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GENÉTICA DA CONSERVAÇÃO E ECOLOGIA MOLECULAR DE ONÇAS-PINTADAS (*PANTHERA ONCA*, FELIDAE)

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“... uma natureza que perdeu seus grandes predadores, seja ele o lobo, o leão ou a onça-pintada, carece de um ingrediente essencial. Eu posso sentir a diferença; há menos vitalidade, menos tensão natural...”

George Schaller

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SUMÁRIO

RESUMO

Com o objetivo de realizar estratégias eficientes de manejo e conservação para a onça-pintada (*Panthera onca*), estudos ecológicos vêm sendo realizados em diferentes áreas ao longo de sua distribuição geográfica. No entanto, análises moleculares complementares a estas abordagens ecológicas são necessárias para garantir a viabilidade deste felídeo a longo prazo. Desta maneira, os estudos aqui apresentados tiveram por finalidade desenvolver metodologias para auxiliar estudos genéticos e ecológicos extremamente necessários para a conservação desta espécie, assim como embasar estudos populacionais com uma perspectiva genético-molecular. No primeiro capítulo, devido à enorme dificuldade de se obter amostras biológicas deste felídeo, foi desenvolvida uma abordagem baseada no sequenciamento de um segmento curto de um gene (*ATP6*) do DNA mitocondrial para identificar de maneira rigorosa amostras fecais de onça-pintada coletadas em campo. Os resultados indicaram a importância de se utilizar um método molecular para a identificação correta de amostras fecais coletadas em campo. No segundo capítulo foi demonstrado que é possível identificar a coloração de onças-pintadas a partir de DNA fecal, através da genotipagem molecular do polimorfismo envolvido no padrão de coloração desta espécie. Embora indivíduos melânicos de onça-pintada sejam relativamente comuns em algumas áreas da sua distribuição, até o momento, nenhum estudo científico envolvendo esta característica foi realizado com populações naturais desta espécie. O uso de amostragem não-invasiva possivelmente é uma das únicas maneiras para estudar diretamente populações de onça-pintada que apresentam esta característica. No terceiro capítulo foi realizado o primeiro estudo envolvendo estruturação e conectividade de populações naturais deste felídeo em uma escala regional. O estudo abrangeu as populações remanescentes de onça-pintada da Ecorregião do Alto Rio Paraná, contida na

Mata Atlântica de Interior. Os resultados indicaram perda de variabilidade genética em populações recentemente isoladas e uma considerável diferenciação entre os fragmentos, sugerindo forte efeito da deriva genética, por sua vez induzida pelo pequeno tamanho efetivo em cada área e o crescente isolamento entre as mesmas. Ao mesmo tempo, análises genéticas identificaram evidência de conexão demográfica recente entre áreas, sugerindo que este processo natural de conectividade deve ser mantido para garantir a viabilidade destas populações em longo prazo. Os resultados serão integrados ao plano de manejo que vem sendo desenvolvido para este felídeo no Alto Rio Paraná, subsidiando a elaboração e efetivação de esforços urgentes para a conservação desta espécie nesta ecorregião.

ABSTRACT

Several ecological studies have been conducted aiming to aid in the design of effective management and conservation strategies for the jaguar (*Panthera onca*) in different areas throughout its geographic distribution. However, molecular analyses complementary to these ecological approaches are necessary to ensure that these strategies secure the long term viability of this felid. The studies presented here aimed to develop methodologies that aid in ecological and genetic studies focusing on the jaguar, which are essential to the conservation of this species, as well as to serve as a basis for in-depth populational analyses employing a molecular genetic perspective. In the first chapter, due to the enormous difficulty in obtaining biological samples of this felid, an approach based on the sequencing of a short segment of a mitochondrial DNA gene (*ATP6*) was developed to accurately identify jaguar faecal samples collected in the field. The results of this study indicated the importance of using a molecular method for the correct identification of faecal samples. In the second chapter, we demonstrated that it is possible to reliably identify the color of a jaguar from faecal DNA by genotyping the molecular polymorphism involved in this coloration variant. Although melanistic individuals are relatively common in some areas of their distribution, so far no scientific study has been conducted addressing this phenotype in the wild. The use of non-invasive sampling is likely one of the few approaches to study natural populations of jaguars exhibiting this characteristic. The third chapter contains the first study involving the structure and connectivity of natural jaguar populations on a regional scale. The study included remnant jaguar populations of the Upper Paraná Atlantic Forest Ecoregion. The results indicated loss of genetic variability in recently isolated populations and considerable genetic differentiation among fragments, suggesting strong effects of genetic drift induced by the small effective size in each area

and increasing isolation among them. At the same time, genetic analyses identified clear evidence of recent demographic connectivity between areas, indicating that gene flow among them should be maintained to ensure the long term viability of these populations. The results will be integrated into the management plan that is being developed for this felid in the Upper Paraná Atlantic Forest, supplying data that is important for the development and implementation of urgent efforts to conserve this species in this ecoregion.

CAPÍTULO I

INTRODUÇÃO

I. 1. A Genética e a Biologia da Conservação

O crescimento populacional humano vem provocando como reflexo uma enorme crise na biodiversidade, com grandes modificações nos sistemas naturais devido à atividade humana. Espécies e populações locais têm sido extintas, enquanto que outras têm diminuído a sua distribuição e densidade, aumentando assim, o seu risco de extinção a curto e longo prazo (Frankham *et al.* 2002).

Em resposta a esta crise surge a Biologia da Conservação, uma ciência multidisciplinar que em conjunto com uma série de outras disciplinas, como a ecologia e a biologia de populações, gera informações para a conservação e o manejo dos recursos naturais (Soulé 1985; Primack & Rodrigues 2001; DeSalle & Amato 2004). Dentro deste âmbito, uma área recente da Biologia, conhecida como Genética da Conservação, tem se tornado uma ferramenta importante na elaboração de programas adequados de conservação e manejo (Johnson *et al*. 2001; Perez-Sweeney *et al.* 2006).

Um dos principais objetivos da Genética da Conservação é avaliar os níveis de diversidade genética existente em uma dada espécie ou população, assim como avaliar a distribuição geográfica desta diversidade, o que é de extrema importância para identificar e priorizar áreas nas quais programas de manejo e conservação devam ser elaborados (Moritz & Faith 1998). Por exemplo, para programas de conservação é fundamental avaliar se a variabilidade apresenta uma distribuição contínua ou se está subdividida, pois, no primeiro caso, qualquer área de sua distribuição é representativa da espécie enquanto que, havendo estruturação, a representatividade de cada subpopulação terá que ser preservada.

