterior Toe Width (ATW)	0.59 (0.06), 0.50 - 0.73	0.53 (0.05), 0.43 - 0.62	< 0.001
22 – Posterior Toe Length (PTL)	1.13 (0.10), 0.98 - 1.4	1.03 (0.08), 0.89 - 1.15	0.001
23 – Posterior Toe Width (PTW)	0.60 (0.06), 0.52 - 0.70	0.55 (0.06), 0.46 - 0.68	0.01
Tooth			
24 – Upper Canine Width (UCW)	0.34 (0.05), 0.27 - 0.42	0.28 (0.03), 0.22- 0.32	< 0.001
25 – Lower Canine Width (LCW)	0.34 (0.04), 0.27- 0.41	0.29 (0.03), 0.24 - 0.33	< 0.001
26 – Upper Fourth Premolar (UFP)	1.19 (0.07), 1.07- 1.34	1.05 (0.06), 0.92 - 1.15	< 0.001

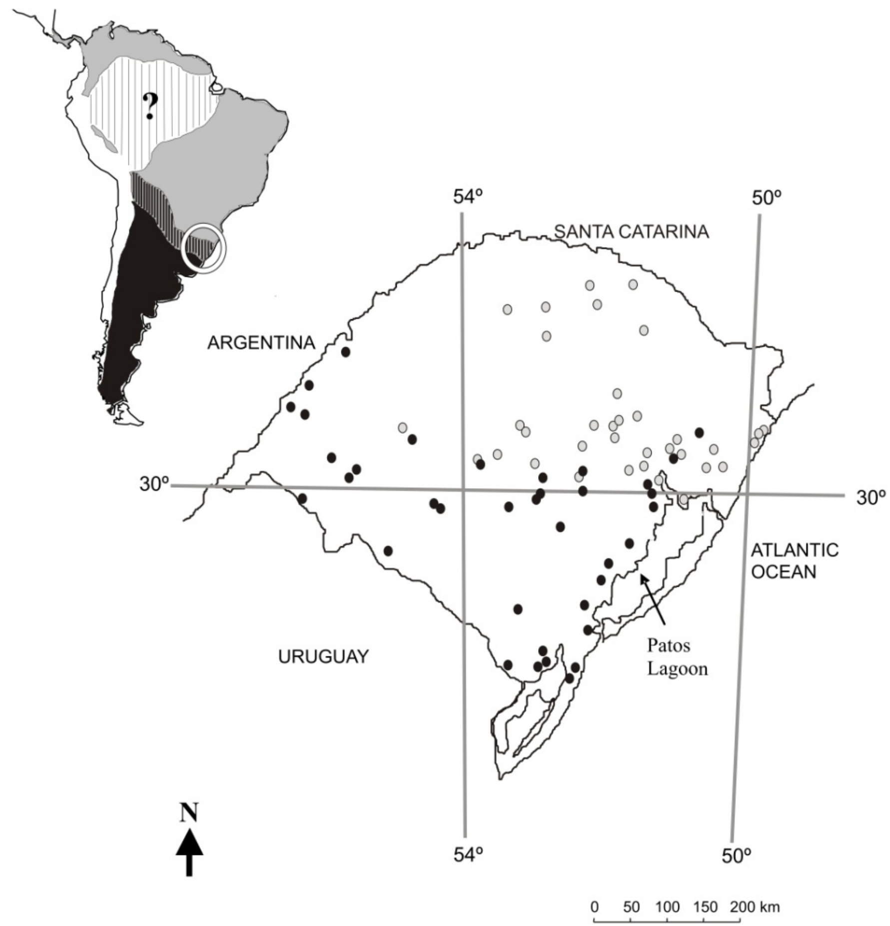


Figure 1 – Capítulo III

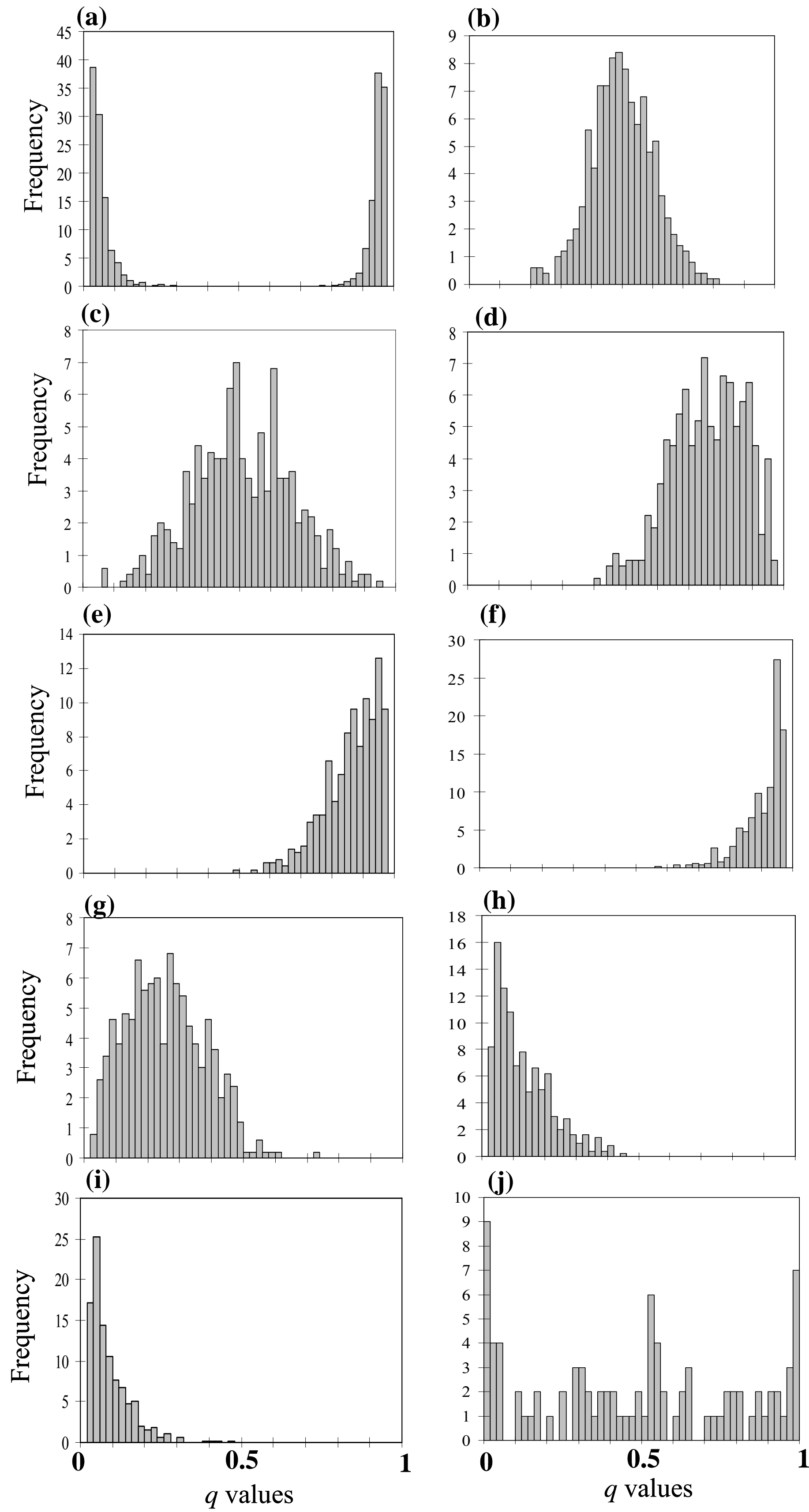


Figure 2 – Capítulo III

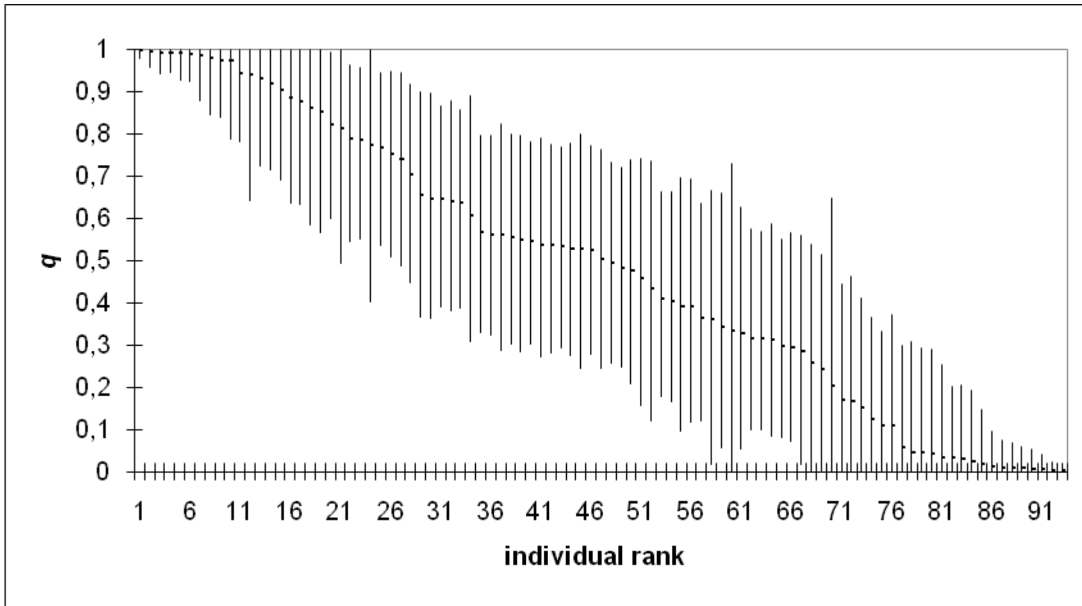
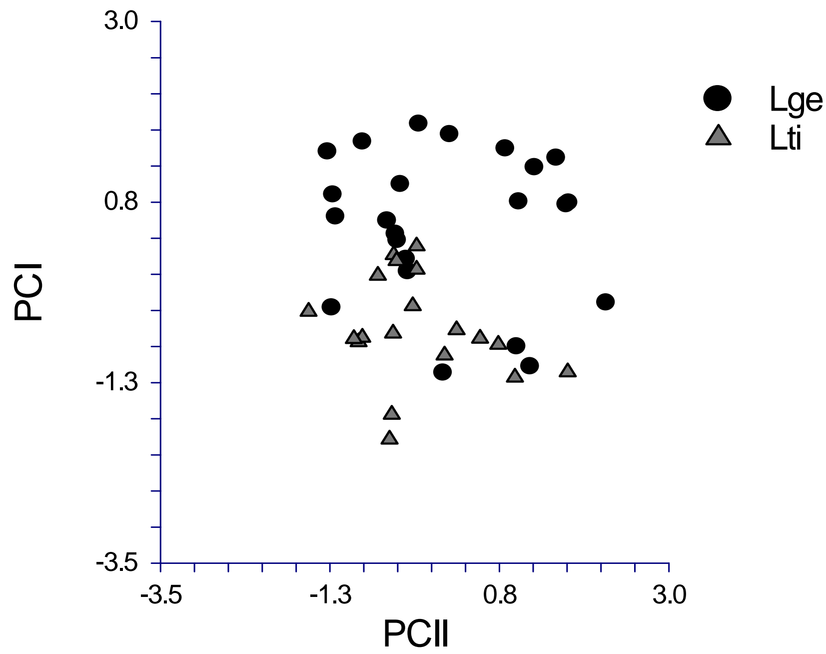


Figure 3 – Capítulo III

A)



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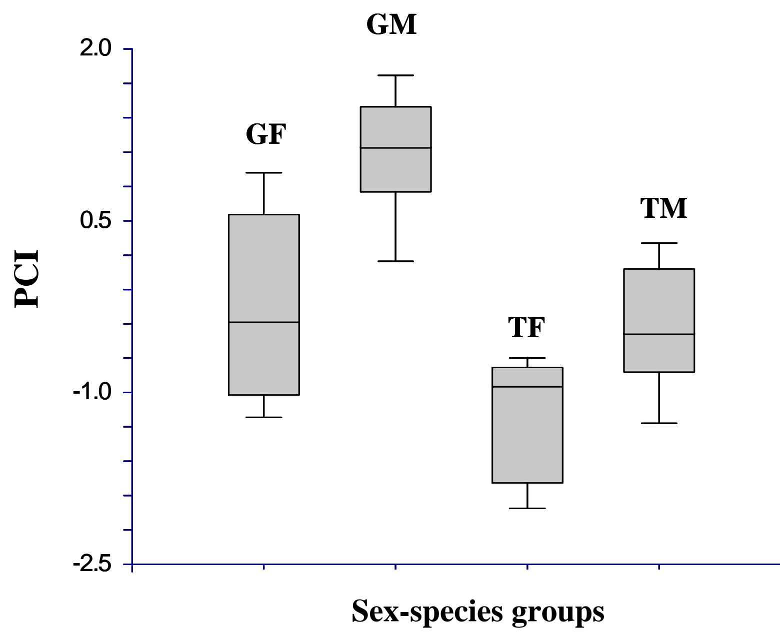

Figure 4 – Capítulo III

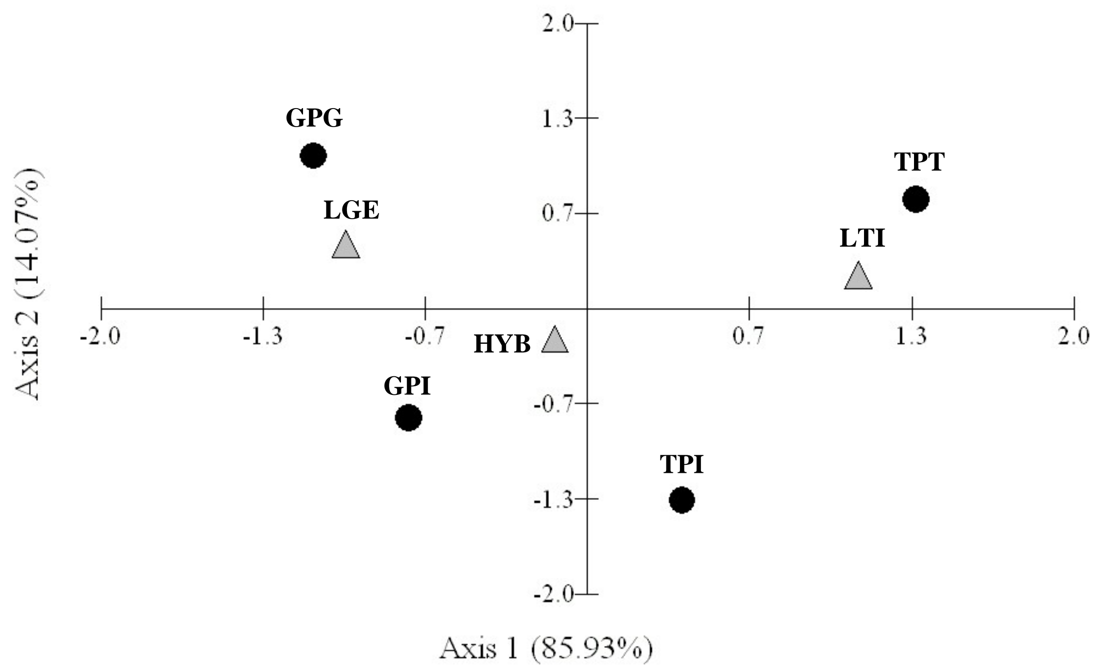

Figure 5 – Capítulo III

CAPÍTULO IV

MANUSCRITO EM PREPARAÇÃO

Spatial distribution, habitat association and trophic niche of *Leopardus tigrinus* and *L. geoffroyi* (Carnivora, Felidae) in their geographic contact zone in southern Brazil

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Running title: Distribution and diet of *L. tigrinus* and *L. geoffroyi* in southern Brazil.

Abstract

Leopardus tigrinus and *L. geoffroyi* are two small cat species that present basically parapatric distributions in the Neotropical Region, with a restricted contact zone in Rio Grande do Sul (RS) state, in southernmost Brazil. This pattern of segregated distribution has been suggested to be maintained mainly by association to different habitats and/or by competitive exclusion. In this study we have compiled 95 geographic records of occurrence of *L. geoffroyi* and 75 of *L. tigrinus* in RS state to evaluate their spatial distribution and to test for differences in habitat association. We also performed a dietary analysis of both species based on 30 digestive tracts, to evaluate their trophic niche overlap in this contact zone and consequently their probability of competing for food resources. Our results show a significant association of *L. geoffroyi* with open vegetations, such as grassland formations, in the southern part of the state, and *L. tigrinus* with more forested environments in its northern portion. The niche overlap between these species' diet was relatively low (O = 0.50), with *L. geoffroyi* preying frequently on animals that are typical of open areas, and *L. tigrinus* focusing on food items that occur mostly in forested habitats. In this geographic area, a hybrid zone between *L. tigrinus* and *L. geoffroyi* has been previously documented, and we included the available hybrid identification (based on genetic analyses) to evaluate patterns of spatial segregation between pure and admixed individuals.

Introduction

The southernmost Brazilian state, Rio Grande do Sul (RS), has the highest diversity of felid species in the country, harboring eight of the ten Neotropical cats: *Leopardus pardalis*, *L. wiedii*, *L. tigrinus*, *L. colocolo*, *L. geoffroyi*, *Puma yagouaroundi*, *P. concolor* and *Panthera onca*. This diversity may leads to extensive range overlap in this area, and may induce character displacement between similar species in the zones of sympatry. The spatial distribution of these species at the RS state has documented by Indrusiak & Eizirik (2003) based on a compilation of the evidence then available. However, current knowledge on most aspects of these species' biology and distribution is still scarce, and available range maps are often derived from a small number of records in many areas. Moreover, in most cases the ecological factors that determine species' distribution have not been assessed or compared. Information regarding inter-specific differences in resource use, which may be critical to understand patterns of spatial segregation, habitat association and niche partitioning, is also extremely scarce for these species in this region and elsewhere in their distribution, hampering the design of adequate conservation plans for these threatened felids (Oliveira 2006).

Two of the small felid species that occur in RS state, *L. tigrinus* and *L. geoffroyi*, have had their exact limits of distribution in the area recently defined by Eizirik *et al.* (2006). These two species, which exhibit an essentially parapatric distribution in the Neotropical region (Figure 1A), have a narrow (usually < 100 Km wide) range overlap in the central area of RS state. *L. tigrinus* is mostly restricted to the northern and central-northern areas of the state, whereas *L. geoffroyi* occurs in the central-southern areas (Figure 1B). In this study we attempt to address the question of what factors are involved in maintaining this spatial segregation, and avoiding a more extensive area of contact and sympatry. Ecological aspects, such as strong associations with different habitats, climates, altitude and prey, as well as the occurrence of competition between the two species, are potential factors involved in the observed pattern of geographic segregation. A difference in habitat association would be the first candidate to explain this pattern. In general, *L. tigrinus* is associated with areas of humid evergreen and subtropical forests that predominate in the northern and eastern regions of South America, while *L. geoffroyi* seems to prefer open areas of scrubby woodland and grassland predominant in southern South America (Nowell & Jackson 1996, Nowak 1999). In agreement with this perceived pattern, in RS state the regions where *L. tigrinus* has been recorded are historically

characterized by forested areas (connected to the Atlantic Forest biome), while the areas of *L. geoffroyi* occurrence are represented by more open habitats (connected to the “pampas” biome continuous with adjacent portions of Uruguay and Argentina). The central area of the state, where the contact between the two species occurs, is exactly the region of transition between these two types of environment (Eizirik *et al*. 2006). However, the existence of occurrence records for both species in a variety of environments, including savannas and semiarid thorny scrub for *L. tigrinus* (Oliveira 1994, Nowell & Jackson 1996) and dense forest regions for *L. geoffroyi* (Ximenez 1975, Johnson & Franklin 1991), pose challenges to this hypothesis of simple habitat preference as a basis for segregation, and suggest that additional factors are required to explain the maintenance of the geographic separation between these two cat species in RS state.

Inter-specific competition for food resources or strong preference for alternative prey items that are themselves geographically segregated are also potential factors conditioning the maintenance of this pattern of spatial distribution in RS state. However, studies describing the diet of *L. tigrinus* and *L. geoffroyi* are so far very scarce, including analyses of stomach material with a very small sample (2-4 individuals each) (Gardner 1971, Ximenez 1982), and few analyses of faecal contents using larger samples (Johnson & Franklin 1991, Olmos 1993, Novaro *et al*. 2000, Manfredi *et al*. 2004). Moreover, those studies tended not to address both species at the same time, nor did they focus on their diets in areas of sympatry. Several of these studies presented the prey item identification at family level (especially in the case of scats), which limits the analysis of trophic niche overlap between closely related and sympatric predators. This limitation reflects the difficulty to identify at a finer level the highly digested material found in faeces, a challenge that can be addressed with the analysis of other kinds of samples, such as digestive tracts (*e.g*. Bisbal & Ojasti 1980, Bisbal 1986, Facure & Monteiro Filho 1996). In the case of contact zones, it is critical to evaluate the local diets of the species involved to understand the level of trophic niche segregation and of competition potential between them, and their consequent influence on ecological and geographical segregation between the species.

The complexity of the relationships between *L. tigrinus* and *L. geoffroyi* in their contact zone is increased due to the extensive hybridization and introgression detected between them in RS state (Trigo *et al*. 2008, Trigo *et al.* in prep. Capítulo II, III). According to these studies, the RS population is characterized by a hybrid swarm, where different recombinant forms predominate relative to pure individuals. This extensive rate

of hybridization may indicate the absence of selection and post-zygotic barriers against the hybrids. However, the detection of morphologic differentiation between the two species in the vicinities of their contact zone suggests that selective pressures may play an important role in maintaining phenotypic integrity of the parental species in spite of the extensive hybridization (Trigo *et al.* in prep. Capítulo III). Similarly, the strong concentration of introgression found by Trigo *et al.* (2008, in prep. Capítulo II) in RS state, which represents a very limited area relative to the broad geographic distribution of both species in South America, may indicates the presence of some type and level of selection against hybrids, and consequently against a stronger geographic spread of the introgression (Barton & Hewitt 1985, Harrison 1993, Barton 2001). The patterns of selection acting on hybrid zones may be related to (i) an endogenous (*i.e.* habitat-independent) selective force, where an intrinsic reduction in hybrid fitness may be maintaining the hybrid zone stability; (ii) exogenous (habitat-dependent) selection, where the hybrids are more adapted to heterogeneous habitats and/or less adapted to the parental habitats; or (iii) a combination of these types of selection. As any information is currently available regarding reduction in fertility and viability of hybrid forms, as well as habitat requirements of the parental and admixed individuals, the characterization of these aspects is one of the major research avenues involved in shedding light on the structure and dynamics of this hybrid zone.