Para fins de manejo duas unidades demográficas intra-específicas podem ser identificadas: (i) Unidades Evolutivamente Significativas (“*Evolutionarily Significant*

Units" - ESUs) e (ii) Unidades de Manejo ("*Management Units*" - MUs). As primeiras são constituídas por unidades demográficas que ocupam áreas geográficas distintas e se apresentam diferenciadas geneticamente (implicando um isolamento histórico) de outras unidades semelhantes contidas na mesma espécie. Já as Unidades de Manejo estão contidas nas ESUs, e são formadas por populações regionais ou locais com restrita conexão demográfica entre si, mas não necessariamente com diferenciação genética profunda. Estas populações provavelmente mantêm algum nível de fluxo gênico entre si, porém sua conectividade demográfica é restrita em uma escala de tempo ecológica. No âmbito de uma avaliação envolvendo poucas gerações, o contato entre elas através de migração ou recolonização pode tornar-se escasso, transformando-as em entidades ecológicas relativamente separadas (Moritz 1994; Eizirik 1996; Crandall *et al*. 2000; Frankham *et al.* 2002; Eizirik *et al.* 2006).

Critérios genéticos para a identificação destas unidades intra-específicas foram propostos por Moritz (1994). Para o reconhecimento de ESUs, estas devem ter monofilia recíproca para haplótipos do DNA mitocondrial (DNAmt) e mostrar divergência significativa nas frequências alélicas de locos nucleares. Já as MUs não mostram monofilia recíproca para alelos do DNAmt mas exibem divergência significativa nas frequências alélicas de locos nucleares. A extinção de uma ESU representaria uma significativa perda de diversidade genética e evolutiva, tendo em vista que este grupo não estaria representado por outra unidade demográfica da mesma espécie. Assim sendo, é necessário implementar áreas protegidas que representem de forma adequada cada uma das ESUs de uma espécie; e da mesma forma, planos de manejo devem levar em conta estas subdivisões demográficas mais profundas. Já o mesmo não se aplica necessariamente às MUs, as quais não se encontram geneticamente tão diferenciadas. Estas devem ser avaliadas caso a caso,

do ponto de vista da manutenção da estruturação genética original, e também da operacionalização de ações de manejo como re-conexão de áreas e possível translocação de indivíduos (Moritz 1994; Eizirik 1996; Crandall *et al*. 2000; Eizirik *et al.* 2006).

É importante salientar que ignorar a diferenciação genética entre populações pode encobrir ou alterar processos evolutivos que têm se desenvolvido ao longo de muitos anos, assim como levar a uma perda importante da biodiversidade. Além disso, se populações separadas há algum tempo tiverem desenvolvido complexos gênicos co-adaptados ou genes que sejam benéficos localmente, promover o fluxo gênico entre estas populações pode resultar no nascimento de indivíduos com um conjunto gênico desvantajoso, reduzindo a viabilidade populacional, um fenômeno denominado de depressão por exocruzamento (Frankham *et al.* 2002).

Atualmente, uma nova ênfase em Genética da Conservação é a utilização de estimativas de variação adaptativa para a definição de unidades intra-específicas, ou seja, identificar a distância adaptativa entre populações e desta maneira auxiliar em medidas de conservação e manejo. Futuramente, o ideal será poder analisar em conjunto com marcadores neutros, genes que envolvam variação fenotípica e adaptação a diferentes ambientes (Storz 2005; Eizirik *et al*. 2006; Edmands 2007; Beebee & Rowe 2008).

Esta abordagem é bastante promissora, porém ainda limitada devido à necessidade de primeiramente investigar polimorfismos fenotípicos com potencial adaptativo em uma dada espécie. Este objetivo é difícil de ser alcançado, pois o genoma da maioria das espécies que são de interesse para a conservação não é bem conhecido. Atualmente, alguns estudos têm iniciado a identificação da base molecular de variantes fenotípicas em populações selvagens (Eizirik *et al.* 2003; Eizirik *et al*. 2006; Beebee & Rowe 2008).

No futuro, estudar genes candidatos a influenciar fenótipos adaptativos pode levar à estimativa de indivíduos com um valor adaptativo maior em certo ambiente, auxiliando, por exemplo, na caracterização da diferenciação evolutiva entre populações, e desta maneira contribuindo para a caracterização detalhada de unidades intra-específicas (Smith & Wayne 1996; Eizirik *et al*. 2006).

Apesar do crescente número de trabalhos em Genética da Conservação, um desafio que permanece é integrar as informações obtidas com aquelas de outras disciplinas, assim como aplicar dados genéticos de maneira efetiva em decisões de manejo e conservação de populações em habitat natural. No entanto, técnicas moleculares e métodos estatísticos aplicáveis a dados genéticos estão cada vez mais disponíveis para estudos de conservação. Desta forma, geneticistas podem e devem utilizar seus dados para auxiliar na definição de estratégias de conservação e manejo, influenciando de maneira ativa nas decisões a serem tomadas (DeSalle & Amato 2004; Vernesi *et al*. 2008).

I. 2. O problema genético das populações pequenas e isoladas

A crescente perda e fragmentação de habitats naturais, principalmente nos últimos séculos, têm contribuído significativamente para o declínio e isolamento populacional de espécies selvagens, ocasionando até mesmo extinções locais (Leite-Pittman *et al.* 2002). Quando reduzidas a pequenas e isoladas populações, espécies são mais suscetíveis a fatores estocásticos demográficos, ambientais e genéticos, assim como a catástrofes ambientais, comprometendo desta maneira sua viabilidade a longo prazo (Frankham *et al.* 2002).

Em termos genéticos, dois fatores de importância fundamental, cujos efeitos aumentam o risco de extinção, são a deriva genética – flutuação ao acaso das frequências alélicas entre gerações – e o endocruzamento – cruzamento entre aparentados – os quais são inevitáveis em populações isoladas e de tamanho reduzido (Lacy 1997; Avise & Hamrick 1996; Frankham *et al*. 2002; Spielman *et al*. 2004).

A deriva genética pode levar à eliminação de certos alelos, ocasionando perda de variabilidade genética e, consequentemente, diminuição da capacidade de populações responderem a mudanças ambientais. Além do que, uma vez que em populações pequenas a deriva genética predomina e os efeitos da seleção natural são tipicamente reduzidos ou até mesmo eliminados, alelos deletérios podem se tornar fixados (Frankham *et al*. 2002).

O endocruzamento, por sua vez, aumenta os níveis de homozigosidade populacional e a probabilidade de expressão de genes recessivos deletérios, levando a uma redução da capacidade de reprodução e sobrevivência de espécies, um fenômeno denominado “depressão por endocruzamento” (Lacy 1997; Frankham *et al*. 2002; Keller & Waller 2002). Hoje, existem evidências claras de depressão por endocruzamento em diferentes espécies selvagens tanto em cativeiro como em vida livre (Avise & Hamrick 1996; Frankham *et al*. 2002; Keller & Waller 2002).

O manejo de populações naturais deve envolver a manutenção de habitats adequados para que a população efetiva local permaneça acima dos níveis em que os efeitos da deriva e do endocruzamento tornam-se pronunciados, permitindo uma ação eficiente da seleção natural. Desta maneira, para a conservação de populações pequenas é fundamental que exista fluxo gênico suficiente para que estas escapem dos efeitos genéticos deletérios (Frankham *et al*. 2002).

Quando o movimento de indivíduos entre populações parece ser insuficiente, devem ser tomados esforços para aumentar a dispersão natural através, por exemplo, da criação de corredores ecológicos. A implementação ou a manutenção de corredores tem sido sugerida como uma das melhores opções para grandes carnívoros, os quais são altamente suscetíveis aos efeitos da fragmentação de habitat e podem usar corredores para se locomover, ao contrário de espécies com limitada dispersão (Dixon *et al*. 2006). Quando a criação de corredores ecológicos não pode ser alcançada, medidas como a translocação de indivíduos ou até mesmo a reprodução assistida, podem ser esforços necessários para mitigar os efeitos genéticos negativos do pequeno tamanho populacional e desta maneira garantir a viabilidade destas populações isoladas (Frankham *et al.* 2002).

Uma abordagem importante é o uso de parâmetros genético-populacionais para estimar o tamanho populacional mínimo requerido para garantir a viabilidade populacional a longo prazo (Frankham *et al*. 2002; DeSalle & Amato 2004). Estimativas variam em torno de 500-7000 indivíduos efetivos (Franklin 1980; Lande 1995; Reed & Bryant 2000; Reed *et al*. 2003). Através destas estimativas é possível concluir que praticamente não há grandes blocos de habitat capazes de suportar por si só populações viáveis a longo prazo de grandes carnívoros, os quais ocupam áreas de vida bastante amplas. Consequentemente, a intervenção humana por meio de esforços de conservação e manejo é de alta prioridade para garantir a sobrevivência destas populações (Reed *et al*. 2003).

I. 3. Microssatélites e suas aplicações em Genética da Conservação

Atualmente, entre os marcadores mais utilizados em estudos populacionais e voltados para a conservação de espécies destacam-se os microssatélites, também

conhecidos como seqüências simples repetidas (SSRs- *Simple Sequence Repeats*) ou repetições pequenas em *tandem* (STRs- *Short Tandem Repeats*) (Kashi *et al*. 1997). Estes marcadores consistem de segmentos de DNA de 1-6 pares de bases com um número variável de repetições em *tandem*, em sua maioria repetições de mono, tetra ou, principalmente, dinucleotídeos. Os microssatélites apresentam-se como locos altamente polimórficos dispersos amplamente em genomas eucarióticos, mas preferencialmente em regiões não codificantes (Goldstein & Schlötterer 1999).

Apesar de amplamente empregados em estudos de Genética da Conservação, um maior problema associado às análises baseadas na utilização de microssatélites é o fato de que ainda não há um modelo evolutivo bem substanciado que represente a dinâmica mutacional destes marcadores (Goldstein & Schlötterer 1999; Ellegren 2004). No entanto, este problema não é tão relevante em se tratando de estudos populacionais que envolvam populações com divergência recente, uma vez que, na maioria dos casos, a diferenciação genética entre estas está associada principalmente à deriva genética, gargalo de garrafa e endocruzamento, nesses casos a mutação teria um menor impacto.

As características apresentadas por estes locos tais como alto nível de polimorfismo, codominância e seletividade neutra (em geral), permitem a sua utilização em uma ampla variedade de estudos que são extremamente necessários para a conservação de espécies ameaçadas. Exemplos seriam aqueles destinados à detecção de hibridação (Randi *et al*. 2001; Trigo *et al*. 2008), bem como os relacionados à filogeografia (Eizirik *et al*. 2001; Luo *et al*. 2004), diversidade genética e estrutura populacional (Wisely *et al.* 2002; Cegelski *et al.* 2003; Ernest *et al*. 2003; Dalén *et al*. 2006; Hájková *et al.* 2007), e determinação de parentesco e estrutura social (Nesje *et al.* 2000; Gottelli *et al*. 2007). Além disso, por envolverem regiões pequenas, podem ser empregados até mesmo em

estudos incluindo DNA muito fragmentado ou disponível em pequenas quantidades (por exemplo, DNA obtido de amostras fecais e pêlos) ou amostras antigas. Os microssatélites têm, portanto, um enorme potencial de aplicação para estudos de populações pequenas e de espécies ameaçadas, uma vez que podem ser realizadas análises a partir de amostras não-invasivas obtidas de populações naturais de difícil acesso (Bruford & Wayne 1993; Smith & Wayne 1996).

I. 4. O uso de DNA não-invasivo obtido de amostras fecais

A utilização de amostras fecais para obtenção de informações ecológicas, especialmente de carnívoros, tem sido empregada de forma ampla em estudos relacionados à distribuição de espécies, abundância, investigação de hábitos alimentares, uso de habitat, parasitismo e nível hormonal (Jenkins & Burrows 1980; Gittleman *et al.* 2001; Moreira *et al*. 2001; Marathe *et al.* 2002; Azevedo 2008). No entanto, a identificação deste tipo de amostra tem sido historicamente baseada em critérios morfológicos como tamanho, forma e cheiro, o que tem mostrado não ser totalmente confiável (Foran *et al*. 1997; Farrell *et al.* 2000, Davison *et al.* 2002, Chame 2003). Isto é particularmente relevante quando as espécies de interesse ocorrem em simpatria e os táxons são relacionados; a morfologia das fezes pode ser similar, podendo gerar resultados enviesados devido à inclusão de amostras erroneamente identificadas como sendo da espécie alvo (Prugh & Ritland 2005).

É, portanto, extremamente necessário um método rigoroso para a identificação correta de amostras fecais coletadas em campo, e a identificação a partir do DNA fecal passa a representar uma ferramenta segura e valiosa, permitindo estudos ecológicos mais rigorosos. Até recentemente, estudos moleculares voltados para espécies ameaçadas eram

bastante limitados devido, principalmente, à grande dificuldade de obtenção de amostras de tecido e/ou sangue para análise (Taberlet & Luikart 1999; Smith & Wayne 1996; DeSalle & Amato 2004).