Considering these gaps in the knowledge of the ecology of these two cat species, the principal goal of this project was to perform a detailed characterization of the spatial distribution of *L. tigrinus* and *L. geoffroyi* in their contact region in RS state, testing for different habitat associations and trophic niche segregation. The latter set of analyses was performed with the objective of verifying the competitive potencial between these two closely species, and of contributing to the understanding of their dynamics of ecological segregation. We also included the information on hybrid identification performed by Trigo *et al.* (in prep. Capítulo III) to evaluate the distribution of genetically identified hybrids relative to vegetational patterns, and to verify the existence of any spatial segregation relative to pure individuals, thus testing the hypothesis of habitat-dependent selection acting against hybrids.

Materials and Methods

Sample collection

For this study we compiled all the available occurrence information for *L. geoffroyi* and *L. tigrinus* in RS state (Eizirik *et al*. 2006, Trigo *et al.* 2008, Trigo *et al.* in prep. Capítulo II, III). The total sample comprised 75 *L. tigrinus* records from 50 geographic points and 95 *L. geoffroyi* from 57 geographic points (Table 1). The records included here involve field collections such as animals captured for ecological studies, individuals killed by rural owners and road killed, captive individuals with known origin, indirect information obtained from verified photographs and confirmed records of specimens deposited in Brazilian museums. Some of the individuals that were collected from road kills or killed by rural owners presented intact digestive tracts, and were then included in the dietary analysis.

Spatial distribution and habitat association

Due to the different kinds of records included in this study, our location points include cases in which precise geographic coordinates were obtained and others where the data on origin was limited to the municipality where the animal had been found (Table 1). For individuals with only municipality information we arbitrarily chose to use coordinates from its center. The geographic coordinates in decimal degrees were primarily obtained and standardized by GPS TRACKMAKER (Ferreira Júnior, O.: http:// www.gpstm.com). For the analysis of spatial distribution and habitat association of *L. tigrinus* and *L. geoffroyi*, the geographic coordinates of individual records were plotted onto maps of RS state vegetation using ARCVIEW 3.2 (ESRI, Redlands, CA, USA). Different classifications of the state's phytoecological regions are described in the literature, including classifications with varying levels of detail. Here we have used a modified classification and characterization from IBGE (1986, 1992, 1993) (Figure 2). According to these documents, RS state is characterized basically by two general plant formations. The southern half of the state is occupied predominantly by grassland formations, while the northern half was historically covered predominantly by forested vegetation.

The grassland formations are represented here by the phytoecological regions of Steppe (STP) and Steppical Savanna (STS). The Steppes are the most common formation in RS state, and similar to the more restricted Steppical Savanna, with the main differences between them related to some characteristics of soil and composition of the grassland

communities. Both regions are characterized by a double seasonality with a cold period that provokes physiological dryness and a short period of hydric deficit during the summer. The predominant vegetation is herbaceous (grasses) with a variable concentration of shrubby and arboreal vegetation. Within the STP formation, despite the high anthropogenic activities (mainly cattle-ranching) in the last few centuries, three subgroups may still be distinguished according to the density of arboreal vegetation: Arboreal Steppe, Park Steppe (parkland or dirty fields) and Grass Steppe (clean fields).

The northern half of the state was originally covered by forest, which includes the phytoecological regions of Dense and Mixed Ombrophilous Forest and Decidual and Semidecidual Seasonal Forests. The Dense Ombrophilous Forest (DOF) or Tropical Pluvial Forest is constituted by a dense and closed arboreal cover influenced by a tropical and humid climate, practically without a dry period. The Mixed Ombrophilous Forest (MOF) or Subtropical *Araucaria* Forest is similar to the DOF, but is characterized mainly by the dominance of *Araucaria angustifolia* in the upper canopy. The Decidual (DSF) and Semidecidual Seasonal Forest (SSF) are characterized by two distinct thermal periods during the year: one tropical with an intense and wet summer, and another subtropical presenting no dry periods but with physiological dryness provoked by intense cold in the winter. Its structure is represented by two distinct arboreal strata: one emergent, open and deciduous, and another continuous with the predominance of species with perennial leaves. The dominant stratum is represented by more than 50% of deciduous trees (*i.e.* shedding their leaves during the cold period) in the DSF and about 20-50% in the SSF.

In addition to these predominant formations, two phytoecological regions with mixed herbaceous, shrubby and arboreal vegetations under particular ecological conditions are mentioned by IBGE (1986, 1992, 1993). The first comprises the Pioneer Formations (PF) on the coast of the state, which is constituted of unstable terrain, constantly renewed with fluvial and maritime depositions, and covered with vegetation in constant succession. The second region comprises the Ecological Tension Areas (ETA) concentrated in the central region of the state, and characterized by the interpenetration of characteristic floras of two or more phytoecological regions (which constitutes in this case the region of contact between STP and DSF).

To investigate association patterns of *L. tigrinus* and *L. geoffroyi* to these different RS's phytoecological regions recognized here, three approaches were used. The first one employed the record points (exact coordinates), where each individual was associated to only one vegetation category. For the second and third analyses we established buffers of 3

Km^2 and 6 Km^2, respectively, around each coordinate point to consider the possible movement of each specimen around the collection site, and to reduce the influence of errors induced by more imprecise geographic coordinates obtained from samples with only municipality-level origin information. In the latter two sets of analyses, each individual can be associated to one or more vegetation category. The range of the buffers was defined based on the few existing studies involving the definition of *L. geoffroyi* home ranges: Johnson & Franklin (1991) documented an average home range of 6 Km^2 for individuals of *L. geoffroyi* at the Torres del Paine Park, Southern Chile; Manfredi *et al*. (2006) found ranges of 2.42 - 3.42 Km^2 in the Argentinean grasslands; and the only study with an *L. geoffroyi* population from RS state (F.D. Mazim, unpublished data), which has documented preliminary data of an average home range of 3.1 Km^2. All the three levels of analysis were tested for differences in species-habitat association using chi-square tests. These tests were followed by a residual analysis, which was performed with the objective to evaluate the habitat categories that were most associated with each of the species.

After the analysis of species-habitat associations, we selected a subset of the records that had been included in the genetic analysis conducted by Trigo *et al.* (in prep. Capítulo III) to evaluate the possibility of habitat segregation between pure and hybrid individuals. This sub-sample comprised 90 individuals from both cat species (48 *L. geoffroyi* and 42 *L. tigrinus*); 34 and 20 of these individuals identified morphologically as *L. geoffroyi* and *L. tigrinus*, respectively, were inferred to be hybrids based on molecular analyses. We included this identification in our data descriptions, and performed the same analyses described above, evaluating the spatial distribution of hybrids and testing for possible habitat segregation between the genetic categories of pure and hybrid individuals. We also performed a Correspondence Analysis with the statistical software MVSP 3.13 (Kovach 1998), as a multivariate technique to simultaneously order the genetic (pure *L. geoffroyi*, pure *L. tigrinus* and hybrids) and the habitat categories. The result of this ordination is presented as a bidimensional plot where the habitats and species are represented by coordinate points. Proximity of habitat categories to genetic categories on the plot indicates which of the former were used with higher frequency by the latter.

Trophic Niche

To perform a dietary analysis of *L. tigrinus* and *L. geoffroyi* in RS state, we collected the entire digestive tract from all available animals that were found dead (mostly road-killed) within the sample included in the spatial distribution and habitat association

analyses. For these individuals (see Table 1 for geographic origin of the samples) we prepared skins for deposit in museum collections, and removed the entire digestive tract (including stomach, intestinal and rectal [fecal] contents). Additionally, we included digestive tracts from two *L. tigrinus* and one *L. geoffroyi* individual collected by collaborators, totaling 13 *L. tigrinus* and 17 *L. geoffroyi* samples. This constitutes the largest sample of this type of material (allowing in-depth dietary analysis relative to feces) that has so far been collected for these species (*e.g.* Ximenez 1982, Novaro *et al*. 2000).

The food contents were washed and sieved (mesh width 0.8 mm) under running water to separate the non-digested material: teeth, jaw fragments, nails, scales, bird feet, beaks, feathers, plant material and in some cases the entire bodies of prey. The retrieved items were initially classified in broad taxonomic categories such as mammals, birds, reptiles, amphibians, fish, invertebrates and plant material, and subsequently subjected to finer analyses aiming to achieve the most detailed identification possible, with the aid of reference collections and consultation with taxon experts.

The dietary data were analyzed by assessing the frequency of occurrence of each item, as well as its relative frequency. The former statistic is the proportion of samples in which a particular item has been found, and is obtained by dividing the number of samples that contain that food item by the total number of samples. The relative frequency of a dietary item is measured as its proportion of occurrence relative to the total number of identified items; it is obtained by dividing the number of times a particular item has been recorded by the total number of items (including multiple occurrences per item). After initial comparisons between the two statistics, the relative frequency was used for the subsequent analyses.

The niche breadth was calculated using Levins's Index (Krebs 1989): $BA = (B - 1)/(n-1)$; where BA = Levins's Index standardized by the number of items, n = total number of recognized prey categories, and $B = 1/\sum pi^2$, where pi is the frequency of the item in the total sample. The values generated from this index can range from 0 (minimum breadth, few types of prey consumed at high frequencies) to 1 (maximum breadth or equi-distribution of the items consumed).

The trophic niche overlap between *L. tigrinus* and *L. geoffroyi* was calculated using Pianka's index (Pianka 1973): $O = \sum p1i\ p2i/(\sum (p1i^2)\ (p2i^2))^{1/2}$, where p1i and p2i represent the relative presence of prey "i" on the diet of predators 1 and 2. This index also takes values between 0 and 1, where a value of 0 indicates no resource sharing between the species, 1 indicates complete overlap, and intermediate values show partial overlap in

resource utilization. We tested the significance of inter-specific niche overlap by comparing observed values with those obtained by randomizing the original matrices (10,000 iterations) using ECOSIM 7 (Gotelli & Entsminger 2001). To distinguish the dietary composition of *L. tigrinus* and *L. geoffroyi* we performed a chi-square test. All analyses were conducted separately considering broad and detailed taxonomic levels of prey identification. Invertebrates and plant items were not included in the computation of relative frequencies, niche amplitude or niche overlap.

Results

Spatial distribution and habitat association

L. tigrinus records were restricted to the central-northern region of RS, while *L. geoffroyi* individuals were restricted to the central-southern area (Figure 2A). Three points were geographic outliers relative to this pattern of segregation: two *L. tigrinus* were recorded in the southeastern portion of the state (in the PF habitat) and one *L. geoffroyi* in the MOF habitat in the northern part of the state. The simultaneous occurrence of both cats was recorded in only ten municipalities, eight of which were located in the central area of the state (Cachoeira do Sul, Eldorado do Sul, Guaíba, Porto Alegre, Rio Pardo, Santa Cruz do Sul, Santa Maria and Triunfo) and two from the southeastern part of RS (Camaquã and Rio Grande).

To perform the habitat association analysis, we grouped all samples from the three steppe categories (represented in the vegetation map) into a single unit named “Steppe” (STP). Likewise, we consolidated the two Ombrophilous Forests (Mixed and Dense) into a singe category named “OMF”. This procedure was performed to enhance the power of statistical comparisons, given the low number of records in the Arboreal and Park Steppe categories, as well as in the DOF habitat. Further analyses were then conducted using seven phytoecological categories: 1) Ombrophilous Forest (OMF), 2) Decidual Seasonal Forest (DSF), 3) Semidecidual Seasonal Forest (SSF), 4) Steppes (STP), 5) Steppical Savanna (STS), 6) Pioneer Formations (PF) and 7) Ecological Tension Area (ETA). Following this classification, the three levels of analysis (point location, 3 Km^2 buffer and 6 Km^2 buffer) yielded very similar results. Significant differences in species-habitat association were detected by all of them (point locations: $\chi^2 = 67.17$; 3 $Km^2 = 65.41$; 6 $Km^2 = 66.34$; d.f. = 6; $p < 0.001$ for all comparisons), with the indication of the same patterns of species-habitat association by the residual analysis (data not shown).

Additionally, we performed a chi-square test to evaluate the minor differences among the three levels of analysis for each species and found no statistical significance (*L. tigrinus*: $\chi^2 = 5.76$, d.f. = 10, $p > 0.20$; *L. geoffroyi*: $\chi^2 = 1.55$, d.f. = 12, $p > 0.20$). The absence of significant differences among these three approaches, and the identical interpretation of the data with any of them, indicates that all three are appropriate to evaluate habitat associations of *L. tigrinus* and *L. geoffroyi* in RS state. However, considering the large number of records in our compiled data that include origin information only to municipality level, we considered it appropriate to use the version with 3 km^2 buffers to perform subsequent analyses. This particular buffer radius was chosen based on the only available telemetry study for these species in RS state (F. D. Mazim unpublished data).

Considering the 3 km^2 buffer option, both species were recorded in all habitat categories, except the STS where just *L. geofroyi* individuals were found. However, the frequency of occurrence was markedly different between the species. *L. tigrinus* was predominantly associated with OMF (30.68%), DSF (29.55%) and ETA (12.05%), while *L. geoffroyi* were more strongly associated with STP (38.78%), SSF (23.47%), PF (10.20%) and STS (8.16%) (Figure 3). Forested habitats were the main vegetation type associated with *L. tigrinus*, totaling 60.23% of its records, while the grassland formations were the main vegetation type associated with *L. geoffroyi* (46.94%). The chi-square test followed by the residual analysis indicated a positive and significant association between *L. tigrinus* and DSF (Raj = 2.92, $p < 0.05$) and OMF (Raj = 5.65, $p < 0.05$) and also between *L. geoffroyi* and STP (Raj = 3.47, $p < 0.05$), SSF (Raj = 3.94, $p < 0.05$) and STS (Raj = 2.74, $p < 0.05$).

We subsequently included the genetic identification of the sampled individuals as putatively pure *L. tigrinus*, pure *L. geoffroyi* and hybrids, according to Trigo *et al.* (in prep. Capítulo III) and performed the same set of analyses. The hybrid distribution was concentrated in the central and the southeastern part of the state, with only two hybrids recorded at the extreme north of RS. The greatest prevalence of hybrids was identified in the central area of the state (76.4% of the identified hybrids) in an approximately 160 Km long geographic stretch (Figure 2B). Significant differences in habitat association were detected in this analysis ($\chi^2 = 28.95$, d.f.=12, $p < 0.01$). However, according to the residual analysis, these differences were associated again to the same significant associations of *L. geoffroyi* and *L. tigrinus* to different specific habitats. The genetically identified hybrids were recorded in all different phytoecological formations and presented no significant

association with any specific habitat category. However, the Correspondence Analysis showed the hybrid category in an intermediate position between the habitats mainly associated to each one of the two cat species (Figure 4).

Trophic Niche

Mammals were by far the main prey items of both species, being present in all samples and represented basically by rodents (Table 2). Birds were recorded for both species, with a higher frequency of occurrence in the *L. tigrinus* sample. Amphibians, reptiles and invertebrates were present at very low frequencies, with the former recorded only for *L. geoffroyi*. Plants were observed in almost all of the *L. tigrinus* samples (92.31%) and at a lower frequency for *L. geoffroyi* (70.59%).

The analyses performed with broad taxonomic categories of prey items revealed very similar frequencies of occurrence in the *L. tigrinus* and *L. geoffroyi* samples (Figure 5A), with no significant differences detected between the diet of the two species ($\chi^2 = 1.53$, d.f. = 3, $p > 0.20$). According to this same categorization, niche breath was slightly broader for *L. geoffroyi* (BA = 0.22) than for *L. tigrinus* (BA = 0.15), and food niche overlap was almost complete (O = 0.99, $p < 0.001$). With the subdivision of the prey items into more specific categories, the niche breath increased to 0.35 for *L. geoffroyi* and 0.33 for *L. tigrinus*, while niche overlap decreased to 0.50 ($p < 0.05$). This more specific categorization allowed the detection of significant differences in the diets of the two cat species ($\chi^2 = 41.25$, d.f. = 18, $p < 0.01$), suggesting a segregation in the taxa that they consumed the most (Figure 5B). Among the mammalian prey items, five taxa were found exclusively in the *L. tigrinus* diet: *Delomys dorsalis*, *Oryzomys* sp., *Oxymycterus* sp., *Rattus rattus* and *Monodelphis* sp., while *Holochilus brasiliensis* and *Calomys* sp. were found only in the *L. geoffroyi* diet. The mammalian prey items that were shared also showed different relative frequencies for the two cats. While *Akodon* sp., *Mus musculus* and *Oligorymozys* sp. were predominantly consumed by *L. tigrinus*, *Cavia* sp. was almost exclusively consumed by *L. geoffroyi*. The same could be noted for the bird, reptile and amphibian classes, where Columbiformes, Cuculiformes, Tinamiformes and Serpentes were exclusively recorded in the *L. tigrinus* diet, and Gruiformes, Passeriformes and Anura found only in the *L. geoffroyi* diet.

Discussion

Patterns of Leopardus tigrinus and L. geoffroyi distribution and habitat association in RS state

The geographic distributions of *L. tigrinus* and *L. geoffroyi* in the Neotropical Region are closed connected to the two principal biogeographic regions characterized for this area: the Brazilian-Guiana region, in which almost all of Brazil is located, and the Andino-Patagonian region (Fittkau 1969). These regions are defined based on many biogeographic aspects, including climate, geology and vegetation elements that permit the occurrence of different species adapted to these specific conditions. RS state is located at the boundary area between these two biogeographic regions, and consequently also at the point of contact between the distributions of *L. tigrinus* and *L. geoffroyi*. This geographic contact zone has been documented to be restricted to the central area of the state by Eizirik *et al*. (2006), with *L. tigrinus* basically restricted to the central-north area and *L. geoffroyi* to the central-south. Our present results, with an extended data set, corroborate the existence of this segregated geographic pattern, with few municipalities recording the occurrence of the both species. As pointed out in that study, one of the main factors that could maintain this basically parapatric distribution and extremely restricted area of sympatry in central RS, is the occurrence of different habitat associations and preferences. In agreement with this hypothesis, our results showed a significant association of the *L. tigrinus* sample to forested habitats (such as the Ombrophilous and the Decidual Seasonal Forests), and of *L. geoffroyi* to grassland formations (such as the Steppes and Steppical Savanna). These results support the hypothesis that spatial segregation between *L. tigrinus* and *L. geoffroyi* at their contact zone may be in part related to different habitat association. However, a strong association of *L. geoffroyi* with a forest formation (Semidecidual Seasonal Forest), basically restricted to the southeastern state, was also detected, challenging the hypothesis of simple habitat segregation between species in their contact zone. Additionally, in spite of the significant association to different habitats, both species were recorded in practically all types of vegetation, even when the hybrid genetic identification was used (data not shown), thus excluding the possibility that this broad habitat use could be associated to hybrid genotypes. These records may indicate some ecological plasticity of these species with respect to habitat utilization. Manfredi *et al.* (2004, 2006), for example, found some variation in the food habitats and habitat utilization by *L. geoffroyi* in different areas of the Argentinean pampas, which suggests that this

species presents some degree of adaptability. Furthermore, the existence of records in various kinds of environment, including human altered habitats (Bisbal 1989, Oliveira 1994, Olmos 1993, Nowell & Jackson 1996, Eizirik *et al.* 2006), also suggests the existence of this flexibility for both species.

The presence of an ecological plasticity in habitat utilization by the two cats suggests that the strict geographic segregation observed in RS may not be exclusively due to historical habitat preferences, but also to an additional ecological mechanism. According to this hypothesis, the two species may have diverged via an allopatric speciation process, and have evolved and remained in separate areas for most of their history; subsequently, one or both of them suffered a range expansion leading to secondary contact between their populations (Eizirik *et al.* 2006). After such an event, incomplete ecological and/or behavioral differentiation between them would have led to direct competition between the two species in their contact zone, preventing their further expansion. Under such a scenario, the specific habitat association detected here might be mostly a consequence of historical distribution and reciprocal interference, rather than strong habitat preference. Taking into account the available evidence for a demographic expansion in the past history of *L. tigrinus*, and possibly also *L. geoffroyi* (Trigo *et al*. 2008), this hypothesis seems fairly plausible. An additional observation favoring this hypothesis is the fact that currently all phytoecological areas of RS state have been profoundly modified by anthropogenic activities, such as cattle-ranching, agriculture and forest plantations (IBGE 1986), which would likely have changed the pattern of geographic segregation between these species had it been strictly determined by habitat preference alone.

On the other hand, it is also possible that the observation made here of both species occurring in practically all types of habitat may be an artifact of our scale of analysis. Ideally, radio-telemetry studies involving habitat selection should be conducted incorporating information on habitat availability, which can be measured at different levels, such within the home range or in the study area as a whole (Steventon & Major 1992). These levels of analyses may lead to different results with respect to the habitat selection by one species, as exemplified by Manfredi *et al.* (2006), who found variation in habitat selection by *L. geoffroyi* in the Argentinean pampas depending on the level of analysis. As our data included only occurrence points at a broader scale of vegetation elements, some specific requirements probably could not be detected. Additionally, it is possible that some points collected do not reflect the exact habitat utilized by the individuals but only an occasional movement through that landscape. More specific studies

including vegetation maps at a finer scale and higher definition and specification of each habitat category are required to verify the degree of plasticity in habitat utilization by these species. Since the natural areas of RS state are currently extremely modified by human activities, the inclusion of maps (or satellite images) that describe the current status of each area is also strongly suggested, so as to verify the degree of adaptability of these species in occupying such environments.

The trophic niche of L. tigrinus and L. geoffroyi in RS state

Our results on the diet of these felids illustrate the relevance of identifying prey items at the most refined level possible, especially in studies comparing the niche overlap between related species. Carnivore food habits have been studied mainly by analyses of faeces (*e.g.* Bonesi 2004, Manfredi *et al.* 2004, Moreno *et al.* 2006, Phillips *et al.* 2007, Vieira & Port 2007), which often allow the identification of prey items only in broad taxonomic classes. The main advantage of working with faeces resides in the larger sample size that may be collected in comparison to stomach contents, allowing a better assessment of the dietary items and their frequencies. However, despite the difficulty in acquiring a substantial sample of stomachs, this analysis generally provides a more precise identification of prey items. The analysis of our data with subdivisions in major groups (such as mammals, birds, reptiles and amphibians) led to highly different results from those obtained with the subdivision of the prey items into more specific categories, especially for the niche overlap estimation. Considering the analysis with the broad categories, the dietary overlap between the two species was almost complete (O = 0.99), with all prey groups consumed at similar frequencies. This first result would imply the occurrence of a strong competitive potencial between *L. tigrinus* and *L. geoffroyi* with respect to food resources, which in turn might be inferred to cause the observed spatial segregation between these species. However, the analysis employing a more refined level of prey identification showed a great reduction of dietary overlap (O = 0.50). This second result, in contrast to the first scenario, suggested an ecological segregation also in prey utilization, at a level that might allow these cat species to coexist (Zaret & Randi 1971).

A connection may be established between the observed habitat association and prey composition of each of the two cat species. Considering the rodent groups that appeared at high frequencies for each species, we could observe differences in prey utilization. The taxa recorded only in the *L. tigrinus* diet, such as *Oryzomys* sp. and *Delomys dorsalis* are mainly associated with forested environments, while groups such as *Calomys* sp., *Cavia* sp.

and *Holochilus brasiliensis*, recorded almost only in the *L. geoffroyi* diet are predominantly associated to wet grasslands (Emmons 1997, Nowak 1999, Weksler *et al*. 2006). Similarly, *Mabuya dorsivittata*, the lizard identified in the diet of *L. geoffroyi*, is a species predominantly associated to open vegetations (Lema 2002). The two groups frequently consumed by both cats (*Akodon* sp. and *Oligoryzomys* sp.) include common and widely distributed species, occurring in a wide variety of habitats (Emmons 1997, Nowak 1999), which may represent an abundant food resource for both species of felids. Although the observed patterns are intriguing, and prompt the design of additional studies (*e.g.* aiming to identify prey at species level), establishing the cause of such ecological associations remains problematic. Some of the same problems detected in the habitat association analysis are also present in the dietary investigation: (i) is the association of each species with different prey an ecological adaptation of these predators, or is it a consequence of their habitat association (*i.e.* prey are selected based on their occurrence and abundance in the preferred habitat)?; (ii) in turn, could this be only a consequence of the historical geographic distribution of each species, leading them to be in different areas and prey on the respectively most abundant prey? The key question here is determining whether habitat and/or prey associations are causes or effects of the geographic segregation between these two species in this area.

The niche breadth detected was low for the two species based on the two levels of analysis, with very similar values to those estimated by Manfredi *et al.* (2004) for three different populations of *L. geoffroyi* in the Argentina grasslands (0.2, 0.3 and 0.36). This niche breadth indicates a high degree of foraging specialization for these species, mainly for *L. tigrinus*, which showed the lowest values of niche breadth based on both types of analysis. The main items consumed by both species (small mammals, especially rodents) were also in agreement with previous data such as those from Manfredi *et al.* (2006) and Novaro *et al.* (2000) for *L. geoffroyi* in the Argentina grasslands and central Patagonian Steppe, and from Gardner (1971) for *L. tigrinus* at Costa Rica. However, other studies documented other prey groups as the main dietary items of these cats. Franklin & Johnson (1991), for example, found that European hares (*Lepus europaeus*) were the main prey utilized by *L. geoffroyi* in southern Patagonia, and Manfredi *et al.* (2006) documented a *L. geoffroyi* population in the Argentinean grasslands that strongly utilizes large aquatic birds (especially Anatidae and Rallidae). These variations on the main prey utilized by *L. geoffroyi* are suggestive of a certain degree of adaptability of this species, which seems to be able to adjust its predatory behavior to exploit alternative food resources that are locally

or temporally abundant (Manfredi *et al.* 2004, Pereira *et al.* 2006). Likewise, this degree of adaptability seems to also be present in *L. tigrinus,* since Ximenez (1982) and Olmos (1993) documented reptiles instead of small mammals as the main prey item utilized by populations of this species in areas of xeromorphic vegetation in the Brazilian northeastern region.

According to our results and the data available for *L. tigrinus* and *L. geoffroyi* in the literature, despite the apparent association and preference for different prey items and habitats, the two species seem to show some ecological flexibility with regard to food resources and habitat utilization. If this is the case, both species might be able to exploit food resources in additional geographic areas of the state, and may be prevented from doing so by reciprocal competition, which leads to the geographic restriction of both of them. On the other hand our data indicate that different prey items are more often consumed by each species, leading to only moderate niche overlap. This observation may be a function of an ongoing process of character displacement, with each species adapting to a different suite of prey to cope with this competition. Additional studies are required to determine whether there is indeed predatory specialization and trophic niche separation between them, which would indicate that dietary competition is not the cause of their geographic segregation. Several ecological factors may interact to promote adaptation of each species to its associated habitat (and respective geographic region), and to maintain its morphological integrity in the face of ongoing hybridization between them (Capítulo III). It is therefore important to consider multiple ecological aspects, as well the genetic identification of pure and hybrid animals in the attempt to characterize the interactions between these species in this area.

Spatial distribution and habitat association of hybrids

The incorporation of the genetic identification of hybrids in the analyses showed the predominance of them in the two areas of the state where ambiguous phenotypic characteristics had been previously reported: the central (Eizirik *et al.* 2006) and the southeastern regions (Mazim *et al.* 2004). The hotspot of hybrid concentration was identified in a stretch of approximately 160 Km around the contact zone in the central area of the state. Considering the extensive area of distribution for both species (see Figure 1A), this hybrid zone extension can be considered to be very restricted, suggesting the existence of selection acting against hybrids that prevents the expansion of this admixture process (Barton 2001).

The exogenous selection (habitat-dependent or ecotone model), where different genetic combinations are favored in different environments, is normally observed to hold at habitat boundaries, where the pure individuals of each of the parental species are adapted to specific habitats, and hybrid genotypes show higher (or sufficient) fitness within a small area of intermediate habitat (Harrison 1993, Barton 2001). The geographic location of the *L. tigrinus* and *L. geoffroyi* hybrid zone seems to be indeed concentrated at the boundary between the types of environment mainly associated to each of the parental species (see Figure 4). This makes the presence of habitat-dependent selection acting against hybrids seem fairly plausible. It is possible that an environmental gradient in the central and southeastern areas of RS state favors (or tolerates) the occurrence of hybrid genotypes in these regions. Although this pattern of habitat segregation was not clearly observed by all of our analyses (since the hybrids were not significantly associated to any type of phytoecological region included in this study), the overall results are still compatible with this pattern, and should be further investigated with additional sampling and more refined habitat characterization. Considering that all the major phytoecological areas of RS are today greatly altered by anthropogenic activities, the inclusion of these aspects of environmental change is also extremely necessary to evaluate the effect of human-induced habitat alteration in the occurrence of hybridizations events, and to assess the use and adaptation of pure individuals of both species to these modified landscapes.

Figure legends

Figure 1 – Geographic maps showing the *L. tigrinus* (in grey) and *L. geoffroyi* (in black) distribution at South America (A) and at Rio Grande do Sul state based on Eizirik *et al.* (2006) (B). At the map A, the dark grey area indicates the potential contact zone between the two cat species; the circle indicates the Rio Grande do Sul state localization and the area with a question mark at the *L. tigrinus* distribution indicates the area with uncertain occurrence.

Figure 2 – Distribution of the coordinate geographic registers obtained at this study on phytoecological regions of Rio Grande do Sul state. A) Distribution of *L. tigrinus* (white circles) and *L. geoffroyi* (black circles) samples; B) distribution of the genetically identified hybrids (black squares) between *L. tigrinus* and *L. geoffroyi*.

Figure 3 – Frequency of occurrence of *Leopardus tigrinus* and *L. geoffroyi* at the seven habitat categories utilized at this study.

Figure 4 – Correspondence analysis showing the association of the three genetic categories (pure *L. tigrinus* = TIG, pure *L. geoffroyi* = GEO and hybrids = HYB) and the seven habitat categories utilized at this study (Ombrophilous Forest = OMF, Decidual Stational Forest = DSF, Semidecidual Stational Forest = SSF, Steppe = STP, Steppical Savanna = STS, Pioneer Formations = PF and Ecological Tension Area = ETA).

Figure 5 – Frequency of occurrence of each prey item category at *Leopardus tigrinus* and *L. geoffroyi* diet, considering the subdivision in major groups (A); and relative frequency of each prey item category considering the subdivision in more specific groups (B).

Table 1 – Description of the register points collected for *Leopardus tigrinus* and *L. geoffroyi*, including informations of the number of individuals at each point, the origin municipality and geographic coordinates in decimal degrees.

Leopardus tigrinus				*Leopardus geoffroyi*			
Origin municipality	Latitude	Longitude	N° of individuals	Origin municipality	Latitude	Longitude	N° of individuals
Arroio do Meio	-29.32	-51.90	1	Alegrete*	-29.77	-55.78	1
Arroio do Meio*	-29.36	-51.97	1	Alegrete	-29.85	-55.93	1
Arroio do Sal	-29.46	-49.84	1	Alegrete	-29.94	-55.46	1
Arroio do Sal	-29.57	-49.95	1	Alegrete	-29.99	-55.53	1
Cachoeira do Sul	-29.84	-53.03	1	Arambaré*	-30.92	-51.50	1
Cachoeira do Sul*	-30.19	-52.96	4	Arroio Grande*	-32.13	-52.93	7
Camaquã*	-30.94	-51.76	1	Arroio Grande	-32.28	-52.88	1
Carazinho*	-28.26	-52.87	1	Arroio Grande	-32.30	-52.93	1
Derrubadas*	-27.25	-53.85	1	Bagé*	-31.33	-54.10	1
Eldorado do Sul	-30.03	-51.40	1	Barra do Ribeiro	-30.34	-51.42	1
Erechim*	-27.63	-52.27	1	Barro Vermelho*	-30.13	-53.15	1
Erval Seco*	-27.55	-53.50	1	Caçapava	-30.37	-53.35	1
Esmeralda*	-28.05	-51.18	1	Cachoeira do Sul	-29.99	-52.92	2
Estância Velha*	-29.65	-51.19	1	**Cachoeira do Sul***	-30.19	-52.96	9
Estrela*	-29.51	-51.92	1	Cachoeira do Sul	-30.23	-53.02	1
Forquetinha*	-29.35	-52.22	1	**Camaquã**	-30.82	-51.74	1
Garibaldi*	-29.25	-51.64	2	Canela*	-29.35	-50.78	1
Getúlio Vargas*	-27.85	-52.19	1	Canguçú*	-31.38	-52.67	1
Glorinha	-29.88	-50.68	1	Cerrito*	-31.72	-52.80	1
Guaíba*	-30.18	-51.44	2	Charqueadas*	-29.95	-51.62	1
Guaporé*	-28.97	-51.91	2	Cristal	-31.05	-52.03	1
Humaitá*	-27.55	-53.97	1	Dom Feliciano*	-30.70	-52.10	1
Ibarama	-29.38	-53.22	1	Dom Pedrito	-30.89	-55.02	1
Ibarama*	-29.42	-53.16	1	Dom Pedrito*	-30.98	-54.67	1
Itapuã	-30.25	-51.00	1	**Eldorado do Sul***	-30.07	-51.49	2
Lagoa Vermelha	-28.20	-51.53	1	Encruzilhada do Sul*	-30.61	-52.67	1
Machadinho*	-27.59	-51.67	2	**Guaíba***	-30.18	-51.44	1
Maquiné*	-29.62	-50.25	1	Herval*	-31.98	-53.52	1
Montenegro*	-29.70	-51.51	1	Itaqui	-28.87	-56.08	1
Morro Reuter	-29.55	-51.10	1	Itaqui	-29.09	-56.39	1

Table 1 (Cont.)

Leopardus tigrinus				*Leopardus geoffroyi*			
Origin municipality	Latitude	Longitude	Nº of individuals	Origin municipality	Latitude	Longitude	Nº of individuals
Nova Esperança do Sul*	-29.40	-54.82	1	Itaqui*	-29.23	-56.15	1
Novo Hamburgo*	-29.74	-51.05	1	Jaguarão	-32.30	-53.36	1
Palmeira das Missões*	-27.92	-53.39	2	Jaguari	-29.55	-54.68	1
Panambi*	-28.30	-53.50	1	Pantano Grande	-30.16	-52.37	1
Passo Fundo*	-28.27	-52.45	7	Pantano Grande*	-30.27	-52.38	1
Porto Alegre*	-30.03	-51.22	1	Pelotas*	-31.57	-52.36	9
Restinga Seca	-29.72	-53.52	1	Piratini	-31.61	-53.24	1
Rio Grande*	-32.03	-52.12	1	**Porto Alegre***	-30.03	-51.22	1
Rio Pardo*	-29.97	-52.41	1	Quaraí*	-30.27	-56.18	4
Rolante	-29.65	-50.58	1	Rio Grande	-31.88	-52.31	1
Santa Cruz do Sul*	-29.61	-52.39	3	**Rio Grande***	-32.03	-52.12	1
Santa Maria*	-29.78	-53.80	1	Rio Grande	-32.36	-52.50	1
Santo Antônio da Patrulha	-29.88	-50.47	1	Rio Grande	-32.44	-52.55	3
São Francisco de Paula*	-29.45	-50.58	9	**Rio Pardo***	-29.97	-52.41	1
São Pedro de Alcântara	-29.41	-49.86	1	Rosário do Sul*	-30.25	-54.92	1
Sarandi*	-27.93	-52.90	2	**Santa Cruz do Sul***	-29.61	-52.39	1
Soledade*	-28.82	-52.53	1	**Santa Maria**	-29.83	-53.77	1
Triunfo*	-29.86	-51.55	1	Santana do Livramento*	-30.88	-55.53	1
Triunfo	-29.92	-51.77	1	São Borja	-28.43	-55.62	1
Viamão*	-30.08	-51.02	1	São Gabriel	-30.26	-54.52	1
				São Gabriel	-30.32	-54.38	1
				São Gabriel*	-30.33	-54.32	1
				São Gabriel	-30.36	-54.32	1
				São Leopoldo*	-29.76	-51.15	1
				São Lourenço do Sul*	-31.25	-52.13	10
				Triunfo*	-29.86	-51.55	1
				Uruguaiana	-29.97	-56.56	1
Total Individuals			75				95

* Origin information only for municipalities; the geographic coordinates were collected for the central area of each municipality.
Notes: Underlined municipalities indicated the localities from digestive tracts included in diet analysis and municipilaties in bold indicates the simultaneous occurrence of both cat species.

Table 2 – Prey items on *Leopardus tigrinus* and *L. geoffroyi* diet at Rio Grande do Sul state, Brazil.

Prey Items		*L. tigrinus* (n = 13)			*L. geoffroyi* (n = 17)		
Major groups	Minor groups	N	FO (%)	FR (%)	N	FO (%)	FR (%)
Vertebrates							
Mammals	**Total mammals**	**35**	**100**	**81.39**	**31**	**100**	**75.61**
Rodentia							
Cricetidae	*Akodon* sp	6	30.77	13.95	3	11.76	7.32
	Calomys sp.	0	0	0	3	17.65	7.32
	Delomys dorsalis	1	7.69	2.33	0	0	0
	Holochilus brasiliensis	0	0	0	6	17.65	14.63
	Oligoryzomys sp	10	46.15	23.25	6	29.41	14.63
	Oryzomys sp	2	15.38	4.65	0	0	0
	Oxymycterus sp	3	15.38	6.97	0	0	0
Muridae	*Rattus rattus*	5	15.38	11.62	0	0	0
	Mus musculus	4	15.38	9.30	1	5.88	2.44
Caviidae	*Cavia* sp	1	7.69	2.33	7	35.29	17.07
Didelphimorphia							
Didelphidae	*Monodelphis* sp.	1	7.69	2.33	0	0	0
Unindentified mammals		2	15.38	4.65	5	29.41	12.19
Aves	**Total Aves**	**7**	**46.15**	**16.28**	**7**	**29.41**	**17.07**
Columbiformes*		1	7.69	2.33	0	0	0
Columbidae	*Columbina talpacoti*						
Cuculiformes*		1	7.69	2.33	0	0	0
Cuculidae	Unindentified						
Gruiformes*		0	0	0	2	11.76	4.88
Rallidae	*Laterallus sp*						
Passeriformes*		0	0	0	3	11.76	7.32
Furnariidae	Unindentified						
Thamnophilidae	*Thamnophilus ruficapillus*						
Unindentified Passeriforme							

Table 2 (Cont.)

Prey Items		*L. tigrinus* (n = 13)			*L. geoffroyi* (n = 17)		
Major groups	Minor groups	N	FO (%)	FR (%)	N	FO (%)	FR (%)
Tinamiformes*		1	7.69	2.33	0	0	0
Tinamidae	Unindentified						
Unindentified aves		4	30.77	9.30	2	11.76	4.88
Reptilia	**Total Reptilia**	**1**	**7.69**	**2.33**	**2**	**5.88**	**4.88**
Squamata							
Sauria* (Lacertilia)		0	0	0	2	5.88	4.88
Scincidae	*Mabuya dorsivittata*						
Anguidae	*Ophiodes* sp						
Serpentes	Unindentified snake	1	7.69	2.33	0	0	0
Amphibia	**Total Amphibia**	**0**	**0**	**0**	**1**	**5.88**	**2.44**
Anura	Unindentified	0	0	0	1	5.88	2.44
Invertebrates	**Total Invertebrates**	**---**	**7.69**	**---**	**---**	**17.65**	**---**
Arachnida	Unindentified	---	7.69	---	---	11.76	---
Unindentified invertebrates		---	0	**---**	---	5.88	
Plants	**Total Plants**	**---**	**92.31**	**---**	**---**	**70.59**	**---**
Liliopsida (Monocotyledoneae)							
Poaceae	Unindentified	---	76.92	---	---	52.94	---
Unindentified Liliopsida		---	7.69	---	---	0	---
Magnoliopsida (Dicotyledoneae)							
Fabaceae (Leguminous)	Unindentified	---	15.38	---	---	11.76	---
Malvaceae	Unindentified	---	0	---	---	5.88	---
Unindentified Magnoliopsida		---	53.85	---	---	35.29	---
Unindentified Plants		---	0	---	---	5.88	---

N = total number of registers for each prey category, FO = frequency of occurrence, FR = relative frequencies.

*N, FO and FR were calculated for the group.