Há, porém, desafios importantes a serem superados no emprego desta metodologia, como a baixa quantidade e qualidade de DNA obtidas e a presença de inibidores de PCR (*Polymerase Chain Reaction*) (Taberlet *et al*. 1999). Como resultado, além de um baixo sucesso de amplificação, há um alto potencial de contaminação e uma elevada taxa de erros de genotipagens. Estes erros, como a ocorrência de "*allelic dropout*" – amostragem aleatória de apenas um dos alelos de um indivíduo heterozigoto – e "falso alelo" – amplificação de artefatos que podem ser interpretados erroneamente como um verdadeiro alelo – podem levar à inferência incorreta de um genótipo.

A necessidade de uma abordagem cautelosa, uma vez que a identificação genética confiável do indivíduo a partir de amostras fecais é de importância fundamental em ecologia molecular e conservação, tem levado muitos autores a utilizar diferentes métodos para detectar e minimizar erros de genotipagem, mas o tempo e o custo requerido ainda são altos (Kohn *et al.* 1999; Ernest *et al.* 2000; Fernando *et al.* 2003; Bonin *et al.* 2004; Broquet & Petit 2004; Maudet *et al.* 2004; Piggott 2004; Buchan *et al.* 2005; Hoffman & Amos 2005). Estes estudos incluem a adoção de uma abordagem de tubos múltiplos, a qual consiste de múltiplas amplificações independentes para cada amostra e loco (Taberlet *et al.* 1996), a comparação do genótipo obtido com aquele a partir de tecidos, ou ainda a repetição de amostras aleatoriamente. Outra forma de minimizar possíveis problemas de genotipagem é quantificar a quantidade de DNA extraído, e utilizar apenas as amostras que estão acima de certo limiar de concentração que garanta uma boa margem de

confiabilidade (Morin *et al*. 2001). Este último método envolve o uso de PCR quantitativo em tempo real, o que torna esta metodologia bastante cara e limitada.

A obtenção de DNA a partir de amostras fecais vem sendo amplamente utilizada e tem mostrado ser um método efetivo para identificar a espécie, o indivíduo e seu sexo (Reed *et al*. 1997; Farrell *et al*. 2000; Kurose *et al.* 2005). Sua aplicação é especialmente importante em espécies noturnas, ameaçadas, raras e que ocupam áreas de difícil acesso (Johnson *et al.* 2001), tendo sido bastante empregada em estudos genéticos e ecológicos de diferentes espécies de carnívoros (Wasser *et al*. 1997; Kohn *et al.* 1995; Lucchini *et al.* 2002; Palomares *et al.* 2002; Adams *et al*. 2003; Ernest *et al.* 2003; Frantz *et al*. 2003; Wan *et al.* 2003; Pilgrim e*t al.* 2005; Prugh *et al*. 2005; Onorato *et al.* 2006; Janecka *et al.* 2008; Napolitano *et al.* 2008).

I. 5. *Panthera onca* (Linnaeus 1758)

A onça-pintada (*Panthera onca*) (Figuras 1 e 2) é a maior espécie de felídeo das Américas e é o único representante vivo do gênero *Panthera* encontrado no Novo Mundo (Nowell & Jackson 1996). A espécie apresenta uma relação filogenética próxima aos outros quatro grandes gatos do gênero *Panthera* (leão, *P. leo*; leopardo, *P. pardus*; tigre, *P. tigris*; e leopardo-das neves, *P. uncia*) e parece ter divergido de um ancestral comum com o leão há pelo menos 2 milhões de anos, chegando às Américas através do Estreito de Bering durante o Pleistoceno (Johnson *et al*. 2006). Este felídeo colonizou diversos ambientes no continente americano e atingiu uma ampla distribuição geográfica possivelmente nos últimos 300.000 – 500.000 anos (Eizirik *et al.* 2001). Registros fósseis datam aproximadamente 850.000 anos e indicam que a espécie ocorria em locais tão ao

norte quanto Washington, Nebraska e Maryland nos Estados Unidos até o sul na Patagônia, Argentina (Seymour 1989; Arroyo-Cabrales 2002).

A espécie é morfologicamente semelhante ao leopardo, sendo, porém, mais robusta, com a cabeça grande e membros vigorosos. Ambos apresentam coloração similar, de fundo amarelado com manchas escuras que desenham uma roseta (ocelos), mas em geral as rosetas da onça-pintada são maiores e menos numerosas, tendo frequentemente um ou mais pontos pretos no seu interior (Figura 1) (Kitchener 1991; Sunquist & Sunquist 2002). O melanismo (escurecimento da coloração de fundo, tendendo ao preto) (Figura 2) ocorre como um polimorfismo comum em várias espécies de felídeos. Na onça-pintada esta característica tem um modo de herança dominante (Deutsch 1975), com todos os indivíduos melânicos apresentando pelo menos um alelo que contém uma deleção de 15 pares de bases no gene *MC1R*, seguido de duas substituições não sinônimas imediatamente adjacentes a esta deleção (Eizirik *et al*. 2003). A ocorrência de uma alta frequência de indivíduos melânicos em certas áreas da distribuição desta espécie parece indicar que este traço tornou-se comum nestas populações devido a uma vantagem adaptativa de indivíduos escuros (seleção natural), ou a um efeito do acaso devido ao tamanho populacional pequeno. Em ambos os casos, a elucidação deste fenômeno tem relevância para a elaboração de estratégias efetivas para a conservação desta espécie.

Historicamente, a distribuição da onça-pintada se estendia do sudoeste dos Estados Unidos ao sul da Argentina (Guggisberg 1975; Swank & Teer 1989). Desde meados de 1900 sua distribuição tem diminuído de maneira considerável, estando hoje restrita a menos de 50% do território original, ocorrendo do norte do México ao norte da Argentina (Sanderson *et al*. 2002) (Figura 3). Alguns poucos indivíduos, ocasionalmente, dispersam para o sul dos Estados Unidos, mas provavelmente partindo de uma população núcleo e

persistente localizada em Sonora, no México (López-González & Brown 2002). A espécie é, atualmente, considerada extinta em El Salvador e no Uruguai (IUCN 2008). Aproximadamente 50% da sua área de ocorrência atual está dentro do território brasileiro, tornando o Brasil uma região fundamental para garantir a viabilidade deste felídeo (Sanderson *et al*. 2002).

A espécie explora uma ampla variedade de habitats (Sunquist & Sunquist 2002; Zeller 2007) ocorrendo desde áreas de floresta tropical densa como a Floresta Amazônica a áreas mais abertas como o cerrado e a caatinga, e áreas sazonalmente alagadas como o Pantanal (Oliveira 1994). A ocorrência da onça-pintada tem sido fortemente associada à presença de água, como também à suficiente disponibilidade de presas e uma preferência à cobertura florestal densa (Mondolfi & Hoogesteijn 1986; Crawshaw & Quigley 1991; Sunquist & Sunquist 2002).