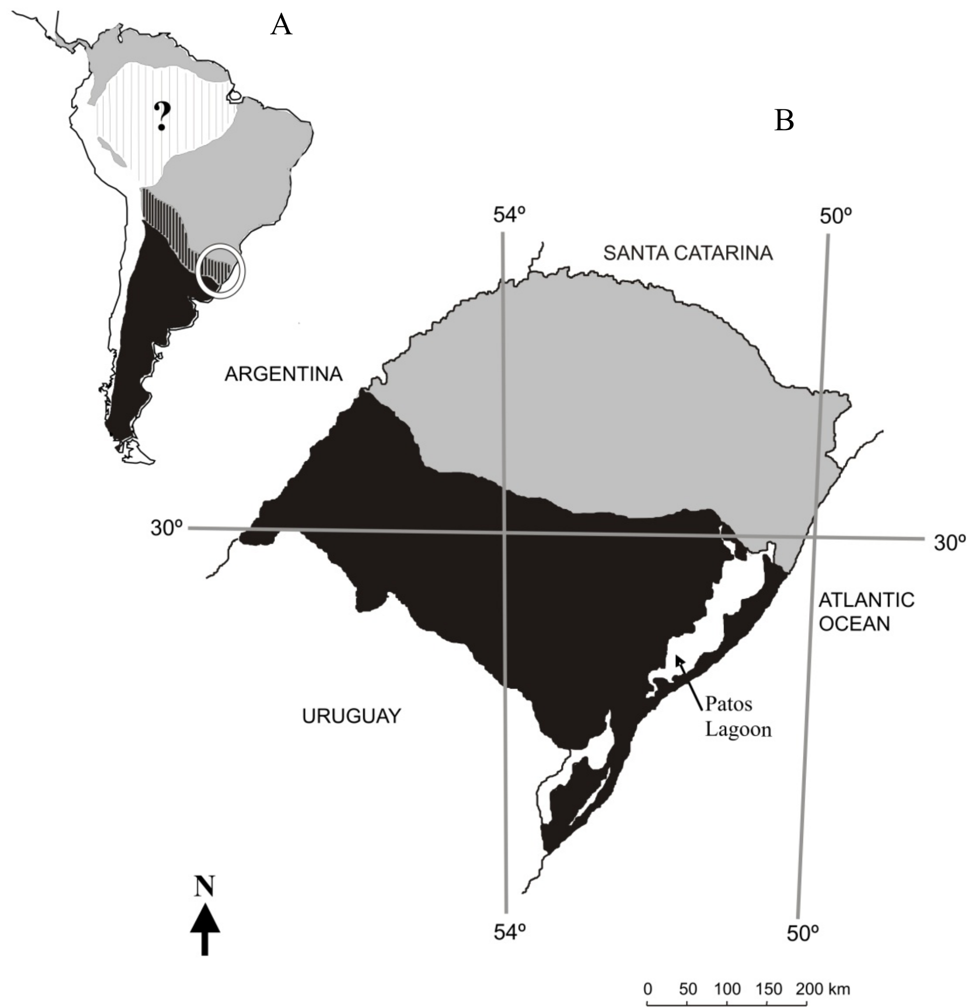


Figure 1 – Capítulo IV

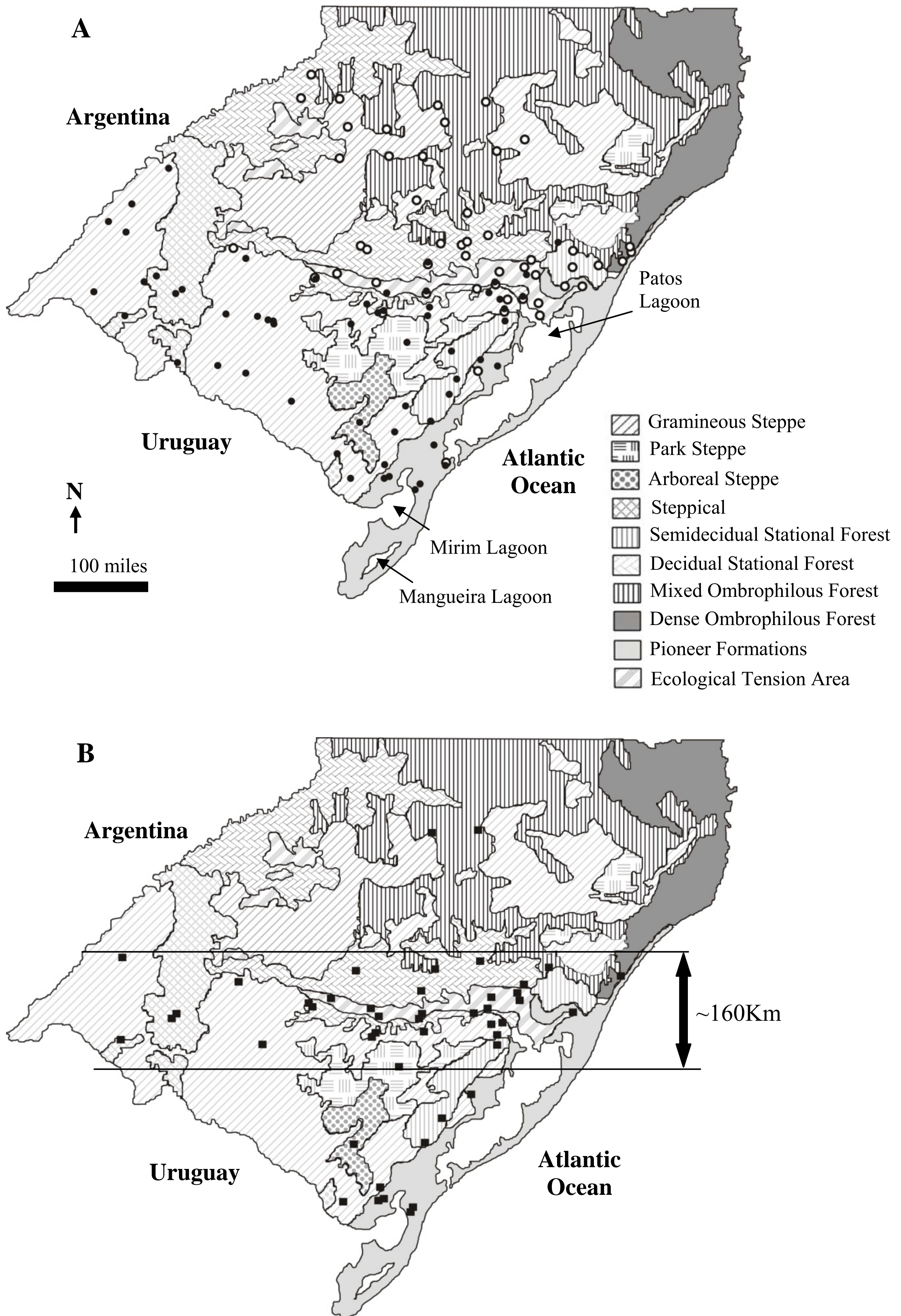


Figure 2 – Capítulo IV

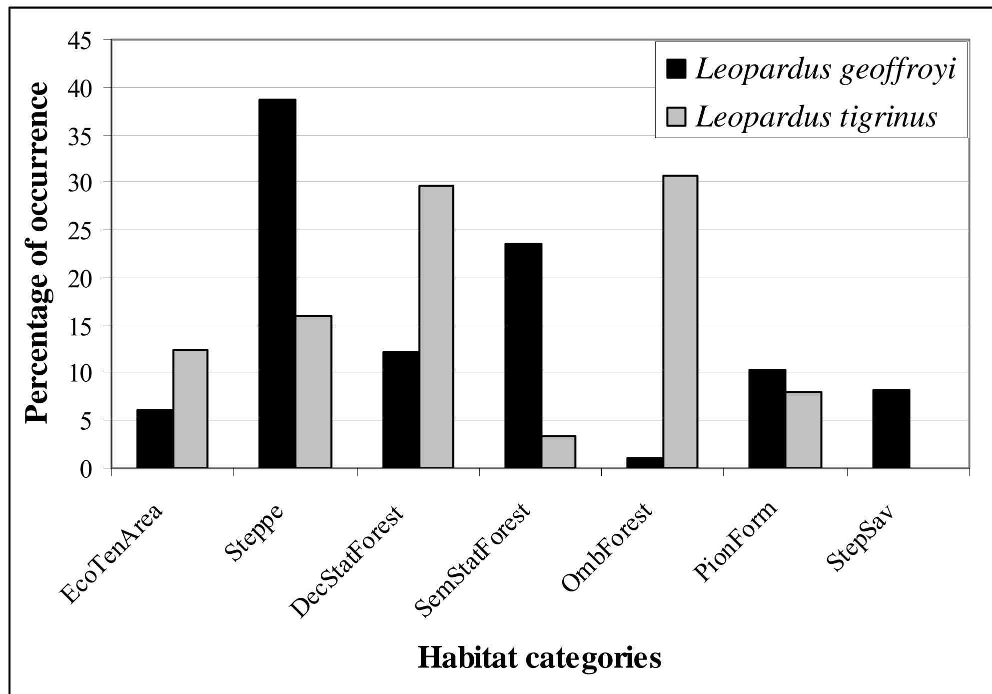


Figure 3 – Capítulo IV

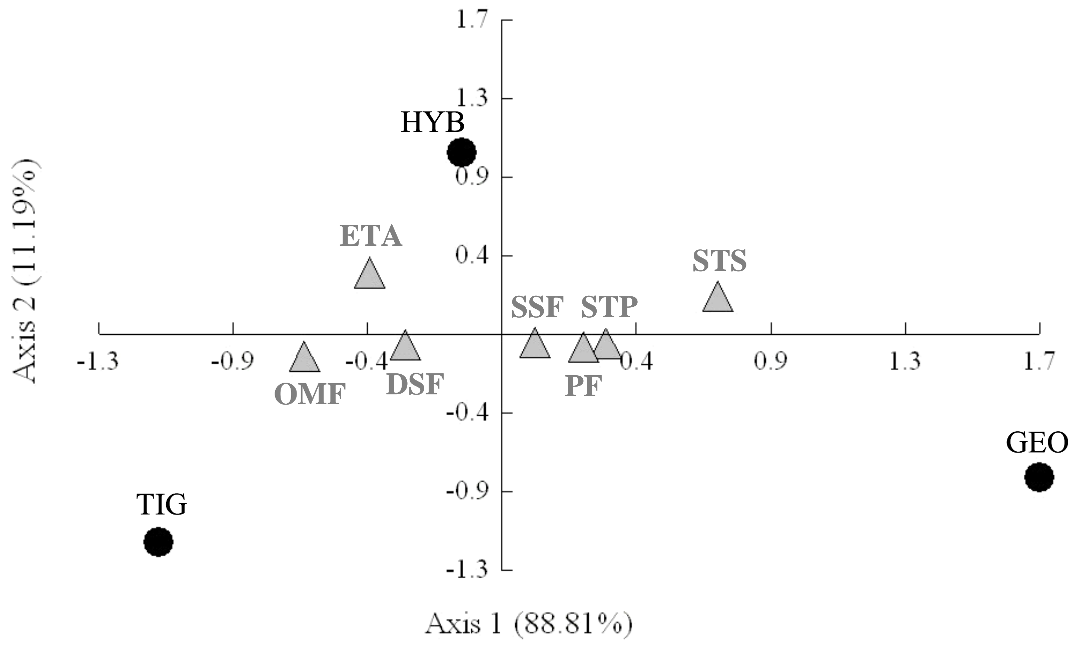
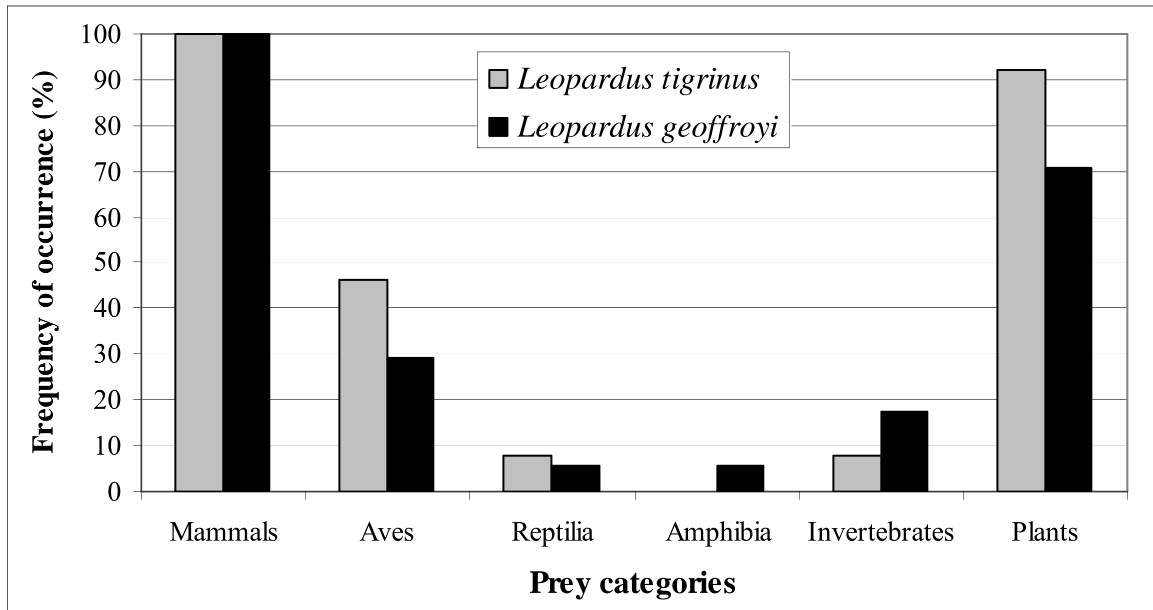
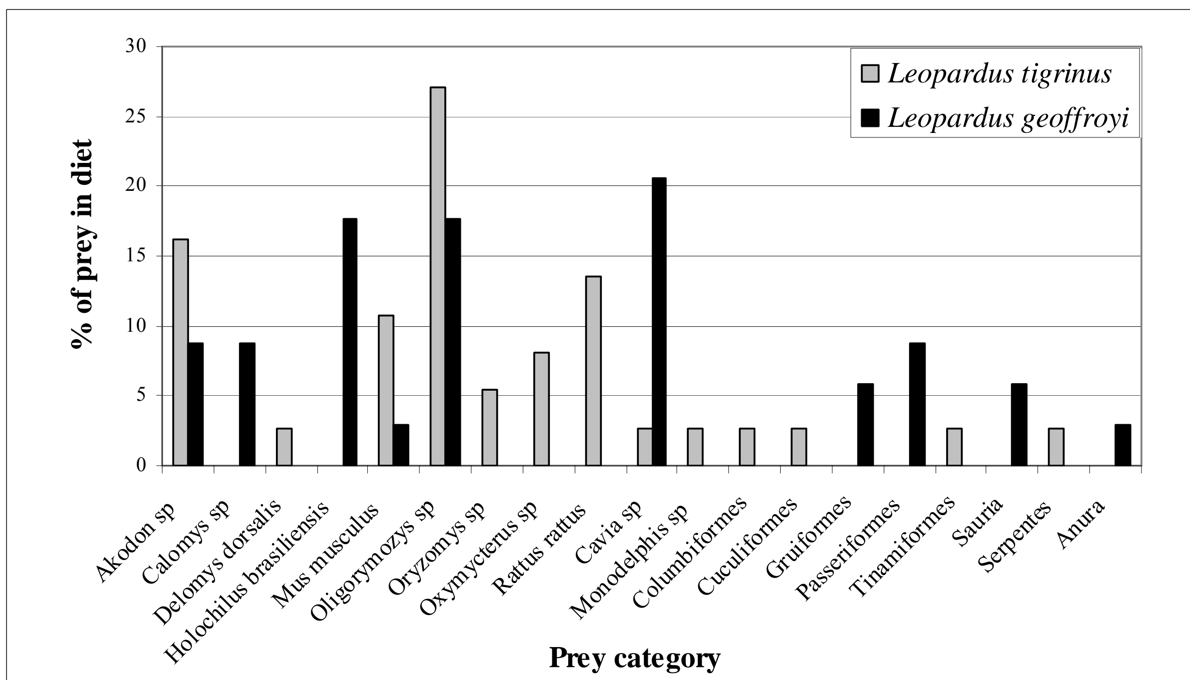


Figure 4 – Capítulo IV



A)



B)

Figure 5 – Capítulo IV

CAPÍTULO V

DISCUSSÃO GERAL

Os primeiros estudos documentando a existência de hibridação entre *Leopardus tigrinus, L. geoffroyi* e *L. colocolo* (Johnson *et al*. 1999, Trigo *et al*. 2008) geraram uma excelente oportunidade para o estudo das relações ecológicas e evolutivas entre estas espécies. Freqüentemente, as zonas híbridas na natureza são vistas como sítios ativos de mudanças evolutivas, ou simplesmente, como um laboratório natural no qual as interações genéticas e ecológicas entre diferentes populações ou espécies possam ser examinadas (Arnold 1992). No presente estudo, as análises genéticas, incluindo diferentes tipos de marcadores moleculares nas três espécies, e as análises morfológicas e ecológicas de *L. tigrinus* e *L. geoffroyi* geraram importantes e inéditas informações sobre os processos de hibridação entre estas e sobre suas interações genéticas e ecológicas.

A hibridação em *L. tigrinus*, *L. geoffroyi* e *L. colocolo*, no presente momento, parece ter se originado a partir de causas naturais. Segundo as análises de nosso primeiro trabalho (Trigo *et al.* 2008), *L. tigrinus* parece apresentar pelo menos um evento de expansão demográfica em sua história evolutiva a cerca de 75.700 (28.700 – 157.000) anos atrás, no final do Pleistoceno. Este período geológico foi marcado por eras glaciais e interglaciais, representadas por extensas modificações climáticas que, aparentemente, contribuíram no estabelecimento dos padrões biogeográficos atuais de muitos organismos (p.ex. Marroig & Cerqueira 1997, Hewitt 1996, 1999, 2000, Hundertmark *et al*. 2002, Lessa *et al*. 2003). Durante o Quaternário, especialmente no final do Pleistoceno e Holoceno, as diversas mudanças climáticas ocorridas foram representadas por alternâncias de períodos secos e frios para quentes e úmidos, tendo os últimos favorecido a expansão e coalescência das florestas tropicais (Ledru *et al.* 1996, Behling *et al*. 1998, De Oliveira 1999, Behling & Negrelle 2001, Behling 2002). Os dados atuais existentes sobre ocorrência de *L. tigrinus* indicam-na como uma espécie predominantemente associada a formações florestais (Oliveira 1994, Nowell & Jackson 1996, Nowak 1999). Considerando-se este padrão de associação com habitat, é possível que a expansão demográfica da espécie tenha sido favorecida pela expansão das áreas florestais durante os períodos de clima mais úmido do final do Quaternário. Esta expansão poderia, então, ter propiciado o contato de *L. tigrinus* com *L. geoffroyi* e com *L. colocolo*. A ausência de barreiras interespecíficas efetivas ao cruzamento, que teriam se desenvolvido devido ao

acúmulo de mutações durante o período de isolamento, teria permitido a ocorrência de eventos de hibridação entre estas espécies. Mudanças ambientais como a eliminação/redução de barreiras geográficas ao fluxo gênico e expansões populacionais, levando a um contato secundário entre espécies que evoluíram em alopatria, têm sido as principais causas sugeridas para a formação de zonas híbridas entre espécies selvagens (Arnold 1992, Harrison 1993, Hewitt 1996, 1999, 2000, Barton 2001). No entanto, a possibilidade de uma origem mais recente e até mesmo propiciada por alterações de habitat de influência antropogênica, principalmente no caso de *L. tigrinus vs. L. geoffroyi*, não pode ser totalmente descartada. O estado do Rio Grande do Sul, particularmente, onde a zona de hibridação entre estas duas espécies foi identificada, apresenta, atualmente, sua paisagem florística extremamente modificada por atividades humanas como a pecuária e a agricultura (IBGE 1986, Rambo 1994), o que poderia, por sua vez, também ter propiciado um maior contato entre as espécies. A datação precisa destes eventos permanece como um dos principais desafios a serem alcançados na compreensão da origem da hibridação entre estes três felídeos na América do Sul.

Do ponto de vista evolutivo, o mais interessante na hibridação entre estas três espécies de felídeos, talvez seja, a existência de padrões diferenciados de hibridação entre uma mesma espécie (*L. tigrinus*) e suas duas espécies relacionadas (*L. geoffroyi* e *L. colocolo*). Primeiramente, os dois processos apresentam-se nitidamente segregados geograficamente. As evidências da ocorrência de hibridação entre *L. tigrinus vs. L. colocolo* aparece restrita às regiões do centro e nordeste do Brasil, onde não há registros da ocorrência de *L. geoffroyi*. Por outro lado, a hibridação entre *L. tigrinus* e *L. geoffroyi* aparece basicamente restrita ao estado do RS, onde as duas espécies apresentam um contato geográfico. O interessante é que o RS apresenta-se como parte integrante da distribuição de *L. colocolo*, no entanto, nenhum registro de hibridação entre esta espécie com *L. tigrinus* foi detectado nesta área. Provavelmente, diferentes eventos e pressões seletivas nas diferentes regiões geográficas, ao longo da história evolutiva destas espécies, tenham propiciado o padrão geográfico de hibridação detectado.

Além da segregação espacial, os eventos de hibridação e introgressão entre cada par de espécies apresentaram padrões contrastantes com diferentes conseqüências demográficas e genéticas para as espécies envolvidas. Enquanto a evidência de hibridação entre *L. tigrinus* e *L. geoffroyi* foi detectada em todos os marcadores moleculares analisados, a hibridação entre *L. tigrinus* e *L. colocolo* foi basicamente sugerida pela análise de segmentos do DNA mitocondrial (DNAmt). Neste último caso, basicamente a

introgressão de seqüências de DNAmt de *L. colocolo* em indivíduos de *L. tigrinus* foi detectada, indicando um padrão unidirecional. Apenas um caso de introgressão na direção oposta, ou seja, de seqüências de *L. tigrinus* em indivíduos de *L. colocolo* foi detectado (Lco02). No entanto, este caso se apresenta controverso, por representar uma amostra de indivíduo mantido em cativeiro, sem procedência geográfica e coletado na década de 80 em um zoológico da Holanda, dificultando dessa maneira, a confirmação de se este evento realmente ocorreu na natureza, ou foi apenas o resultado de um cruzamento em cativeiro. A introgressão, além de unidirecional, apresentou-se basicamente restrita a indivíduos amostrados na região central e nordeste do Brasil. Surpreendentemente, 100% da amostra de *L. tigrinus* do nordeste brasileiro (onde teoricamente a espécie *L.* colocolo não ocorre) apresentou a introgressão de seqüências de DNAmt específicas de *L. colocolo*. Este padrão de introgressão do DNAmt, considerado isoladamente, seria altamente sugestivo da existência de uma extensa zona de hibridação entre estas espécies. No entanto, nossas análises indicaram a existência praticamente exclusiva de genes específicos de *L. tigrinus* em locos de microssatélite autossômicos e introns ligados aos cromossomos X e Y nesta mesma amostra. Apenas um indivíduo de *L. tigrinus* (bLti81) apresentou evidências de hibridação em outros marcadores moleculares. No entanto, este caso também se apresentou controverso. A associação deste indivíduo à população de *L. colocolo,* revelada por todos os marcadores avaliados, sugere a possibilidade de uma identificação morfológica errônea, principalmente, considerando-se que este espécime apresentava uma pelagem melânica que geralmente dificulta a identificação de espécies de felídeos de pequeno porte.

A ausência de evidências de hibridação em marcadores nucleares tornou difícil a aceitação de uma zona híbrida atual e extensa entre estas espécies, pois se este fosse o caso, esperar-se-ia uma mistura de alelos específicos de *L. tigrinus* e de *L. colocolo* ao nível nuclear. Casos como este, de forte dissociação citonuclear, foram também detectados em outras espécies de mamíferos, como em coiotes (*Canis latrans*) e elefantes africanos (*Loxodonta cyclotis e L. africana.*) (Adams *et al*. 2003, Roca *et al*. 2004). No caso dos coiotes, o estudo conduzido com populações do sudeste dos Estados Unidos, revelou a existência de um haplótipo de DNAmt associado à cães domésticos distribuído em alta freqüência na população de coiotes, juntamente com a ausência absoluta de evidência de introgressão em marcadores nucleares. Os autores deste trabalho sugerem que a população de coiotes teria se expandido na direção do sudeste dos EUA no passado, e a hibridação com cães domésticos poderia ter ocorrido na população ancestral que iniciou a colonização desta região. Os machos, como os primeiros dispersores em eventos de expansão

demográfica, teriam encontrado dificuldades em encontrar parceiras reprodutivas, favorecendo assim o cruzamento entre machos de coiotes e fêmeas de cães domésticos, mais abundantes na área. O processo de expansão demográfica em si teria sido o responsável pela disseminação do haplótipo de DNAmt específico de cães domésticos na população de coiotes. No caso dos elefantes africanos, evidências de hibridação entre as duas espécies *Loxodonta africana* (associada ao habitat de savana) e *L. cyclotis* (associada às regiões florestais) foram encontradas também somente ao nível do DNAmt. A introgressão deste material genético demonstrou-se estritamente unidirecional, com uma extensa distribuição de haplótipos específicos da espécie florestal em populações da espécie da savana, incluindo populações amostradas em áreas muito distantes das zonas de contato entre as duas espécies. Este padrão foi associado a um processo de hibridação e introgressão unidirecional, envolvendo primeiramente fêmeas florestais e machos de savana, seguido de retrocruzamentos de fêmeas híbridas com machos de savana. Em elefantes, sabe-se que o sucesso reprodutivo dos machos está associado ao tamanho corporal, assim, machos da savana que são quase duas vezes maiores que machos da floresta, teriam vantagens reprodutivas sobre machos florestais nas zonas de contato e também sobre machos híbridos. Os padrões de hibridação iniciais estritamente unidirecionais, seguidos de múltiplas gerações de retrocruzamento com apenas uma das espécies envolvidas, em ambos os casos, teriam diluído a proporção de genes nucleares de cães domésticos e de *L. cyclotis* nas espécies de coiote e *L. africana*, respectivamente. Segundo Roca *et al*. (2004), cerca de apenas 10 gerações sucessivas de retrocruzamentos unidirecionais são capazes de repor praticamente 100% dos genes nucleares, apagando, desta maneira, a evidência de uma hibridação ocorrida no passado, que ficaria registrada apenas no DNAmt não recombinante. Um cenário similar pode ser inferido para explicar os padrões observados entre *L. tigrinus* e *L. colocolo*. É provável que a expansão demográfica de *L. tigrinus* tenha favorecido o contato inicial de machos desta espécie com fêmeas de *L. colocolo*, primeiramente, na região central do país que parece carregar os haplótipos ancestrais desta expansão. A presença de determinadas pressões seletivas, como, por exemplo, uma menor fertilidade dos híbridos machos, pode ter favorecido o retrocruzamento de fêmeas híbridas com machos de *L. tigrinus,* que em algumas gerações praticamente eliminaram a evidência de hibridação nos marcadores nucleares. Os descendentes destes cruzamentos poderiam, então, ter ampliado sua distribuição, colonizando novas áreas disponíveis no centro e nordeste do Brasil, onde uma população geneticamente diferenciada de *L. tigrinus* foi documentada.

Em contraste ao padrão contraditório de indícios de hibridação encontrados entre *L. tigrinus* e *L. colocolo*, a ocorrência de hibridação entre *L. tigrinus* e *L. geoffroyi* foi sugerida por todos os marcadores utilizados e em ambas as populações fenotípicas, indicando a existência de introgressão bidirecional. As evidências de uma origem híbrida foram encontradas predominantemente em indivíduos provenientes do estado do RS, onde uma zona de contato entre as duas espécies foi previamente identificada por Eizirik *et al*. (2006). Avaliando em conjunto todos os marcadores utilizados, cerca de 60% da população total amostrada para o estado foi considerada como de origem híbrida, constituindo uma das mais extensas zonas de hibridação detectada até o momento em carnívoros (Gotelli *et al*. 1994, Vilà & Wayne 1999, Beaumont *et al*. 2001, Randi *et al*. 2001, Lecis *et al*. 2006, Verardi *et al*. 2006). Diversas combinações entre marcadores foram identificadas nos indivíduos analisados das duas espécies, indicando uma extensiva e atual zona de hibridação, caracterizada por várias gerações de cruzamentos em todas ou praticamente todas as direções, que levaram a uma intensa introgressão de componentes genéticos de uma espécie para a outra. Esta grande complexidade de combinações genéticas possíveis, provavelmente, contribuiu para a dificuldade encontrada na definição precisa das categorias híbridas a que os indivíduos amostrados pertenciam. A distinção entre híbridos F2 e retrocruzamentos, assim como entre indivíduos puros e retrocruzamentos, não foi plenamente possível, mesmo com a grande quantidade e diversidade dos marcadores moleculares utilizados. A dificuldade de identificação de híbridos e definição de categorias híbridas torna-se progressivamente difícil à medida que sucessivas gerações de cruzamentos entre diferentes formas híbridas e parentais vão ocorrendo, sendo necessário um número extremamente extenso de marcadores para sua adequada classificação (Boecklen & Howard 1997, Anderson & Thompson 2002, Vähä & Primmer 2006). Este intenso padrão de hibridação parece ter provocado conseqüências genéticas profundas nas populações locais de ambas as espécies, onde pudemos claramente detectar uma maior homogeneidade genética entre estas do que entre populações de ambas as espécies situadas em áreas mais distantes da zona de contato.

Apesar de bidirecional, a introgressão detectada entre *L. tigrinus* e *L. geoffroyi* parece apresentar algum nível de assimetria, representado por uma maior taxa de introgressão de segmentos genéticos de *L. tigrinus* na população de *L. geoffroyi.* Padrões de introgressão assimétrica podem ser favorecidos por aspectos demográficos, como diferenças nas densidades locais das espécies hibridizantes, que poderia levar ao aumento da pressão de introgressão genética em uma das direções; por aspectos sociais ou

fisiológicos diferenciais entre as espécies envolvidas, incluindo diferentes períodos de estro, cuidados parentais e socialização (p. ex. Roy *et al*. 1994, Vilà & Wayne 1999); ou ainda, por pressões seletivas diferenciais em cada espécie atuando contra a introgressão de novos alelos (*e.g.* Cianchi *et al*. 2003). No caso de seleção diferencial, as variantes genéticas poderiam apresentar uma menor redução em sua viabilidade e fertilidade quando retrocruzadas com a espécie parental *L. geoffroyi*, favorecendo assim a introgressão de genes nesta população. Da mesma maneira, o cruzamento de formas híbridas com as espécies parentais pode gerar novas características que não estejam sujeitas à mesma pressão seletiva nos dois lados da zona de contato (Vallender *et al*. 2006). Neste caso, uma mesma nova variação genética, gerada a partir do cruzamento entre híbridos e as formas parentais, poderia ser favorável na região de ocorrência de *L. geoffroyi,* mas desfavorável na de *L. tigrinus*, propiciando, assim, a sua proliferação na primeira população.

A existência de algum nível de seleção atuando sobre as formas híbridas constitui uma das principais questões que se mantém em aberto a respeito do processo de hibridação entre *L. tigrinus* e *L. geoffroyi*. A intensa taxa de introgressão detectada entre estas espécies poderia ser sugestiva da ausência de seleção ou da presença de uma seleção relativamente fraca contra os híbridos. Neste caso, as duas espécies poderiam estar caminhando para um processo de intensa homogeneização, que no futuro poderia levar a sua miscigenação total. Por outro lado, a elevada concentração dos eventos de hibridação no estado do RS, particularmente em uma área de cerca de 160 km de extensão em sua região central, onde cerca de 76% dos híbridos identificados foram detectados, poderia ser sugestiva da existência de um padrão de equilíbrio entre dispersão e algum tipo de seleção contra determinadas formas híbridas. Comparada à extensa área de distribuição das duas espécies envolvidas, a extensão da zona híbrida pode ser considerada como bastante restrita sugerindo a existência de algum tipo de seleção, atuante sobre os híbridos, que estaria mantendo esta zona estável ao longo do tempo e evitando, assim, sua maior expansão. Segundo Barton & Hewitt (1985), a grande maioria das zonas híbridas conhecidas são realmente mantidas estáveis em limitadas áreas geográficas próximas à zona de contato entre as espécies por este tipo de equilíbrio entre seleção e dispersão, onde a amplitude da zona está diretamente relacionada à força da seleção atuante. Nestes casos, dois tipos gerais de seleção contra híbridos são possíveis, incluindo a seleção endógena independente do ambiente e a seleção exógena ou habitat-dependente (Barton & Hewitt 1985, Arnold 1992, 1993, Harrison 1993, Barton 2001). A ausência atual de informações sobre fertilidade e viabilidade dos híbridos F1, F2 e retrocruzamentos entre estas espécies,

torna difícil a avaliação da existência de um padrão de seleção endógena atuante sobre esta zona de hibridação. Por outro lado, a presença de uma seleção exógena dependente do ambiente, onde as espécies parentais seriam favorecidas em diferentes ambientes, enquanto os híbridos seriam selecionados negativamente nestes, mas favorecidos em ambientes com gradientes de heterogeneidade, seria plausível de aceitação na manutenção de uma zona híbrida estável entre *L. tigrinus* e *L. geoffroyi*. Todavia, os primeiros testes conduzidos para avaliação de uma possível segregação ambiental entre indivíduos puros e parentais e apresentados no Capítulo IV, não demonstraram claramente a existência deste padrão. Apesar da ausência de comprovação deste tipo de segregação, pelos dados e análises apresentadas, a hipótese de seleção habitat-dependente não deve ser totalmente descartada neste momento, sendo necessária a condução de estudos mais específicos de utilização de habitats que possam detector padrões não perceptíveis por nosso nível de análise.

A definição de forças seletivas atuantes em zonas híbridas constitui uma difícil tarefa em estudos de hibridação entre espécies. No caso, de *L. tigrinus* e *L. geoffroyi*, avaliações comparativas dos níveis de fertilidade entre indivíduos puros e as diversas formas híbridas definidas geneticamente, poderiam auxiliar na investigação da existência de seleção endógena atuante nesta zona. Por outro lado, tendo em vista a maior concentração de amostras obtidas neste estudo para a região central do estado do RS, que poderia estar superestimando a taxa de hibridação nesta área em relação às outras áreas do estado, a inclusão de uma amostragem mais homogênea das duas espécies ao longo de toda a extensão do estado poderia contribuir para a confirmação deste padrão de distribuição das formas híbridas. Juntamente, a inclusão de uma amostra mais representativa de toda a área de distribuição de *L. geoffroyi* poderia auxiliar na definição da amplitude geográfica do gradiente genético existente entre as duas espécies e, assim, propiciar uma melhor averiguação da existência ou não de seleção atuante na manutenção desta zona híbrida.

Apesar da intensa introgressão genética detectada entre *L. tigrinus* e *L. geoffroyi* em sua zona de contato do estado do RS, as análises morfológicas conduzidas neste estudo indicam a existência de populações morfologicamente diferenciadas. As evidências morfológicas dos eventos de hibridação entre estas espécies parecem estar restritas (de acordo com as análises realizadas), predominantemente, a variações nos padrões de pelagem, podendo sugerir assim, a existência de mecanismos que continuam mantendo a diferenciação fenotípica entre estas espécies. A maioria dos estudos envolvendo testes de identificação genética e morfológica de indivíduos híbridos e puros, como no caso de *L. tigrinus* e *L. geoffroyi*, demonstra a freqüente falha na identificação destas categorias

utilizando-se apenas caracteres morfológicos (p. ex. Gaubert *et al*. 2005, Chan *et al*. 2006, Lecis *et al*. 2006, Vallender *et al*. 2006). A identificação de híbridos baseada somente na morfologia é dificultada pelo fato de que nem toda variação morfológica apresenta uma base genética e de que nem toda introgressão de genes será refletida morfologicamente. Além disso, em populações extensivamente hibridizantes como *L. geoffroyi* e *L. tigrinus*, várias gerações de retrocruzamento podem apagar completamente as evidências morfológicas destes eventos (Rhymer & Simberloff 1996, Allendorf *et al*. 2001). Além disso, a manutenção da distinção morfológica entre espécies altamente hibridizantes, como parece ser o caso de *L. tigrinus* e *L. geoffroyi* pode ser um indicativo de que as características morfológicas das espécies envolvidas possam ser controladas por genes que estejam sob forte seleção diferencial.

Finalmente, a intensa introgressão de genes seletivamente neutros entre *L. tigrinus* e *L. geoffroyi* no estado do RS contrasta com a manutenção de uma aparente distinção morfológica entre as duas espécies e uma aparente distinção ecológica refletida pelos diferenciados padrões de associação com habitat e utilização de recursos alimentares apresentados no Capítulo IV. Estes contrastantes aspectos da biologia destas espécies, nas proximidades de suas áreas de contato geográfico, ilustram a complexidade dos processos evolutivos atuantes em zonas híbridas, onde a manutenção da distinção entre espécies depende de um delicado equilíbrio entre diferentes forças evolutivas.

Perspectivas futuras

Muitos aspectos importantes sobre os padrões de hibridação entre *L. tigrinus*, *L. geoffroyi* e *L. colocolo* foram esclarecidos neste estudo. No entanto, um número ainda maior de questões permaneceu em aberto para novas investigações.

Em primeiro lugar, as primeiras inferências sobre os padrões de hibridação entre *L. tigrinus* e *L. colocolo* foram apresentadas aqui, sendo necessária a condução de inúmeros novos estudos para ampliar o conhecimento existente sobre os processos evolutivos atuantes sobre estas espécies. Estudos básicos de definição exata de suas áreas de ocorrência e limites de distribuição nas regiões central e nordeste do Brasil, incluindo a verificação da existência de possíveis zonas de contato, como a identificada para *L. tigrinus* e *L. geoffroyi*, são primordiais. A condução de novos estudos genéticos, incluindo um maior número e tipos de marcadores, além de uma amostragem mais representativa tanto de *L. tigrinus* quanto de *L. colocolo* provenientes desta região, é fundamental para a verificação dos padrões de hibridação aqui sugeridos. Além disso, a indicação de uma

população geneticamente diferenciada de *L. tigrinus* no centro-nordeste brasileiro amplia a necessidade, já existente, da condução de estudos morfológicos e ecológicos detalhados nesta população, principalmente em comparação às populações de *L. tigrinus* do sul e sudeste brasileiro.

No que diz respeito à hibridação entre *L. tigrinus* e *L. geoffroyi* no sul do Brasil, investigações sobre os padrões específicos de requerimento de habitats e/ou recursos alimentares por indivíduos puros e híbridos, assim como, sobre níveis de fertilidade e/ou viabilidade das diferentes categorias híbridas são extremamente necessárias para auxiliar no entendimento dos padrões de manutenção desta zona. Estudos envolvendo aspectos morfológicos mais detalhados das duas espécies, com a inclusão de uma amostra mais representativa e novas metodologias, não só nas áreas de contato, mas também ao longo de toda a distribuição das espécies, seriam extremamente importantes para a elucidação da influência dos processos de hibridação sobre a diferenciação morfológica destas. A inclusão de novos marcadores e de uma amostragem mais representativa tanto das duas espécies no estado quanto da distribuição mais ao sul de *L. geoffroyi* poderá auxiliar em análises mais específicas de datação dos eventos de hibridação e da existência ou não de pressões seletivas atuando sobre a manutenção de uma zona híbrida estável. E finalmente, apesar das evidências atuais indicarem uma causa natural para os processos de hibridação entre estas espécies, a análise da influência de alterações ambientais de origem antropogênica sobre o comportamento e distribuição destas, deve ser levada em consideração. A possibilidade de que estas alterações ambientais poderiam, na atualidade, estar favorecendo o processo de hibridação entre estas espécies não pode ser totalmente descartada no momento, e merece uma atenção especial por poder implicar em importantes medidas para conservação destas espécies em ambiente selvagem.

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ANEXO

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Inter-species hybridization among Neotropical cats of the genus *Leopardus*, and evidence for an introgressive hybrid zone between *L. geoffroyi* and *L. tigrinus* in southern Brazil

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Inter-species hybridization among Neotropical cats of the genus *Leopardus*, and evidence for an introgressive hybrid zone between *L. geoffroyi* and *L. tigrinus* in southern Brazil

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Abstract

Natural hybrid zones between distinct species have been reported for many taxa, but so far, few examples involve carnivores or Neotropical mammals in general. In this study, we employed mitochondrial DNA (mtDNA) sequences and nine microsatellite loci to identify and characterize a hybrid zone between two Neotropical felids, *Leopardus geoffroyi* and *L. tigrinus*, both of which are well-established species having diverged from each other *c.* 1 million years ago. These two felids are mostly allopatric throughout their ranges in South America, with a narrow contact zone that includes southern Brazil. We present strong evidence for the occurrence of hybridization between these species and identify at least 14 individuals (most of them originating from the geographical contact zone) exhibiting signs of interspecific genomic introgression. The genetic structure of Brazilian *L. tigrinus* populations seems to be affected by this introgression process, showing a gradient of differentiation from *L. geoffroyi* correlated with distance from the contact zone. We also corroborate and extend previous findings of hybridization between *L. tigrinus* and a third related felid, *L. colocolo*, leading to an unusual situation for a mammal, in which the former species contains introgressed mtDNA lineages from two distinct taxa in addition to its own.

Keywords: Carnivora, hybridization, introgression, *Leopardus geoffroyi*, *Leopardus tigrinus*, South America

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Introduction

The role of hybridization in the evolution of living organisms has been extensively discussed among evolutionists (e.g. Arnold 1992; Harrison 1993; Dowling & Secor 1997; Barton 2001; Fitzpatrick 2004). The classical view of zoologists is that the evolutionary significance of hybridization is small, in most cases consisting of occasional sterile hybrid individuals with no relevant contribution to future generations. In contrast, botanists frequently see hybridization as a common phenomenon, acting as an important source of new variation and potentially new species (Harrison 1993). This apparent dichotomy has been challenged in recent decades, with the development and implementation of diverse molecular techniques allowing for in-depth genetic analyses of natural populations. These approaches have led to the conclusion that interspecies hybridization is quite common in animals and frequently include the production of fertile hybrids which may have considerable importance for future adaptation and even speciation (Barton & Hewitt 1985; Harrison 1993; Allendorf *et al*. 2001). Several hybrid zones have recently been

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documented in vertebrates, ranging from cases in which few hybrid individuals are detected (Schwartz *et al*. 2004) to extensive introgressive zones leading to the production of hybrid swarms (Nolte *et al*. 2006), or even suggested as possibly responsible for the formation of new species (Roy *et al*. 1994; Reich *et al*. 1999).

Even though it appears that natural hybridization is more common in animals than previously thought, it is still unclear how widespread its occurrence is, and whether some zoological groups or biogeographical regions may be more prone to foster such processes. Moreover, very few cases of animal hybridization have been described in detail, so that the investigation of the underlying causes and evolutionary significance of these processes remains in its infancy. A more thorough understanding of this phenomenon would be important not only to assess its evolutionary relevance (e.g. in terms of illuminating the various aspects of the speciation process), but also as a conceptual basis to design adequate conservation strategies for endangered populations showing signs of admixture with other taxa.

Several cases of hybridization involving mammalian carnivores (Mammalia, Carnivora) have been reported. So far, the examples that have received the most attention are those of North American wild canids (e.g. Lehman *et al*. 1991; Wayne & Jenks 1991; Roy *et al*. 1994; Reich *et al*. 1999; Miller *et al*. 2003), domestic dogs vs. wild canids (e.g. Gottelli *et al*. 1994; Vilà & Wayne 1999; Randi & Lucchini 2002; Adams *et al*. 2003; Vilà *et al*. 2003) and domestic cats vs. European wildcats (e.g. Beaumont *et al*. 2001; Randi *et al*. 2001; Lecis *et al*. 2006). The latter example, as well as most cases involving domestic dogs, describes situations in which the hybridizing populations are conspecific, i.e. domestic and wild forms of the same species. With the exception of the complex case of North American canids, little attention has been devoted to interspecific hybridization in carnivores, although some interesting examples have recently emerged in the literature (e.g. Schwartz *et al*. 2004; Lancaster *et al*. 2006). Virtually nothing is known about the occurrence of hybridization among Neotropical carnivores, a very diverse assemblage including several sets of closely related species. At least some of these sets are likely the product of rapid radiations following a single invasion after the closure of the Panama Isthmus and, so far, the ecological, evolutionary and biogeographical processes underlying their diversification have not been thoroughly characterized.

Of the 10 species of wild cats occurring in the Neotropics (Sunquist & Sunquist 2002), seven are known to comprise a monophyletic lineage endemic to this region (Johnson *et al*. 2006), herein referred to as the genus *Leopardus* (Wozencraft 2005; Johnson *et al*. 2006; E. Eizirik *et al*., unpublished data). The basal divergence among these seven species has been estimated to have occurred *c*. 2.9 million years ago (Johnson *et al*. 2006), which is consistent with a rapid radiation following a single invasion of South America via the Panama isthmus in the Pliocene. Within this clade, a well-supported subgroup includes the little spotted cat or oncilla (*Leopardus tigrinus*), Geoffroy's cat (*L. geoffroyi*), the kodkod (*L. guigna*), the pampas cat (*L. colocolo*) and possibly also the Andean mountain cat (*L. jacobita*). The species-level delimitation among these felids has been supported by reciprocal monophyly in mitochondrial DNA (mtDNA) analyses including multiple individuals (Johnson *et al*. 1999), corroborating the classical, morphology-based definition of these taxa. Interestingly, our previous analyses using mtDNA and Y-chromosome sequences identified some individuals that appeared to be natural hybrids between *L. tigrinus* and *L. colocolo* (Johnson *et al*. 1999). In spite of the intriguing nature of this finding, further analyses of this issue have so far been hampered by the difficulty in sampling a larger number of individuals from natural populations of these species, especially *L. colocolo*.

Within this group, *L. tigrinus* and *L. geoffroyi* are morphologically similar, exhibiting similar body proportions and general appearance. The main characters used to distinguish them are body size and coat colour, with *L. geoffroyi* being larger and more robust (total length: 690–1250 mm; weight 2.2–7.8 kg) and usually showing a gray background colour with solid black spots (Ximenez 1971, 1973, 1975; Sunquist & Sunquist 2002; Lucherini *et al*. 2006). *L. tigrinus* tends to be smaller (total length: 710–936 mm; weight 1.75–3.5 kg) and more gracile in appearance, with usually yellowish/ochre pelage bearing rows of dark spots and open rosettes (Kitchener 1991; Oliveira 1994; Eisenberg & Redford 1999; Sunquist & Sunquist 2002).

These two species show an essentially allopatric distribution in South America (Fig. 1): *L. geoffroyi* occurs from Bolivia, Paraguay, northern Argentina and southern Brazil to the southern tip of South America (Oliveira 1994; Eisenberg & Redford 1999; Wozencraft 2005), overlapping with the distribution of *L. tigrinus* at the northern end of its range. The latter species ranges from Costa Rica to southern Brazil and northeastern Argentina (Oliveira 1994; Nowell & Jackson 1996; Eisenberg & Redford 1999), but its current distribution is not completely defined and may be discontinuous, mainly due to the lack of detailed evidence of its occurrence throughout the Amazon basin (Nowell & Jackson 1996; Oliveira 2004).