As onças-pintadas são animais territorialistas e solitários, capazes de se dispersar eficientemente por grandes áreas e atravessar até mesmo grandes rios (Seymour 1989; Oliveira 1994). Animais monitorados no Parque Estadual das Várzeas do Rio Ivinhema (MS), mostraram uma movimentação de até 30 km em 3-4 dias por uma paisagem altamente fragmentada (Sana *et al*. 2006). Já no Parque Nacional do Iguaçu (PR), um macho subadulto locomoveu-se uma série de vezes entre Brasil, Argentina e Paraguai, percorrendo grandes distâncias e atravessando o Rio Iguaçu, bem como o Rio Paraná na fronteira destes países (Crawshaw 1995).

Estudos de radiotelemetria têm mostrado que este felídeo apresenta um padrão de atividade bastante variável. No Peru, Bolívia e Brasil apresentaram-se ativos durante o dia e a noite, mas em Belize e na Venezuela foram primariamente noturnos (Emmons 1987;

Rabinowitz & Nottingham 1986; Crawshaw & Quigley 1991; Crawshaw 1995; Scognamillo *et al.* 2003; Maffei *et al.* 2004).

A área de vida varia amplamente para a espécie, com machos ocupando áreas maiores do que as fêmeas. Em geral, a área de um macho pode conter a área de vida de duas ou mais fêmeas, mas, pode haver também sobreposição entre as áreas de vida de indivíduos do mesmo sexo (Sunquist & Sunquist 2002). Em Belize, a área de vida média de duas fêmeas foi calculada em torno de 10 km^2 e a de quatro machos ao redor de 33 km^2 (Rabinowitz & Nottingham 1986). Em contrapartida, no Pantanal, as áreas de vida foram bem maiores, com quatro fêmeas ocupando em média 140 km^2 e um único macho 152 km^2 (Crawshaw & Quigley 1991). Na Mata Atlântica, a área de vida média de duas fêmeas no Parque Estadual das Várzeas do Rio Ivinhema (MS) foi de 212 km^2 e a de um único macho de 299 km^2 (Sana *et al.* 2006). Já em uma área de ambiente mais fechado, como no Parque Estadual Morro do Diabo (SP), dois machos ocuparam uma área média de 132 km^2 e cinco fêmeas de 87 km^2 (Cullen *et al*. 2005).

A densidade de onças-pintadas varia geograficamente devido à densidade de presas, composição do habitat e exploração humana (Quigley & Crawshaw 1992). Principalmente no Brasil esta variação é bastante grande, com estimativas em torno de 0,84 a 6,7 adultos/100 km^2 para diferentes locais (Schaller & Crawshaw 1980; Crawshaw 1995; Cullen *et al*. 2005; Sana *et al*. 2006; Silveira 2004; Soisalo & Cavalcanti 2006).

A dieta da onça-pintada apresenta uma base de presas extremamente diversa, que varia de acordo com a disponibilidade e a localização geográfica (Sunquist & Sunquist 2002). Ao longo da sua distribuição, mais de 85 espécies diferentes de presas foram registradas, sendo que ela consome preferencialmente mamíferos de médio a grande porte (> 1 kg; Seymour 1989) como porcos-do-mato, capivaras, antas, veados, pacas, cutias e

tatus; eventualmente elas também predam tartarugas, jacarés, lagartos, peixes e aves (Emmons 1987; Seymour 1989; Oliveira 1994).

A predação sobre o gado doméstico pode ser considerável em algumas áreas (em geral ela é associada à redução populacional ou à extinção antropogênica de suas presas naturais, bem como à modificação dos habitats originais), o que gera sérios conflitos com pecuaristas, assim como intensa mortalidade das onças-pintadas (Sunquist & Sunquist 2002). Embora esta espécie tenha sido bastante caçada no passado para servir de troféu ou para suprir o mercado de peles, no momento, a retaliação ou perseguição direta por pecuaristas, em conjunto com a acentuada perda e fragmentação de seus habitats remanescentes, são as principais ameaças à sua sobrevivência (Nowell & Jackson 1996). A espécie é listada no apêndice I da CITES (*Convention on International Trade in Endangered Species of Wild Fauna and Flora*) e é considerada "quase ameaçada" pela IUCN (*The International Union for Conservation of Nature and Natural Resources*) (Sunquist & Sunquist 2002). No Brasil, a onça-pintada é listada como vulnerável na lista dos animais ameaçados de extinção do IBAMA (2003). Segundo Mace & Lande (1991), que adotam o critério da probabilidade de extinção em um determinado período de tempo, a espécie encontra-se em situação crítica na Floresta Atlântica, Floresta Subtropical e Cerrado, e está ameaçada no Pantanal e Amazônia (IUCN/CAMP 1994).

Análises preliminares de viabilidade populacional indicam que para garantir a viabilidade deste felídeo a longo prazo seria necessária uma população de aproximadamente 650 indivíduos (Eizirik *et al.* 2002). Dado que a maioria das áreas ao longo da distribuição da espécie é pequena demais para sustentar uma população viável, isso implica que elas irão depender do fluxo gênico para manter o seu potencial evolutivo. Assim sendo, como já salientado na seção I.2 em termos gerais, são extremamente

necessários planos de manejo que envolvam o fluxo entre áreas isoladas através de corredores ecológicos, translocação de indivíduos ou fertilização assistida (Rodrigues & Oliveira 2006). Eizirik *et al.* (2002) enfatizaram que remanescentes populacionais da espécie, particularmente os da Mata Atlântica Costeira, têm uma probabilidade de extinção muito alta para as próximas décadas, ressaltando a urgência da implementação de estratégias efetivas para sua conservação.

Especialmente a partir da década de 1990, começaram a ser realizados estudos no Brasil com o objetivo de avaliar aspectos da ecologia, distribuição e conservação da onça-pintada em uma escala regional. Essas pesquisas vêm sendo desenvolvidas no Pantanal (Quigley & Crawshaw 1992; Dalponte 2002; Silveira 2004; Soisalo & Cavalcanti 2006; Azevedo & Murray 2007), Cerrado (Silveira & Jácomo 2002), Mata Atlântica (Crawshaw 1995; Garla *et al.* 2001; Leite *et al.* 2002; Conforti & Azevedo 2003; Cullen *et al.* 2005; Sana *et al*. 2006; Paviolo *et al.* 2008), Amazônia (Michalski & Peres 2007) e Caatinga (Perez 2008; RG Morato, comunicação pessoal). Em todas essas regiões a sobrevivência atual e futura deste felídeo encontra-se ameaçada por diversos processos antropogênicos.