Field-based surveys of the precise geographical distribution of *L. tigrinus* and *L. geoffroyi* in Rio Grande do Sul (RS) state, southernmost Brazil, conducted between 1993 and 2004, revealed a narrow contact zone (≤ 100 km in width) between these species (Eizirik *et al*. 2006). In this region, we and others have observed individuals bearing atypical coat colour patterns, seemingly 'intermediate' between the two species (Mazim *et al*. 2004; Eizirik *et al*. 2006). Many of these animals were recorded in the Central Depression region of RS state (which consists of a mosaic of grassland, riparian forests

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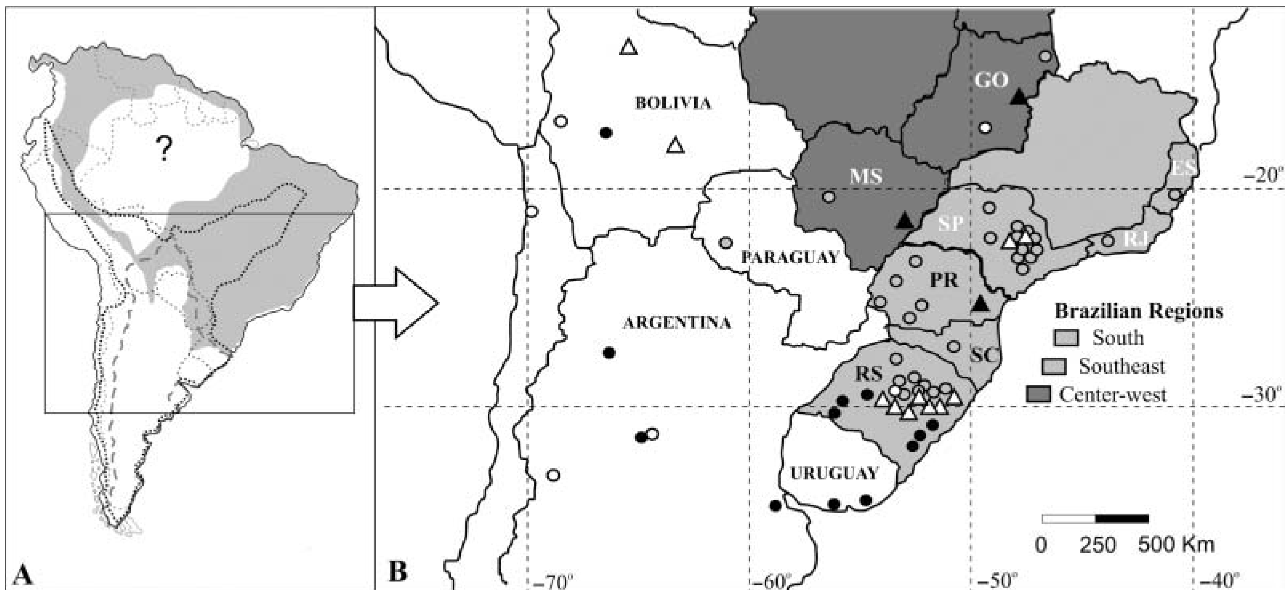

Fig. 1 (A) Geographical distribution of *Leopardus tigrinus* (grey-shaded area), *L. geoffroyi* (area defined by the grey broken line) and *L. colocolo* (area defined by the black dotted line) in South America (modified from Oliveira 1994; Nowell & Jackson 1996; Eisenberg & Redford 1999). (B) Map of the study area showing approximate sample collection sites for *L. tigrinus* (grey circles), *L. geoffroyi* (black circles) and *L. colocolo* (white circles). Each symbol represents one sampling locale and may include one or more individuals (only individuals with known collection locales are included). The Central American sample of *L. tigrinus* (Lti13) and the samples from other felid species (*L. guigna*, *L. pardalis*, *L. wiedii*) are not shown in the figure. White triangles indicate the sampling sites of hybrids between *L. tigrinus* and *L. geoffroyi*, while black triangles indicate collection locales for hybrids between *L. tigrinus* and *L. colocolo*. Abbreviations of Brazilian states: RS (Rio Grande do Sul), SC (Santa Catarina), Paraná (PR), MS (Mato Grosso do Sul), SP (São Paulo), RJ (Rio de Janeiro), ES (Espírito Santo), GO (Goiás).

and marshland fragments amidst a matrix of agricultural landscapes), exactly where contact between the two species occurs (Eizirik *et al*. 2006). This atypical colour pattern has led us to hypothesize the existence of a hybrid zone between these species in this area.

In this context, the goals of the present study were (i) to test the field-based hypothesis that *L. tigrinus* and *L. geoffroyi* hybridize in the wild, and to investigate whether the genetic patterns are consistent with a hybrid zone; (ii) if this hypothesis was supported, to characterize this hybrid zone, assessing the magnitude of admixture and the occurrence of genomic introgression in one or both directions; (iii) to further investigate the evidence of hybridization between *L. tigrinus* and *L. colocolo*; and (iv) to test the possibility of hybridization between *L. geoffroyi* and *L. colocolo*. We employed mtDNA sequences and nuclear microsatellite markers to address these issues, and interpreted these molecular data in a comparative fashion, as well as in the light of the morphologically-based identification of each sampled individual. Our results corroborate and expand the previous inference of hybridization between *L. tigrinus* and *L. colocolo* and strongly support the hypothesis of a hybrid zone between the former and *L. geoffroyi* in southern Brazil, leading to interesting inferences regarding the evolutionary history of these species in South America.

Materials and methods

Sample collection and laboratory procedures

Biological material (blood and tissue samples) of Neotropical felids was obtained from captive animals of known origin, road-killed individuals or wild animals captured by farmers. Samples of 57 *Leopardus tigrinus* were obtained from three major Brazilian regions, comprising eight Brazilian states: the southern region, including RS ($n = 16$), Paraná (PR) ($n = 9$) and Santa Catarina (SC) ($n = 1$) states; the southeastern region, including São Paulo (SP) ($n = 23$), Rio de Janeiro (RJ) ($n = 1$) and Espírito Santo (ES) ($n = 1$) states; and the center-west region, including Mato Grosso do Sul (MS) ($n = 2$) and Goiás (GO) ($n = 4$) states (see Fig. 1 for sample collection locales and Supplementary material for details). In addition, two samples from Paraguay, one from Costa Rica and one Brazilian sample with unknown state origin were included. Samples of 41 *L. geoffroyi* individuals were obtained from Argentina ($n = 5$), Bolivia ($n = 7$), Uruguay ($n = 5$) and RS state in Brazil ($n = 22$), along with two samples of unknown origin. Seven samples of *L. colocolo* were also included in the study (two from Argentina, one from Bolivia, one from Chile, two from Brazil and one with unknown origin). Finally, two samples of *L. guigna* and one

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each of ocelot (*L. pardalis*) and margay (*L. wiedii*) were used for comparison in some of the analyses. DNA extraction from all samples was performed using standard phenol/chloroform protocols (Sambrook *et al*. 1989; Palumbi *et al*. 1991; Hillis *et al*. 1996).

Three mtDNA segments were amplified by polymerase chain reaction (PCR) from these samples, using primers developed or adapted for improved performance in carnivores: a segment of the *ND5* gene including *c*. 750 bp [using primers ND5-DF1 (TTGGTGCAACTCCAAATAAAAGT) and ND5-DR1 (AGGAGTTGGGCCTTCTATGG)]; a ~400-bp segment including the *ATP8* gene and part of the *ATP6* gene [using primers ATP8-DF1 (AGAAGCTAAATAAGCATTAACCTTTTA) and ATP6-DR1 (CCAGTATTTGTTTTGATGTTAGTTG)], and the 5′ portion of the control region (CR) [using primers MTLPRO2 and CCR-DR1 (Tchaicka *et al*. 2007)]. For all three segments, PCR reactions were performed in a 20–25 μL final volume containing 1.5–2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 U of Taq DNA polymerase (Invitrogen) or Taq GOLD (ABI), and 0.2 μM each of the forward and reverse primers. Thermocycling used a touchdown profile as described in Tchaicka *et al*. (2007), with the annealing temperature decreasing from 60 °C to 51 °C in 10 cycles, followed by 30–35 cycles in which it was kept constant at 50 °C.

PCR products were analyzed on an ethidium-bromide-stained 1% agarose gel and then purified using either Polyethyleneglycol-8000 or the enzymes exonuclease I and Shrimp alkaline phosphatase. Purified PCR products were sequenced using either the DYEnamic ET kit (Amersham) or Big Dye chemistry (ABI) and subsequently analyzed in a MegaBACE 1000 or an ABI-PRISM 3700 automated sequencer, respectively. Sequence electropherograms were verified and corrected by eye using SEQUENCHER (Gene Codes) or CHROMAS (http://www.technelysium.com.au/chromas.html) and then aligned using the CLUSTALW algorithm implemented in MEGA 3.1 (Kumar *et al*. 2004); the alignment of each mtDNA segment was checked and edited by hand separately.

In addition to the mtDNA sequences, nine microsatellite markers [six tetranucleotide (FCA391, FCA424, FCA441, FCA453, F42, F124), two trinucleotide repeat loci (F98 and F146) and one dinucleotide (FCA723)], developed originally for the domestic cat (Menotti-Raymond *et al*. 1999, 2005) were selected for use in this study. Each microsatellite locus was amplified individually by PCR (Saiki *et al*. 1985); reactions were performed in a 15 μL volume containing 1.5–3.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 U of Taq DNA polymerase, and 0.1 μM each of the forward and reverse primers. The thermal profile was: 94 °C for 3′, and 30 cycles of 45″ at 94 °C, 45″ at 48–60 °C (annealing temperature varied among loci), and 1′ at 72 °C, followed by 10′ of final extension.

PCR products for microsatellite loci were analyzed by vertical electrophoresis in 6% nondenaturing polyacrylamide gels, and the microsatellite alleles were detected by silver nitrate staining (Tegelstrom 1992). Genotypes were scored manually using a 25-bp size ladder (Gibco BRL), as well as an allelic ladder constructed with all alleles found for each locus in this study. Aiming to thoroughly verify and confirm the observed genotypes, 25% to 30% of the samples were reanalyzed two to three times per locus, resulting in 100% concordance among replicates. This effort also included five cases in which the same captive animal was collected twice by different people at different times, and each of the samples was genotyped separately, again resulting in complete concordance of results. Finally, part of our data set (15 *L. tigrinus* and 29 *L. geoffroyi* individuals) was generated using fluorescently labelled primers and an ABI 373 A automated sequencer, employing the computer programs GENESCAN 2.1 (ABI) and GENOTYPER 2.1 (ABI) to precisely calibrate allele sizes. Of these individuals, seven of each species were also genotyped in silver-stained polyacrylamide gels to verify and calibrate the allelic correspondence between the two detection methods, resulting in full agreement of all replicated genotypes. All genotypes were thus integrated into a single data set, with the exact size of each allele based on the more precise estimation using the automated sequencer.

Data analysis

mtDNA data. Exploratory phylogenetic analyses were initially performed for each mtDNA segment separately using the distance-based neighbour-joining (NJ) algorithm (Saitou & Nei 1987) implemented in MEGA, to assess any occurrence of incongruence among these data sets. Support for inferred nodes was assessed using 100 replicates of nonparametric bootstrap. As no incongruence was identified at any well-supported node, the three segments were concatenated into a single data set, which was used for all subsequent analyses.

Phylogenetic analyses of the final data set were performed using four optimality criteria: maximum likelihood (ML), maximum parsimony (MP), distance-based (with the NJ algorithm) and Bayesian Inference (BI). The ML, MP and NJ approaches were performed using PAUP*4.b10 (Swofford 1998), while the BI method employed MRBAYES 3.1 (Huelsenbeck & Ronquist 2001). The best-fit model of nucleotide evolution for the concatenated data set was estimated using the Akaike Information Criterion (AIC) implemented in MODELTEST 3.7 (Posada & Crandall 1998). This model (or an approximation of it) was implemented in the ML and BI analyses, as well as the NJ search (which used ML distances). The ML analysis employed a heuristic search started from a NJ tree and followed by NNI branch-swapping. The final MP phylogeny was based on a heuristic search using simple taxon addition and TBR branch-swapping, limiting the procedure to store a maximum of 10 000 trees. Nodal support

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for the ML, MP and NJ methods was assessed with 100 replicates of bootstrapping (in the case of MP limiting the search to store a maximum of 1000 trees per replicate). The Bayesian analysis used two independent replicates of the Metropolis-Coupled Markov chain Monte Carlo procedure, each containing four chains (one cold, three heated) run for 3 000 000 generations, with trees and parameters sampled every 100 steps, and the first 25% of the samples discarded as burn-in. Trees were rooted using *L. pardalis* and *L. wiedii* as outgroups (see Johnson *et al*. 2006).

In addition to phylogenies, haplotype networks were built using the median-joining approach (Bandelt *et al*. 1999) implemented in NETWORK 4.2.0 (www.fluxus-engineering.com), allowing for ambiguous connections as well as direct ancestor-descendent relationships among haplotypes. Measures of mtDNA diversity were calculated with DnaSP 4.10.0.8 (Rozas *et al*. 2003), which was also employed to make inferences regarding historical changes in population size using a mismatch distribution analysis and several neutrality tests (Tajima's D, Fu & Li' D* and F*, Fu's Fs). In addition, ARLEQUIN 3.11 (Excoffier *et al*. 2005) was used to assess the magnitude of mtDNA-based species-level differentiation, employing an Analysis of Molecular Variance (AMOVA) approach (Excoffier *et al*. 1992), the results of which were tested for statistical significance with 10 000 permutations.

The age of each of the observed mtDNA clades was estimated using the Bayesian approach implemented in the program BEAST 1.4.4 (Drummond *et al*. 2002; Drummond & Rambaut 2006), employing a molecular calibration point for the divergence between *L. tigrinus* and (*L. geoffroyi* + *L. guigna*). The age of this node was estimated in a previous study (using a Bayesian relaxed clock method applied to a 18.7-kb nuclear supermatrix and incorporating multiple fossil constraints; Johnson *et al*. 2006) to be 930 000 years ago, with a credibility interval of 560 000–1 480 000 years ago. To apply this molecular calibration in a conservative fashion, we used the minimum and maximum ages in this credibility interval as boundaries in a uniform prior distribution for this node's age. The MCMC procedure was run for 50 million generations, with samples taken every 1000 steps; results were analyzed with the program TRACER (Rambaut & Drummond 2004) removing the initial 5 million steps as burn-in.

Microsatellite data. Microsatellite diversity was evaluated separately for *L. tigrinus*, *L. geoffroyi* and *L. colocolo* based on the number of polymorphic loci, alleles per locus and private alleles. We used ARLEQUIN to compute values of observed (H_O) and expected (H_E) heterozygosity and to test for deviations from Hardy–Weinberg Equilibrium (HWE) for each locus, using the exact test of Guo & Thompson (1992). The microsatellite data set was tested for genotyping errors due to stuttering, short allele dominance and null alleles using a Monte Carlo simulation of expected allele-size differences using MICRO-CHECKER (Van Oosterhout *et al*. 2004). Allele size-difference frequencies were determined to deviate from expectations if they fell outside the Bonferroni-corrected 95% confidence interval generated by the simulation. The distributions of allele frequencies, presence of private alleles and linkage equilibrium (LE) at all loci for each of the species were evaluated using GENEPOP 3.1d (Raymond & Rousset 1995). Significance levels of HWE and LE were adjusted using the sequential Bonferroni method to take into account multiple tests on the same data set (Rice 1989). To test for the occurrence of genetic introgression, the same methodology was conducted with the *L. tigrinus* sample subdivided into regional groups (see Results). The level of genetic differentiation between species and among regional groups of *L. tigrinus* was assessed with an AMOVA, as implemented in ARLEQUIN, using an F_{ST} analogue (Weir & Cockerham 1984). Statistical significance of the observed values was tested using 10 000 permutations.

We applied the Bayesian clustering method implemented in the program STRUCTURE 2.2 (Pritchard *et al*. 2000), incorporating the 'correlated allele frequencies' model (Falush *et al*. 2003), to assign individuals to populations and to identify hybrids between *L. tigrinus*, *L. geoffroyi* and *L. colocolo*. Three different sets of analyses were performed, in every case using 500 000 MCMC iterations following a burn-in period of 200 000 steps. We initially evaluated the approximate probability of each of a varying number of K populations for the pooled data, by empirically setting prior values of K = 1–10, and evaluating the Ln likelihood of the data (for each value of K, five independent runs were performed to confirm the stability of the likelihood estimates). For this analysis, we did not use the phenotypic information for species assignment, so that the most likely number of populations was thus determined from the genetic data alone. This approach was employed for two separate data sets; one of them including all three species and a second one focusing exclusively on the comparison between *L. geoffroyi* and *L. tigrinus*. A third set of STRUCTURE analyses, conducted only for the *L. geoffroyi* + *L. tigrinus* data set, employed the phenotype-based species identification; in this case the program estimates the probability of each individual belonging to one of the assumed clusters, or to have partial ancestry in one of them in previous generations (see Results for more details on the different sets of STRUCTURE runs).

Results

mtDNA

Genetic variation and phylogenetic relationships. The three investigated species exhibited moderate to high levels of variability in the concatenated mtDNA data set (Table 1). Haplotype (gene) diversity was highest in *Leopardus tigrinus*

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Table 1 Genetic diversity assessed for *Leopardus tigrinus*, *L. geoffroyi* and *L. colocolo* samples using a concatenated mtDNA data set containing segments of the control region and the *ND5* and *ATP8* genes

Clade	N	No. of haplotypes	No. of variable sites	Nucleotide diversity (± SE)	Haplotype diversity (± SE)
L. tigrinus	54	25	27	0.003866 (± 0.002269)	0.9266 (± 0.0247)
L. geoffroyi	38	18	28	0.006247 (± 0.003462)	0.8990 (± 0.0341)
L. colocolo	10	6	18	0.009462 (± 0.005638)	0.8444 (± 0.1029)

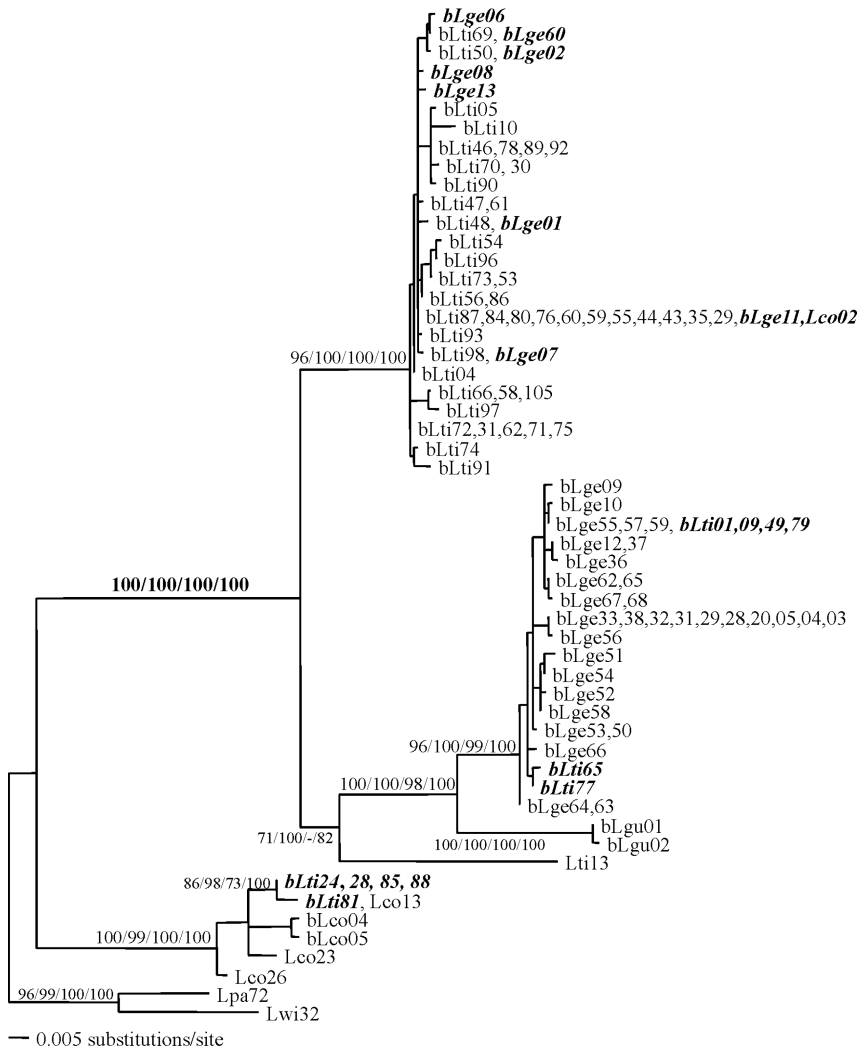

Fig. 2 Maximum likelihood (ML) phylogeny of mitochondrial DNA haplotypes (concatenated control region, *ND5* and *ATP8* segments, totaling 1024 bp) sampled in multiple individuals of *Leopardus tigrinus* (bLti), *L. geoffroyi* (bLge), *L. colocolo* (Lco), *L. guigna* (bLgu), *L. wiedii* (Lwi) and *L. pardalis* (Lpa) (see Supplementary material for details on sample ID and collection data). Individuals shown in bold italic fonts are inferred to be interspecific hybrids carrying an introgressed mtDNA haplotype from a different species. Values above or below branches indicate support for the subsequent node based on ML/MP/NJ/BI (Bayesian posterior probabilities are indicated as percentages); support is depicted only for nodes defining major clades relevant for our analyses.

and lowest in *L. colocolo*, correlating with the included sample size and number of variable sites for each species. In contrast, nucleotide diversity was considerably higher in *L. colocolo* than in the other two species, in spite of the small sample size available for the former, which only partially represented its geographical range.