Vários destes projetos têm implementado programas de monitoramento utilizando armadilhas fotográficas e/ou cães treinados para encontrar rastros e capturar indivíduos. Estes estudos são fundamentais para o desenvolvimento de planos de conservação e manejo para esta espécie, mas para que possam ser desenvolvidas estratégias adequadas, garantindo a viabilidade das populações a longo prazo, são necessários estudos genéticos complementares a estas abordagens ecológicas. Em 1999, um encontro de especialistas no México (“El Jaguar em el Nuevo Milenio”) apontou como uma das ações prioritárias para a conservação deste felídeo, a necessidade de análises genéticas para determinar o grau de diferenciação entre suas populações, bem como a otimização do uso de DNA obtido

através de amostras não-invasivas, viabilizando o seu uso em diferentes estudos ecológicos e genéticos com a espécie (Taber *et al*. 2002).

Estudos moleculares sobre a onça-pintada são bastante recentes e ainda escassos. Eizirik *et al*. (2001) apresentaram uma análise filogeográfica e enfatizaram questões voltadas para a evolução deste felídeo de uma forma geral. As análises foram baseadas em cerca de 40 indivíduos amostrados ao longo da distribuição da espécie, sendo utilizados 29 locos de microssatélites e parte da região controladora do DNAmt. O estudo indicou a ausência de subdivisões acentuadas, propondo a divisão da espécie em quatro grupos filogeográficos não totalmente isolados. Esta análise molecular contrastou com estudos taxonômicos anteriores, baseados em características morfológicas, que haviam proposto a existência de oito subespécies (Pocock 1939; Seymour 1989). Uma reavaliação destas características morfológicas também não encontrou diferenças significativas entre as supostas subespécies (Larson 1997).

Em um estudo posterior, Ruiz-Garcia *et al.* (2006), com dados de 12 locos de microssatélites, não encontraram diferenciação acentuada entre as supostas subespécies propostas para a Colômbia, embora para alguns locos a heterogeneidade tenha sido significante. O estudo utilizou também amostras provenientes da Guatemala, Venezuela, Bolívia, Peru, Paraguai e Brasil e encontrou alelos exclusivos para algumas destas regiões geográficas.

Outros dois estudos utilizando microssatélites foram realizados, os de Moreno *et al*. (2006) e Soares *et al.* (2006); porém, o primeiro estimou apenas a variabilidade genética existente em indivíduos em cativeiro, enquanto o último envolveu uma análise de paternidade para testar um suposto caso de infanticídio que teria ocorrido na população de onças-pintadas do Parque Nacional das Emas, no cerrado brasileiro.

Desta maneira, embora existam remanescentes de populações de onça-pintada em várias regiões brasileiras e muitas destas estejam em perigo de extinção, ainda não foram realizados estudos genéticos mais detalhados envolvendo uma amostragem maior em nível local e regional para que seja possível investigar o grau de variabilidade genética existente nestas populações, assim como o grau de conectividade entre elas.

A investigação destes aspectos é extremamente importante para que se avalie a viabilidade destas populações e para que planos de manejo locais adequados para este felídeo possam ser colocados em prática. No entanto, estes estudos são limitados principalmente devido à dificuldade de se obter amostras locais desta espécie, dado o seu comportamento elusivo, noturno e sua baixa densidade, determinando que este animal seja extremamente difícil de ser capturado ou até mesmo visualizado. Atualmente, os estudos ecológicos que vêm sendo desenvolvidos em diferentes regiões brasileiras (citados anteriormente), assim como o uso de amostras não-invasivas, permitem que se inicie em nível local a determinação da variabilidade genética e estrutura populacional deste felídeo, colaborando assim para a sua conservação.

Figura 1. Foto de um indivíduo da espécie *Panthera onca* com coloração selvagem. Fotografia gentilmente cedida por Adriano Gambarini.

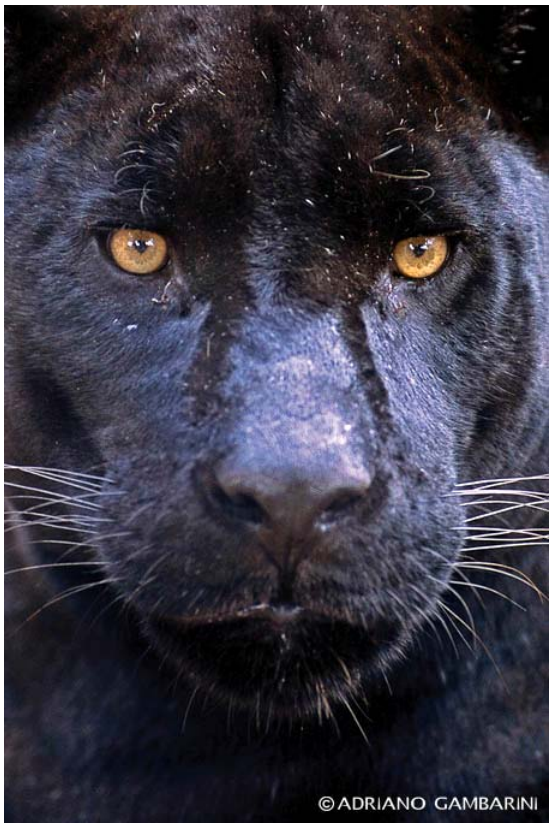

Figura 2. Foto de um indivíduo melânico da espécie *Panthera onca.* Fotografia cedida por Adriano Gambarini.

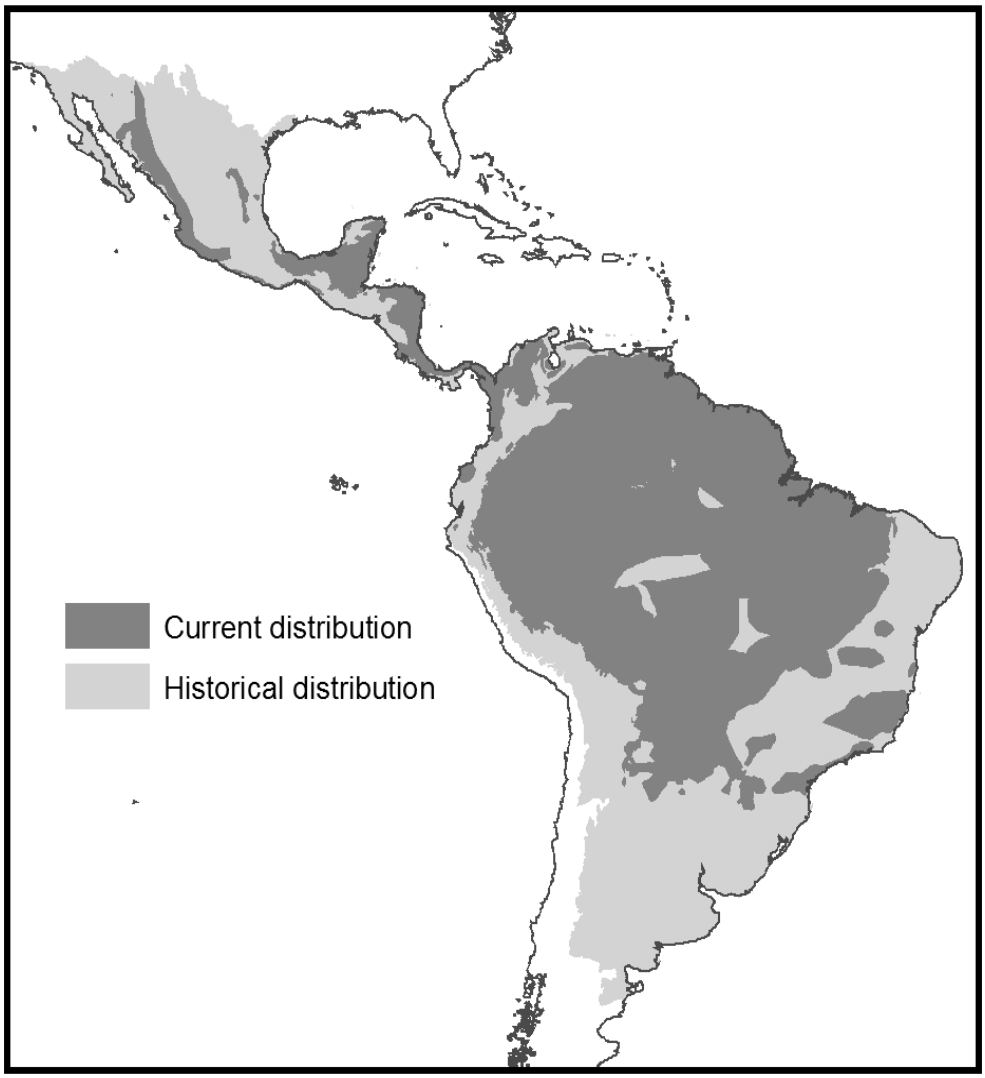

Figura 3. Distribuição histórica (cinza claro) e atual (cinza escuro) da onça-pintada nas Américas (modificado de Sanderson *et al.* 2002 e Zeller 2007).

CAPÍTULO II

OBJETIVOS

II. 1. Objetivos

1. Desenvolver um método baseado no DNA para identificar de forma confiável amostras fecais de onça-pintada coletadas em campo, viabilizando o seu uso em estudos genéticos e ecológicos;

2. Testar a possibilidade de identificar a coloração de onças-pintadas a partir de amostras fecais coletadas em campo e cativeiro;

3. Investigar a diversidade genética existente em populações remanescentes de onça-pintada da Mata Atlântica de Interior (Ecorregião do Alto Rio Paraná), assim como caracterizar a distribuição geográfica desta variabilidade, analisando processos de fluxo gênico e diferenciação entre fragmentos;

4. Relacionar os resultados obtidos com informações disponíveis sobre a biologia e ecologia desta espécie, contribuindo assim para a elaboração e efetivação de estratégias adequadas para a sua conservação.

CAPÍTULO III

1° ARTIGO

DEVELOPMENT AND TESTING OF AN OPTIMIZED METHOD FOR DNA-BASED IDENTIFICATION OF JAGUAR (*PANTHERA ONCA*) AND PUMA (*PUMA CONCOLOR*) FAECAL SAMPLES FOR USE IN ECOLOGICAL AND GENETIC STUDIES

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Development and testing of an optimized method for DNA-based identification of jaguar (*Panthera onca*) and puma (*Puma concolor*) faecal samples for use in ecological and genetic studies

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Abstract The elusive nature and endangered status of most carnivore species imply that efficient approaches for their non-invasive sampling are required to allow for genetic and ecological studies. Faecal samples are a major potential source of information, and reliable approaches are needed to foster their application in this field, particularly in areas where few studies have been conducted. A major obstacle to the reliable use of faecal samples is their uncertain species-level identification in the field, an issue that can be addressed with DNA-based assays. In this study we describe a sequence-based approach that efficiently distinguishes jaguar versus puma scats, and that presents several desirable properties: (1) considerably high amplification and sequencing rates; (2) multiple diagnostic sites reliably differentiating the two focal species; (3) high information content that allows for future application in other carnivores; (4) no evidence of amplification of prey DNA; and (5) no evidence of amplification of a nuclear mitochondrial DNA insertion known to occur in the jaguar. We demonstrate the reliability and usefulness of this approach by evaluating 55 field-collected samples from four locations in the highly fragmented Atlantic Forest biome of Brazil and Argentina, and document the presence of one or both of these endangered felids in each of these areas.

Keywords Faecal DNA · Mitochondrial DNA · *Panthera onca* · *Puma concolor* · Species identification

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Introduction

The jaguar (*Panthera onca*) and puma (*Puma concolor*) are the only large felids currently present in the Neotropics. Both species are now threatened by habitat loss and fragmentation, along with direct persecution by ranchers due to conflict over livestock depredation (Nowell and Jackson 1996). Resilience to human disturbance varies between them, with the jaguar being considerably more sensitive to anthropogenic threats (Polisar 2002; Novack 2003; Silveira 2004). To maintain viable populations of these felids, urgent conservation efforts are needed in several areas, and

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a first step towards this goal is the establishment of reliable methods to assess the presence and abundance of each of these species in different areas, so as to allow the adequate design of ecological, behavioral and genetic studies.

A major impediment to the development of such efforts is the difficulty and cost of obtaining direct information on these animals throughout their geographic range, given their low density and elusive behavior (Johnson et al. 2001). As a consequence, intensive studies such as those based on capture and radio-telemetry data are expensive and restricted to some focal areas, which in most cases still lack long-term monitoring of populations. In recent years, technical and analytical advances in approaches such as camera trapping have allowed a substantial increase in the number of studies investigating the presence of these species. In spite of the relevance of this approach, it still lacks the ability to provide biological samples of the identified individuals, or to provide information on important ecological and behavioral aspects such as diet, hormonal levels or interactions with pathogens. Biological samples are also critical for the development of genetic studies, which have the potential to illuminate issues such as loss of allelic diversity in fragmented populations, evolutionary history of demographic units, social interactions among individuals and patterns of territoriality and dispersal. In this context, recent advances in molecular biology have permitted the use of noninvasive samples (e.g. scats, hairs) as a reliable source of DNA, allowing genetic studies of free-ranging animals to be performed without having to capture or even observe them (Taberlet et al. 1999).