All phylogenetic analyses performed with various methods led to congruent results with respect to major topological features of the mtDNA tree (Fig. 2). Clades representing *L. geoffroyi* and *L. colocolo* were strongly supported, as was the placement of the kodkod (*L. guigna*) as the sister-group of *L. geoffroyi*. All Brazilian samples of *L. tigrinus* also formed a highly supported clade, corresponding to a morphologically well-defined species occurring in this region. The deep divergence between these lineages and the Central American sample of *L. tigrinus* (Lti13), which had been observed in a previous study (Johnson *et al*. 1999), was corroborated by this larger mtDNA data set. Moreover, in several of the analyses performed here (e.g. Fig. 2), the Lti13 lineage was found to be more closely related to the (*L. geoffroyi* + *L. guigna*) clade than to the remaining *L. tigrinus*, which would challenge the monophyly of this species. Additional

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Table 2 Characteristics of nine microsatellite loci analyzed in 60 *Leopardus tigrinus*, 41 *L. geoffroyi* and six *L. colocolo* individuals. The size range, number of alleles and expected heterozygosity (H_E) are given for each locus. The mean values across loci are shown in bold at the bottom

		L. tigrinus		*L. geoffroyi*		*L. colocolo*	
Locus	Size range	No. alleles	H_E	No. alleles	H_E	No. alleles	H_E
FCA391	207–247	7	0.795	8	0.773	5	0.712
FCA424	166–198	6	0.669	4	0.353	5	0.909
FCA441	131–151	5	0.674	5	0.693	5	0.848
FCA453	186–210	7	0.695	5	0.717	3	0.818
FCA723	243–343	20	0.907*	24	0.932*	4	0.803
F42	219–259	9	0.858	11	0.884	7	0.894
F98	163–187	6	0.402*	3	0.572	5	0.803
F124	160–216	13	0.800	9	0.843*	7	0.924
F146	151–169	7	0.645	5	0.658	5	0.939
Mean		**8.89**	**0.716**	**8.22**	**0.714**	**5.11**	**0.85**

*Significant departure from HWE ($P < 0.05$).

sampling of *L. tigrinus* individuals in Central America and northern South America will be required to further investigate the possibility of a species-level distinction involving these populations.

Identification of hybridization and introgression events. The phylogenetic trees revealed the presence of several 'misplaced' individuals, i.e. animals whose morphological identification did not match their respective mtDNA clade (Fig. 2). These included eight *L. geoffroyi* individuals (bLge01, 02, 06, 07, 08, 11, 13, 60) placed in the *L. tigrinus* clade; one *L. colocolo* individual (Lco02) also placed in the *L. tigrinus* clade; six *L. tigrinus* individuals (bLti01, 09, 49, 65, 77, 79) placed in the *L. geoffroyi* clade; and five *L. tigrinus* samples (bLti24, 28, 81, 85, 88) placed in the *L. colocolo* clade. Several of these misplaced individuals were available for re-inspection (e.g. frozen carcass from road-killed animals); in all such cases the original morphological identification was affirmed by congruent re-assessments by the authors as well as independent scrutiny by other experts. To verify if contamination could explain the results, DNA from these individuals was re-extracted, and the relevant mtDNA segments were independently amplified and sequenced, in every case confirming the original result. Interestingly, four of the *L. geoffroyi* found to contain 'misplaced' mtDNA haplotypes (bLge02, 07, 08, 11) presented coat colour patterns that seemed to be ambiguous or intermediate with respect to *L. tigrinus*.

Given the observation of clear species-level distinction on the basis of tree topology, combined with individual 'swaps' suggestive of secondary contact, we performed a pairwise AMOVA to assess the magnitude of overall mtDNA differentiation among the samples defined by morphology as belonging to each species. This approach aimed (i) at assessing the net mtDNA differentiation among species; and (ii) to serve as a baseline for comparison with the microsatellite data set (see below). Various sequence-based distance measures were explored, as well as a traditional F_{ST} using only haplotype frequencies. The latter approach led to a severe underestimate of species-level differentiation (overall $F_{ST} = 0.06$) likely due to the high intraspecific haplotype diversity (see Table 1) masking the evident occurrence of distinct clades (see Fig. 2). In contrast, all AMOVA comparisons incorporating haplotype differences produced high and significant ($P < 0.001$) F_{ST} values, in spite of the inclusion of 'swapped' individuals in their morphology-based cluster. For example, an AMOVA using p-distances led to F_{ST} estimates of 0.61 for *L. geoffroyi* vs. *L. colocolo*; 0.53 for *L. tigrinus* vs. *L. geoffroyi* and 0.47 for *L. tigrinus* vs. *L. colocolo*.

Demographic history and implications for hybridization. The phylogenetic trees indicated the existence of very little internal structure within the *L. tigrinus* and *L. geoffroyi* clades, with shallow branches, no robust support for any grouping and no clear geographical clustering of haplotypes (Fig. 2). This very shallow and unstructured pattern, which approximates a star-like appearance in an unrooted tree, is suggestive of a recent population expansion. In contrast, the *L. colocolo* clade did present some internal structure, in agreement with our previous results (Johnson *et al*. 1999). Interestingly, there was a well-supported inner group joining the single Brazilian sample of *L. colocolo* included in the mtDNA data set (Lco13) and all *L. tigrinus* samples that clustered in this clade.

The next set of mtDNA-based analyses focused exclusively on the *L. tigrinus* and *L. geoffroyi* clades and aimed to investigate two issues: (i) the occurrence and age of historical population expansions in these groups; and (ii) the relationship between these inferred past events and the haplotype-swaps indicative of interspecies hybridization. To test the hypothesis of recent population expansions in

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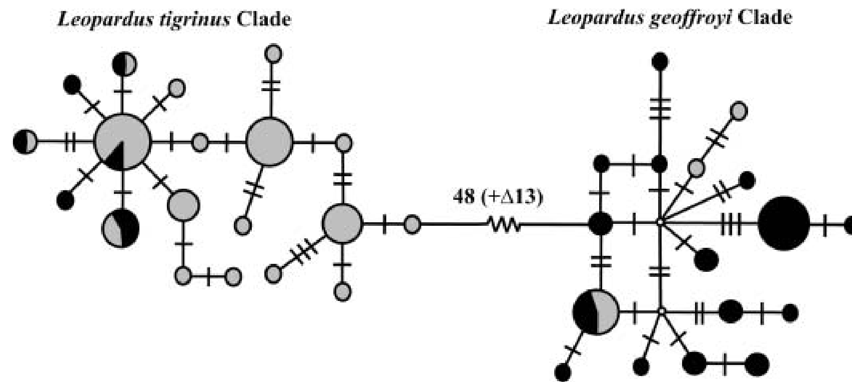

Fig. 3 Median-joining network of mtDNA haplotypes sampled in *Leopardus tigrinus* (grey) and *L. geoffroyi* (black) individuals. Only control region (CR) and *ND5* sequences were used in this analysis (totaling 795 bp), and all sites containing missing information or gaps were excluded. The area of each circle is roughly proportional to the haplotype frequency. Haplotypes shared between the two species are represented by circles with mixed colours, in which the relative frequency is indicated by the proportion of black and grey. Bars placed on connecting lines indicate the exact number of nucleotide differences between haplotypes. The branch connecting the two main clades contains 48 nucleotide differences and a synapomorphic deletion of 13 nucleotides in the CR defining the *L. geoffroyi* clade.

these groups, we performed mismatch distribution analyses and neutrality tests for each of these mtDNA clades. The mismatch distribution was smoothly unimodal for the *L. tigrinus* clade (see Supplementary material), supporting the inference of a recent demographic expansion in this species. Although the pattern was also roughly unimodal for the *L. geoffroyi* clade, in this case, the curve was not completely smooth, suggesting a more complex demographic history for this felid. All neutrality tests produced negative values for both clades, but most were nonsignificant ($P > 0.05$). The only exception was Fu's Fs test for the *L. tigrinus* clade (–12.179), which was significantly negative ($P < 0.05$). Overall, these results are supportive of the hypothesis of a recent population expansion in both species, especially in *L. tigrinus*.

We then performed a molecular dating analysis to assess the age of the haplotype coalescence (Time to the Most Recent Common Ancestor, TMRCA) in each of the two focal clades. This coalescence age would be an upper bound to the time since the inferred expansions occurred. Moreover, given the shallow, unstructured pattern observed in the phylogeny, it can be assumed that the coalescence age approximates the expansion age, especially in the case of *L. tigrinus* (for which the signal of recent expansion is clearer). The Bayesian molecular dating analyses performed with BEAST yielded a smooth posterior probability distribution (indicating a reliable parameter estimate) for the TMRCA of both the *L. tigrinus* and *L. geoffroyi* mtDNA clades. Interestingly, the ages were remarkably concordant for the two clades, with median TMRCA estimates of 75 700 and 70 000 years ago, respectively. The credibility interval around these estimates, indicated by the 95% highest probability density interval, was also very similar in the two species: 28 700–157 000 years ago for the *L. tigrinus* clade, and 26 800–144 000 years ago for the *L. geoffroyi* clade.

Finally, to gain a more detailed understanding of the genealogical relationships among mtDNA haplotypes in these two focal clades, we constructed a median-joining network, using a concatenation of the *ND5* and control region segments (the *ATP8* segment was excluded due to its having more missing data) and removing from the analysis all sites with missing or ambiguous information (Fig. 3). The two species-level groups were again apparent, separated by 48 substitutions and one synapomorphic indel. A star-like pattern could be observed in a portion of the *L. tigrinus* cluster, with one common haplotype in a central position connected by short branches (one mutational step each in all but one case) to multiple rarer sequences. No such pattern could be discerned in the *L. geoffroyi* phylogroup, which is congruent with the other results, indicating that the signal for a recent sudden expansion is less clear in this species than in *L. tigrinus*. Five haplotypes were shared between the two species, and four others seemed to be 'swapped' between them (i.e. they were only sampled in the species which did not correspond to their containing cluster). Interestingly, these haplotypes were always nested within each of the well-defined groups, i.e. they occupied internal rather than basal positions within each cluster, supporting the interpretation that the 'swaps' are due to secondary contact. In the case of the *L. tigrinus* clade, all haplotypes sampled in *L. geoffroyi* individuals were associated with the star-like portion of the cluster (see Fig. 3).

Microsatellites

Patterns of allelic diversity. All nine microsatellite loci were polymorphic for *L. tigrinus*, *L. geoffroyi* and *L. colocolo*. All individuals presented unique multilocus composite genotypes. Levels of genetic diversity and allele frequency distributions were similar for the three species (Table 2; see

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Supplementary material for details). There were 36 private alleles, 14 of which in *L. tigrinus*, 12 in *L. geoffroyi* and 10 in *L. colocolo*. A significant departure from HWE was observed at two loci each for *L. tigrinus* and *L. geoffroyi* after a Bonferroni correction ($\alpha = 0.05$) (Table 2).

These deviations indicated the possible presence of null alleles or other locus-specific genotyping errors. Analysis of the microsatellite data set with MICRO-CHECKER showed no evidence for genotyping errors due to stuttering or large-allele dropout, suggesting the presence of nonamplifying alleles as a probable source of genotyping errors. Estimated null frequencies varied by population and locus, with *L. geoffroyi* presenting two significant results of general excess of homozygotes for most allele size classes (F124, FCA723), *L. tigrinus* three (F98, F146, FCA723) and *L. colocolo* one (FCA391). The only locus with evidence of null alleles detected at more than one population was FCA723, suggesting that this locus is prone to genotyping errors that may lead to deviations from HWE. All pairwise locus combinations were in LE for *L. geoffroyi* and *L. colocolo* (no information was available for the analysis of the combinations including loci F42, F124 and F146 for *L. colocolo*; $\alpha = 0.05$, after Bonferroni correction for 36 comparisons). However, two combinations of loci were in linkage disequilibrium for *L. tigrinus* (FCA424 × F42 and FCA441 × F98). Additional analyses were performed after exclusion of inferred hybrids (see below), leading to a better understanding of the mechanisms underlying the observed departures from equilibrium.

The microsatellite-based AMOVA indicated that genetic diversity was significantly partitioned between the three species, although the magnitude of interspecies differentiation was modest. There was a higher degree of differentiation observed for *L. colocolo* relative to the other two ($F_{ST} = 0.162$ with *L. geoffroyi* and $F_{ST} = 0.140$ with *L. tigrinus*; $P < 0.001$). The genetic differentiation between *L. tigrinus* and *L. geoffroyi* was remarkably low for an interspecies comparison ($F_{ST} = 0.064$; $P < 0.001$), especially given that these two species clearly form separate evolutionary units on the basis of the mtDNA data (see Fig. 2 and text above for mtDNA-based AMOVA).

Admixture analyses. To investigate whether hybridization among these species could be an underlying cause for the low levels of observed interspecific microsatellite divergence, we performed a more detailed assessment using the Bayesian approach implemented in the program STRUCTURE. In the initial sets of analyses we utilized all genotyped samples of *L. tigrinus* and *L. geoffroyi*, as well as six *L. colocolo* individuals (see Supplementary material). We began by evaluating the most likely subdivision scenario without using the phenotype-based information, and the probability of the observed data was minimal with $K = 10$ and maximal with $K = 3$ (see Supplementary material for mean ± SD values of –Ln Likelihoods). Evaluating the results with $K = 3$, we observed that each species was assigned predominantly to one of the three clusters. However, while the *L. colocolo* population was assigned to Cluster 3 with a mean probability higher than 0.9, the *L. geoffroyi* and *L. tigrinus* populations were assigned to Clusters 1 and 2 with mean probabilities of 0.558 and 0.642, respectively. These results suggested that it was more difficult to assign the *L. geoffroyi* and *L. tigrinus* individuals to exclusively one cluster based on the genetic information alone. One *L. tigrinus* individual (bLti81) was assigned to the *L. colocolo* cluster with a probability of 0.95, in agreement with the mtDNA results (Fig. 2). Intriguingly, four other individuals (bLti24, 28, 85, 88) whose mtDNA indicated a hybrid ancestry with *L. colocolo,* were instead simultaneously (and partially) assigned to the *L. tigrinus* and *L. geoffroyi* clusters.

The exclusion of these five *L. tigrinus* individuals identified by the mtDNA data as hybrids with *L. colocolo* eliminated the linkage disequilibrium for the former species at all nine analyzed loci, even though the hybrids with *L. geoffroyi* still remained in the data set. In contrast, HW disequilibrium persisted in the *L. tigrinus* and *L. geoffroyi* population, even after the exclusion of all individuals putatively identified as hybrids by the present analyses (see below).

The next sets of analyses were focused on investigating the hybridization between *L. tigrinus* and *L. geoffroyi*, and therefore we excluded all *L. colocolo* individuals as well as the five samples (mentioned above) inferred to be hybrids with this species. To dissect the genetic composition of all individuals in this data set ($n = 96$), we performed a detailed analysis using STRUCTURE. In the main set of runs, we did not use phenotype-based prior information on species assignment, so as to let the clusters be assessed solely on the basis of the genetic data. We initially investigated the most likely number of distinct populations included in this data set. For this set of analyses we only tested $K = 1$–4, since we had observed a substantial decrease in the likelihood of the data with $K > 4$ in the previous runs (which had also included *L. colocolo*). The probability of the data was maximal for $K = 3$ (–Ln likelihood = 2738.44; see Supplementary material for mean ± SD of all likelihood values), followed closely by $K = 2$ (2743.84). Since the correlated frequencies model has been reported to overestimate K in some cases, reflecting deviations from random assortment that are not caused by genuine population subdivision (Falush *et al*. 2003), we followed Pritchard *et al*.'s (2000) recommendation that, when different values of K have similar probability estimates, we should be sceptical about the reliability of the ones implying a higher degree of subdivision. This would especially be the case when the assignments were roughly symmetrical to multiple populations, with almost no individuals strongly assigned to one of the three clusters, and with no clear biological interpretation for these assignments. As this pattern was clearly recognizable in our case when assuming $K = 3$, we chose to employ $K = 2$ for this set of

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Table 3 Microsatellite-based population assignment and ancestry allocation of inferred hybrids between *Leopardus tigrinus* and *L. geoffroyi*. Only individuals conservatively identified as hybrids based on the mtDNA data set are shown. See text and Supplementary material for discussion on additional individuals whose microsatellite genotypes also suggest a hybrid origin. Abbreviations for individual ID and geographical origin are the same as in Figs 1 and 2

		Without phenotypic information*		With phenotypic information†	
Sample	Origin	Cluster 1 (bLge)	Cluster 2 (bLti)	Cluster 1 (bLge)	Cluster 2 (bLti)
L. geoffroyi		0.85	0.15	0.97	0.03
bLge01	RS	0.98 (0.89–1.00)	0.02 (0.00–0.11)	0.99	0.00–0.00–0.01
bLge02	RS	0.91 (0.42–1.00)	0.09 (0.00–0.58)	0.98	0.00–0.00–0.02
bLge06	RS	0.73 (0.00–1.00)	0.27 (0.00–1.00)	0.94	0.00–0.02–0.04
bLge07	RS	0.45 (0.00–1.00)	0.55 (0.00–1.00)	0.81	0.01–0.07–0.11
bLge08	RS	0.63 (0.00–1.00)	0.37 (0.00–1.00)	0.91	0.00–0.02–0.07
bLge11	RS	0.40 (0.00–1.00)	0.60 (0.00–1.00)	0.73	0.09–0.08–0.10
bLge13	RS	0.40 (0.00–1.00)	0.60 (0.00–1.00)	0.79	0.05–0.04–0.12
bLge60	RS	0.47 (0.00–1.00)	0.53 (0.00–1.00)	0.93	0.01–0.02–0.04
L. tigrinus		0.18	0.82	0.05	0.95
bLti01	RS	0.37 (0.00–1.00)	0.63 (0.00–1.00)	0.00–0.07–0.10	0.83
bLti09	RS	0.90 (0.54–1.00)	0.10 (0.00–0.46)	0.15–0.61–0.14	0.10
bLti49	RS	0.93 (0.59–1.00)	0.07 (0.00–0.41)	0.59–0.16–0.09	0.16
bLti65	SP	0.04 (0.00–0.24)	0.96 (0.76–1.00)	0.00–0.00–0.02	0.98
bLti77	SP	0.03 (0.00–0.18)	0.97 (0.82–1.00)	0.00–0.00–0.01	0.99
bLti79	RS	0.81 (0.34–1.00)	0.19 (0.00–0.66)	0.24–0.49–0.17	0.10

*These columns contain the 'q' value (mean across five runs) for each individual in each population cluster, i.e. the probability that its genomic ancestry lies in that group, disregarding any prior assumption based on morphology (see text for details). A conservative estimate of the credibility interval (lowest observed lower bound to highest upper bound among five independent runs) is given in parentheses. The top line for each species indicates the overall assignment of its samples (identified morphologically) to each genetically defined cluster.
†These columns contain the q value for each individual in either its own assumed cluster (single number) or as a result of admixture with the other group (three numbers). In the latter case, the numbers are the probabilities that the individual's ancestry lies in the other genetic cluster in the first, second or third past generation, respectively (see text for details).

analyses, as this seemed to capture most of the genetic structure in the sampled individuals and was biologically more reasonable.

With $K = 2$, the *L. geoffroyi* samples were assigned predominantly to Cluster 1 (with $q1 = 0.85$), and those of *L. tigrinus* to Cluster 2 (with $q2 = 0.82$) (Table 3). Of the 41 *L. geoffroyi* individuals, 29 were assigned to Cluster 1 ('geoffroyi' cluster) with $q1 \geq 0.9$, while 11 had intermediate $q1$ values between 0.818 and 0.396 (Fig. 4), i.e. they had a considerable portion of their genome inferred to be of *L. tigrinus* ancestry (see Supplementary material for a full list of q-values for all individuals). One additional individual (bLge67, from Bolivia) had a probability of 0.92 of belonging to the *L. tigrinus* population instead of its own, indicating that it also possessed hybrid ancestry. The 12 samples with $q1 < 0.90$ were all from RS state, mostly from its central region, with only one exception from Argentina (bLge50) (see Fig. 1). Of the 55 *L tigrinus* samples, 34 were assigned to Cluster 2 ('tigrinus' cluster) with $q2 \geq 0.9$, while 19 had intermediate $q2$ values (between 0.897 and 0.113) and two were highly associated with the *L. geoffroyi* cluster (bLti09: $q2 = 0.099/q1 = 0.901$ and bLti49: $q2 = 0.072/q1 = 0.928$) (Fig. 4). Of the 21 individuals assigned to Cluster 2 with $q2 < 0.90$, 12 (57%) were from RS state, while the remaining ones originated in different Brazilian states: Paraná (6), São Paulo (2) and Goiás (1).

All samples from both species with $q \leq 0.90$ showed very broad credibility intervals for their q-values, often encompassing both ends of the 0–1 range (see Fig. 4 and Supplementary material). This type of pattern has also been reported for other highly hybridizing species (e.g. Beaumont *et al*. 2001; Nielsen *et al*. 2003). Assuming a q-value of 0.9 as the threshold for distinguishing pure from hybrid individuals, as reported in similar hybridization studies (e.g. Lancaster *et al*. 2006; Vähä & Primmer 2006; Oliveira *et al*. 2008), and considering the broad intervals for q found in the intermediate individuals in our sample, our microsatellite data set identified 12 putative hybrids among the morphologically defined *L. geoffroyi* individuals, and 21 among the *L. tigrinus* (see Supplementary material).

To further assess the genetic ancestry of the inferred hybrids so as to determine the occurrence and extent of genomic introgression in our sample, we performed an additional set of STRUCTURE analyses using the phenotype-based information. In this case, the analysis allows the inference, for each individual, of the probability that its

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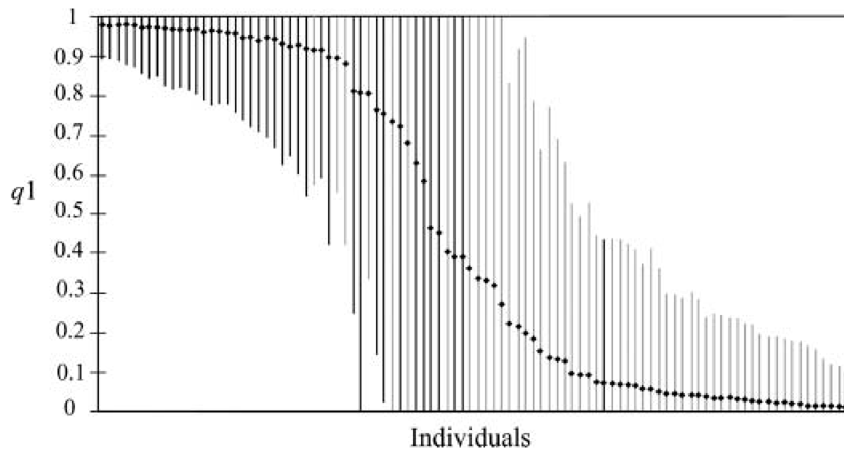

Fig. 4 Graph depicting the results of the Bayesian admixture analysis focusing on the hybridization between *Leopardus geoffroyi* and *L. tigrinus* (performed with STRUCTURE, with $K = 2$, employing the correlated frequencies model and no use of prior population information, i.e. only the genetic data were used to infer population assignment). All *L. colocolo* individuals and the five identified hybrids with that species were excluded from this data set. Diamonds represent the mean $q1$ value for each individual (averaged over five independent runs). Vertical lines represent a conservative estimate of the credibility interval (CI) for each individual, i.e. the range between the lower and upper bounds of the CIs observed across the five runs. Thicker black lines correspond to individuals morphologically identified as *L. geoffroyi*, while thinner gray lines indicate individuals identified as *L. tigrinus*. Individuals are sorted according to their mean $q1$ value (see Supplementary material for a complete list of q-values and their CIs for all individuals).

ancestry lies in a different population in the first, second or third past generations (Pritchard *et al*. 2000). In this case, these three possibilities are equivalent to the sample originating from a misidentified individual (i.e. totally belonging in the other genetic population), or an F_1 hybrid, or a second-generation hybrid, respectively. As expected, the use of phenotype-based priors greatly enhanced the assignment of individuals to their assumed population (Table 3): the *L. geoffroyi* sample was now assigned with $q1 = 0.97$ to Cluster 1 and the *L. tigrinus* sample with $q2 = 0.95$ to Cluster 2. All individuals inferred to be hybrids in the previous set of analyses were carefully inspected, revealing no case suggestive of misidentification. The majority of the hybrid individuals presented high probabilities of belonging to their assumed phenotype-based population while having admixed ancestry from the other species, predominantly in the second and third past generations. Five of the twelve *L. geoffroyi* presenting q-values ≤ 0.90 of belonging to its own cluster in the previous set of analyses (without phenotype-based information), and ten of the 21 *L tigrinus*, still presented evidence of admixed ancestry when maintaining a threshold of $q = 0.9$ for these runs. In addition, none of the inferred hybrid individuals presented a very high probability of being an F_1, indicating the occurrence of advanced introgression and complex patterns of admixed ancestry (see Table 3 and Supplementary material).

There were several cases of congruence in the detection of hybrids using the mtDNA and microsatellite data sets (e.g. bLge07, bLge11, bLge13, bLti01, bLti09, bLti49 and bLti79; see Table 3). However, some individuals bearing 'misplaced' mtDNA haplotypes were only identified as admixed in the microsatellite-based STRUCTURE analysis without phenotypic information, while others were not recognized as being admixed by any microsatellite-based assessment. Finally, some individuals presented intermediate q-values in the microsatellite-based analyses, suggestive of admixture, but there was no evidence of hybridization with mtDNA. Given this complex set of patterns, which is expected under a scenario of multigenerational admixture, we conservatively defined as inferred hybrids only the 14 individuals bearing conclusive mtDNA-based evidence (Table 3), while the actual number may be much higher; up to 33 considering the microsatellite data alone (see above and Supplementary materialfor details).

To investigate whether we could detect a geographical pattern in this observed genetic introgression, we focused on the *L. tigrinus* sample, for which a broader spatial coverage was available. We hypothesized that admixture with *L. geoffroyi* would be more prevalent in the vicinity of their geographical contact zone than farther north in Brazil, as indicated by the individual-based analyses described above. Our goal was to test whether this trend could be detected at the population level with our microsatellite data set. For this analysis, we only used *L. tigrinus* samples with a known geographical origin and excluded regions for which our sample size was very small (Brazilian center–west and Paraguay). This resulted in a sample of 50 *L. tigrinus*, which were subdivided into three geographical subpopulations, arranged in a south-to-north sequence: (i) RS state; (ii) SC + PR states; and (iii) SP + RJ + ES states (see Fig. 1 and Supplementary material). We assessed the genetic differentiation among these regions, as well as each of them vs. the

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Table 4 Genetic differentiation among three geographical subpopulations of *Leopardus tigrinus* and the *L. geoffroyi* sample, estimated using an F_{ST} analogue calculated via an AMOVA approach. The overall F_{ST} value for this subdivision scenario was 0.075 ($P < 0.05$). Abbreviations of species designation and Brazilian states are as in Figs 1 and 2

	bLge	bLti-RS	bLti-SC/PR
bLge	—	—	—
bLti-RS	0.049*	—	—
bLti-SC/PR	0.069*	0.027*	—
bLti-SP/RJ/ES	0.117*	0.057*	0.009

*Statistically significant F_{ST} value ($P < 0.05$).

entire sample of *L. geoffroyi* ($n = 41$), using an AMOVA-based estimate of F_{ST}. All comparisons yielded significant F_{ST} values, except between the two northernmost *L. tigrinus* populations (Table 4). The RS partition was thus the most divergent among the three *L. tigrinus* subgroups and was found to be more similar genetically to the *L. geoffroyi* sample than to the SP + RJ + ES population of its own species. There was a clear trend of increased differentiation from *L. geoffroyi* as the sampling got more distant from the contact zone between the two species, supporting the inference of a geographical gradient of introgression affecting the genetic composition of *L. tigrinus* in the surveyed areas.

Discussion

A hybrid zone between Leopardus tigrinus *and* L. geoffroyi *in southern Brazil*

The results presented here provide strong evidence for the occurrence of hybridization between *Leopardus tigrinus* and *L. geoffroyi*, which seems to be concentrated in their region of geographical contact in southern Brazil (see Fig. 1). The joint inference from the mtDNA and microsatellite data sets, in combination with the morphology-based assessments, suggests that a hybrid zone between these species occurs in this area. Our analyses also identified a geographical gradient of introgression of *L. geoffroyi* genomic components into *L. tigrinus* populations sampled at varying distances from the inferred hybrid zone.

The magnitude of *L. tigrinus* and *L. geoffroyi* differentiation based on our microsatellite data set was quite low, relative to what is usually observed in other studies focusing on carnivores (e.g. Johnson *et al*. 1999; Randi *et al*. 2001; Randi & Lucchini 2002). This pattern might be explained by incomplete evolutionary separation between these two cat species, due to recent common ancestry. However, this hypothesis is rejected by the mtDNA results presented here (see Fig. 2), as well as previous analyses indicating that these species do present clear evolutionary distinctiveness (Johnson *et al*. 1999, 2006). The extensive interspecific allele sharing observed here could instead be influenced by the occurrence of rampant homoplasy at the examined loci, leading to the co-occurrence of alleles that are identical in state, though not by descent (Jarne & Lagoda 1996). This phenomenon tends to homogenize allele frequencies between distantly related species or populations and has been reported even at the intraspecific level (Nauta & Weissing 1996; Culver *et al*. 2001). It is probable that homoplasy does play a role in the low microsatellite-based interspecies divergence observed here (as may be inferred from the low F_{ST} differentiating *L. colocolo* from *L. geoffroyi*, in spite of the lack of any evidence of hybridization between them). However, it seems unlikely that the magnitude of interspecies allele overlap observed here could be achieved by homoplasy alone, especially in light of the geographical pattern seen in Table 4, as well as the mtDNA-based analyses supporting hybridization. More likely, the observed pattern of extensive allele sharing and weak genetic differentiation between the two species reflects a combination of some level of homoplasy and considerable introgressive hybridization between these species, which has eroded their allelic differences at these markers.

The evidence of hybridization and introgression between these two cat species is supported by the observation of 'misplaced' mtDNA haplotypes and by a typical cline in microsatellite-based genetic differentiation between them (see Fig. 4). However, the precise identification of all hybrid individuals is challenging, due to the complex pattern of admixture inferred by the combination of the two types of molecular markers. Fourteen individuals were identified as hybrids due to their 'swapped' positions in the mtDNA phylogeny (see Fig. 2), including animals from both species (based on morphological criteria). Although most individuals could be unambiguously assigned to one species using morphological criteria, some of the animals identified as putative hybrids (e.g. bLge08 and bLge11) bore unusual coloration patterns, seemingly intermediate between the two taxa. With the microsatellite data, several individuals of both species presented intermediate *q*-values assigning them to either cluster, with very broad credibility intervals that hampered a conclusive inference of their allocation (see Table 3 and Supplementary material). There was no clear correlation between the extent of microsatellite-based admixture and the presence of an introgressed mtDNA lineage (e.g. when comparing both types of data for bLge01, bLge02, bLti65 and bLti77 in Table 3 and Fig. 2). Nevertheless, there were some cases of concordance between mtDNA introgression and a high level of nuclear admixed ancestry, which possibly indicate individuals descended from recent (and/or multiple) episodes of hybridization.

Considering the combined evidence from the mtDNA and microsatellites, up to 33 individuals may be identified as hybrids between *L. tigrinus* and *L. geoffroyi* (assuming

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$q = 0.9$ as a threshold; see Supplementary material), which represents 34% of the final sample ($n = 96$). This would be one of the most extensive levels of hybridization reported for carnivores up to now, similar to what has been observed in the intensely hybridizing populations of wild and domestic cats in Hungary (Lecis *et al*. 2006). However, in the case of the European wild and domestic cats (*Felis silvestris*), the hybridizing populations are very closely related and currently regarded as conspecific (Driscoll *et al*. 2007), whereas the Neotropical pair described here consists of distinct species separated *c.* 1 million years ago (Johnson *et al*. 2006). Our mtDNA data support the evolutionary distinction of the two lineages (see Fig. 2), in stark contrast with the extremely low level of differentiation observed with the microsatellite markers.

Most of the individuals identified here as probable hybrids based on both molecular markers have a geographical origin compatible with the presence of a hybrid zone. Of the 14 cats identified as hybrids by the mtDNA data, 12 originate from RS state, particularly from its central region near parallel 30°S, the only area in Brazil where sympatry of the two species has been documented (Eizirik *et al*. 2006). Despite the difficulty in precisely defining hybrids with our microsatellite data, we could observe a similar pattern of concentration of intermediate individuals in this geographical contact zone, with few implicated animals sampled far from it. As a whole, these observations indicate that the admixture between these species is quite concentrated in the areas surrounding their contact zone, suggesting either that the hybridization process is extremely recent or that there is some selective restriction on the geographical spread of admixed descendants. In-depth studies involving ecological as well as genetic analyses will be required to further understand the underlying causes of this pattern.

Although we could only document the clear occurrence of genomic introgression from *L. geoffroyi* to *L. tigrinus*, it remains plausible that it also occurs in the opposite direction, given the observation that morphologically defined *L. geoffroyi* bear genomic segments and/or mtDNA haplotypes originating from *L. tigrinus*. If affirmed by further scrutiny, it would remain to be determined whether the magnitude of introgression is symmetrical between these species. This would produce an interesting comparison to the patterns found in several studies of hybridization between other pairs of carnivore taxa, which often identify asymmetric introgression (e.g. Roy *et al*. 1994; Vilà & Wayne 1999; Randi & Lucchini 2002; Lancaster *et al*. 2006; Lecis *et al*. 2006). A common and plausible explanation for the asymmetry observed in other systems is the difference in local density between the two hybridizing populations, leading to the increased pressure of genomic introgression in one direction vs. the other. The uneven presence of males and females from different hybridizing populations may also affect the directionality of the process (e.g. Lancaster *et al*. 2006), especially if associated with mating systems that favour one of the possible hybrid pairs. Although very little is known about the mating system of *L. geoffroyi* and *L. tigrinus* in the wild, or their relative densities in this hybrid zone, preliminary field observations suggest that both are relatively common in RS state (the former in the south and the latter in the north, Eizirik *et al*. 2006), suggesting that the genomic influx may be similar in both directions.

The results presented here allow us to propose a hypothesis for the genesis of this hybrid zone, which postulates that the two species evolved in allopatry from a common ancestor that lived *c.* 1 million years ago, and they only recently entered in geographical contact due to a population expansion in one or both of them. Our analyses are compatible with the inference of recent population expansions in both species, though the signal is stronger and clearer for *L. tigrinus*. The shapes of the mtDNA phylogeny and haplotype network, along with results from the mismatch distribution analysis and Fu's Fs test, all indicate that this species bears the signature of a recent demographic expansion, which was inferred to have occurred near the coalescence of its haplotypes *c.* 76 000 years ago. An intriguing finding was the very concordant coalescence date of the *L. geoffroyi* clade, raising the possibility that the demography of both species was similarly affected by the same historical events. Most interestingly, the network positions of all *L. tigrinus* mtDNA haplotypes introgressed into the sampled *L. geoffroyi* were associated with this inferred expansion (see Fig. 3), supporting the speculation that the hybrid zone may be a consequence of this historical process of population growth.

There are several examples of hybrid zones that seem to be the result of secondary contact between previously allopatric populations that meet due to demographic expansions caused by responses to climatic and habitat changes (Barton & Hewitt 1985; Harrison 1993). Although this scenario is plausible for the case of *L. tigrinus* and *L. geoffroyi*, with a demographic expansion playing an important role in the geographical encounter between the two species, the exact age of the hybridization process still cannot be determined. Although the observed pattern is consistent with a natural hybrid zone formed *c.* 70 000 years ago, it is still possible that the actual admixture between the species is much more recent and could have been influenced by human activities. Anthropogenic habitat alteration has been rampant in some areas of RS state for over two centuries, and it is conceivable that these populations were not in direct contact prior to human disturbance. Depending on the intensity of interspecies breeding per generation, it is not impossible that two centuries of hybridization could lead to the observed pattern of admixture (e.g. see Mank *et al*. 2004). The distinction between these two historical scenarios is one of the major challenges ahead in the effort to characterize this hybrid zone.

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The broader picture: hybridization among L. tigrinus, L. geoffroyi *and* L. colocolo

Hybridization between *L. tigrinus* and *L. colocolo* had been previously reported (Johnson *et al*. 1999), and three of the implicated individuals identified in that paper (bLti24, 28 and 85) are included in this study, corroborating our initial findings with an expanded data set. In addition, here we identified two more *L. tigrinus* individuals that share *L. colocolo* ancestry, and one animal (Lco02) that seems to be a hybrid in the opposite direction (*L. tigrinus* mtDNA introgressed into a *L. colocolo*). The latter individual was a captive animal, whose sample was collected in 1981, so it is difficult to ascertain whether the implied hybridization event (involving a female *L. tigrinus* and a male *L. colocolo*) happened in the wild. The remaining animals involved in this hybrid combination can be ascertained to have a wild origin, implying that the underlying events did occur *in situ*. Interestingly, the mtDNA data supported a phylogenetic connection between these individuals and the *L. colocolo* sample collected in central Brazil, whose regional origin (Goiás state) is the same as that of three of these hybrid animals (see Fig. 2 and Supplementary material). We can thus infer that a hybrid zone between *L. tigrinus* and *L. colocolo* occurs in central Brazil, even though more sampling is still required to characterize it in more detail.

These results therefore reveal a remarkable pattern of complex interspecies admixture, which can be graphically observed in our mtDNA-based phylogenetic tree (see Fig. 2): individuals identified morphologically as *L. tigrinus* may bear one of three very distinct mitochondrial lineages, i.e. (i) that of their own species; (ii) that of *L. geoffroyi*; or (iii) that of *L. colocolo*. This situation of a double hybrid zone is quite unusual, especially involving medium-to-large mammals. Among the few reported cases of hybridization involving wild populations of three different mammal species, two include carnivores. The case of North American canids includes the observation that coyotes (*Canis latrans*) have expanded their range in the last century and in that process have hybridized at different levels with congeneric species, namely the grey wolves (*C. lupus*) and the eastern wolf-like populations often recognized as separate taxa (*C. rufus* and/or *C. lycaon*) (e.g. Lehman *et al*. 1991; Roy *et al*. 1994; Kyle *et al*. 2006). The second case is that of three species of fur seals of the genus *Arctocephalus* re-colonizing a sub-antartic island after historical extinction in the 19th century (Lancaster *et al*. 2006). The influence of human disturbance is clear in the latter case (as the historical extinction occurred due to over-hunting), and the three-species hybridization that can currently be observed on that single island is likely to decrease over time (Lancaster *et al*. 2006). In the case of canids, human impact has also played a major role, in the form of habitat alteration fostering coyote expansion, and as persecution of wolves leading to their decline in eastern North America (Kyle *et al*. 2006). The main source of complexity in this case lies in the persisting debate over the historical distinctiveness of eastern/red wolves, and whether they did represent a unique taxon prior to hybridization with coyotes (Roy *et al*. 1994; Murray & Waits 2007). The case reported here thus seems to present some relevant differences with respect to one or both of these other examples: (i) the lineages involved seem to be distinct enough to represent well-accepted species; (ii) the historical population expansion that may have been involved in this process is much older than the human presence in the region; (iii) there seem to be two geographically defined hybrid zones instead of a single site of admixture (as is the case in fur seals); (iv) bidirectional introgression may be occurring (although not yet demonstrated conclusively), at least in the case of *L. tigrinus* vs. *L. geoffroyi*; and (v) the causative role of recent human impact on the genesis of the hybrid zone is not as clear (or likely) as in the other two cases, although it remains a possibility.

Finally, our analyses showed no evidence of hybridization between *L. geoffroyi* and *L. colocolo*, in sharp contrast with the observed admixture of *L. tigrinus* with both of those species. If this observation is affirmed by further sampling of these felids, it is likely to reflect an important difference with respect to the reproductive isolation mechanisms acting in the various possible pairs formed by these species. Since *L. colocolo* and *L. geoffroyi* are sympatric over most of their geographical ranges (see Fig. 1), it can be hypothesized that they have evolved fully effective mechanisms for avoidance of hybridization with each other. The fact that this is not the case of *L. tigrinus* with respect to either of these two other species supports the inference that it has evolved in allopatry and only more recently entered in contact with these congeners, inducing the formation of a double hybrid zone.

Implications for conservation

The elucidation of hybridization processes between wild species in nature is critically important for the conservation of the involved taxa (Allendorf *et al*. 2001). Both *L. tigrinus* and *L. geoffroyi* are listed in Appendix I of CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora), while *L. colocolo* is in Appendix II (UNEP-WCMC 2004); all three species are considered to be near-threatened by the World Conservation Union (IUCN; IUCN/SSC Cat Specialist Group 2002). In Brazil, *L. tigrinus* and *L. colocolo* are considered vulnerable and *L. geoffroyi* near-threatened (IBAMA 2003), while in RS state, *L. colocolo* is listed as endangered and the others two as vulnerable (Marques *et al*. 2002). For the three species, lack of information on their biology, ecology, genetic structure and evolutionary history pose challenges to the design and implementation of adequate management and conservation strategies (Nowell & Jackson 1996).

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In this context, our results allow several recommendations regarding the conservation of these felids. Captive breeding of animals originating from areas where hybridization has been detected should be managed carefully, so as not to artificially increase the representation of introgressed genomic segments in the *ex situ* gene pool. In particular, we showed here that RS state constitutes a genetically distinct *L. tigrinus* population, which is more similar to *L. geoffroyi* than to conspecific populations located farther from the contact zone. Since it is still conceivable that human-induced habitat alteration has exacerbated (or even caused) this hybridization process, we recommend that this population be managed separately (e.g. in captive breeding programs and possible translocation operations), so as to not to compromise the genetic integrity of *L. tigrinus* in areas located farther from the hybrid zone. Furthermore, it is critical to perform in-depth ecological and genetic studies attempting to dissect the causes and current consequences of these hybridization processes, including the possible influence of human-induced habitat change and the role of natural selection in restricting the spread of introgression beyond the hybrid zone detected in southern Brazil.

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This study was performed within a broader research program on genetic, ecological and evolutionary aspects of the contact zone between *Leopardus tigrinus* and *L. geoffroyi* in southern Brazil. The microsatellite-based analyses were the subject of Tatiane C. Trigo's MSc thesis at the Graduate Program in Genetics and Molecular Biology of UFRGS, coadvised by Drs Thales Freitas and Eduardo Eizirik. The mtDNA-based analyses were performed by the research teams of Dr Eizirik and Dr Sandro Bonatto, also with direct participation of Ms Trigo. Dr Freitas is an evolutionary biologist working on several Neotropical mammals, especially fossorial rodents. Ms Gilis Kunzler and Ms Luana Cardoso were undergraduate research assistants that participated in the mtDNA-based analyses. Dr Jean Carlos R. Silva works on conservation medicine of Neotropical wildlife, with emphasis on felids. Drs Stephen O'Brien and Warren Johnson are involved in several research projects addressing evolution and population genetics of felids and other mammals. Dr Bonatto works on phylogenetics and molecular population genetics of various taxa, especially Neotropical animals. Dr Eizirik is an evolutionary and conservation geneticist working mostly on Neotropical carnivores.

Supporting Information

Additional Supporting Information may be found in the online version of this article.

Fig. S1 Histograms showing the frequency distribution of microsatellite alleles in *Leopardus tigrinus* (grey bars), *L. geoffroyi* (black bars) and *L. colocolo* (white bars). Each graph depicts alleles from a single microsatellite locus, identified at the top.

Fig. S2 Mismatch distribution analysis of the *Leopardus tigrinus* and *L. geoffroyi* clades, based on concatenated mtDNA control region and *ND5* sequences (795 bp, excluding all sites with missing information or gaps). The dashed line represents the observed pattern, while the continuous line depicts the pattern expected under a model of sudden demographic expansion.

Table S1 Samples of Neotropical felids analyzed in the present study

Table S2 Bayesian clustering analyses performed with STRUCTURE using the correlated frequencies model and no phenotype-based information. Each value corresponds to the average among five different runs

Table S3 Population assignment of *Leopardus tigrinus* and *L. geoffroyi* individuals using a STRUCTURE analysis with no phenotype-based information. The membership proportion for each individual is expressed as a *q*-value followed by its respective credibility interval in parentheses. Individuals with probabilities ≤ 0.90 of belonging to their phenotype-based species are shown in bold. Abbreviations for individual ID and geographical origin are the same as used in Figure 1 and Table S1, Supplementary material.

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