Analyses based on faecal DNA are now widespread, and have been applied to a broad array of taxa to address a variety of questions (Reed et al. 1997; Wasser et al. 1997; Kohn et al. 1999; Sloane et al. 2000; Parsons 2001; Palomares et al. 2002; Adams et al. 2003; Ernest et al. 2003; Wan et al. 2003; Pilgrim et al. 2005; Bergl and Vigilant 2007). Once scats are collected in the field, a first step for their use in genetic or ecological studies is their identification at species level. Morphology-based criteria have been shown to often be unreliable (Farrell et al. 2000; Davison et al. 2002; Reed et al. 2004), leading to a growing concern regarding the development of rigorous approaches for species-level assignment of field-collected scats. This is particularly relevant when the focal species for a field study occurs in sympatry with related taxa, whose scat size, morphology and scent may be quite similar. In such cases, molecular methods based on DNA sequences, nuclear VNTRs, PCR-RFLP or haplotype-specific Polymerase chain reaction (PCR) have been shown to successfully identify carnivore species, leading to a reliable alternative to traditional means of identification (Farrell et al. 2000; Davison et al. 2002; Palomares et al. 2002; Bhagavatula and Singh 2006; Lucentini et al. 2007; Pilot et al. 2007).

In the specific case of jaguar and puma, these felids are sympatric over almost all the range of the former species. It is thus unlikely that field studies focusing on jaguars will be carried out in areas devoid of pumas, so that both species will probably be sampled in most scat collection efforts. This issue is compounded by the established knowledge that it is difficult to distinguish jaguar and puma scats on the basis of their morphological features (Emmons 1987; Farrell et al. 2000; Chame 2003), rendering the problem of species-level identification a critical impediment for reliable field studies on these felids. It is thus very important to devote attention to the development of DNA-based assays that reliably discriminate these species, and that also present other desirable features such as high PCR success rate and no co-amplification of prey DNA.

Although several assays based on mitochondrial DNA (mtDNA) data have so far been applied to carnivore scat identification (Farrell et al. 2000; Novack et al. 2005; Bhagavatula and Singh 2006; Weckel et al. 2006; Lucentini et al. 2007; Miotto et al. 2007), very few studies have included Neotropical species, and puma and jaguar in particular. Farrell et al. (2000) developed primers targeting the mtDNA *cytochrome b gene* (*cyt b*) to identify carnivore scats in the context of an ecological study in Venezuela investigating four species (*P. concolor*, *P. onca*, *Leopardus pardalis* and *Cerdocyon thous*). In that study, 20 of 34 scats (59%) were successfully amplified and sequenced, allowing species-level identification. More recently, Miotto et al. (2007) used those same primers in a study to determine the presence of pumas and their estimated minimum population in two protected areas in Brazil, and also achieved 60% success rate in amplification and sequencing. Higher amplification success rates (83% and 85%, respectively) were observed with *cyt b* markers in the studies of Adams et al. (2003) and Onorato et al. (2006) in different areas of the United States. However, both of these papers reported a certain amount of prey DNA amplification using these primers (13 and 8%, respectively), which suggests that additional marker development is desirable to maximize the efficiency of assays capable of identifying carnivore species.

In the case of the jaguar, a complicating factor for the development of mtDNA-based assays is the presence in all five *Panthera* species of a large nuclear insertion (*numt*) containing most of the mitochondrial genome. A detailed study investigating this *Panthera numt* suggested that it encompasses a long segment spanning eight protein coding genes (including *cyt b*), two rRNA genes, 17 tRNA genes, and the control region (Kim et al. 2006). Since the amplification (or coamplification) of a *numt* is a known complication that can hamper or confound genetic analyses (Zhang and Hewitt 1996; Kim et al. 2006), including DNA-based identification, it is important to develop markers that

target mtDNA regions that are not contained in this translocation.

In this paper we describe a DNA-based assay for the identification of jaguar and puma faecal samples that bears the following assets: (1) high specificity, as sequence-based identification leads to multiple diagnostic characters between the two species; (2) high sensitivity, as the amplified fragment is short and leads to high amplification rates; (3) avoidance of *numt* amplification by targeting a mtDNA region not included in this translocation; and (4) no detected amplification of prey DNA. In addition to accomplishing successful discrimination between jaguar and puma scats, the method proposed here has a potential for much broader application in carnivores, as its sequence-based diagnosis allows for multiple sympatric species to be reliably identified.

Materials and methods

In order to develop an effective molecular approach for reliable identification of *P. onca* scats, especially with respect to distinguishing it from and *P. concolor*, we used 52 jaguar reference samples that spanned the geographic distribution of this species (Table 1), including blood ($n = 15$), tissue ($n = 1$), hair ($n = 2$) and faeces ($n = 34$) collected from captive animals. For comparison, we analyzed nine reference samples of pumas (eight blood samples and one tissue), representing multiple geographic regions where it is sympatric with the jaguar. Additional analyses included a marker test using a faecal sample of domestic cat (*Felis catus*) collected in Argentina, paired samples of blood and scat collected from four captive jaguars (see Table 1), and comparisons with multiple sequences available in GenBank or generated in our laboratory for other purposes.

In addition to samples originated from known specimens, we analyzed 55 scats collected by field researchers in different areas, and identified as "large felid" based on morphological features such as diameter, size and shape, as well as associated tracks. "Large felid" scats are usually interpreted as originating from either jaguar or puma, and discriminating between the two is a known and recurrent challenge in most cases. All 55 samples were collected from areas in the Atlantic Forest biome where both species are thought to occur, and where field projects addressing ecological aspects of one or both of them are currently being carried out. The field sites were: Reserva Natural Vale, Espírito Santo State, Brazil (RV; $n = 9$); Parque Estadual do Rio Doce, Minas Gerais State, Brazil (RD; $n = 1$); Parque Estadual das Várzeas do Rio Ivinhema, Mato Grosso do Sul State, Brazil (PI; $n = 3$), and several forested locations in the Misiones Province, Argentina (MP; $n = 42$).

Blood samples were preserved in a salt-saturated solution (100 mM Tris, 100 mM EDTA, 2% SDS) and tissues and hairs were kept in ethanol 96%. Approximately 6 g of faeces were collected and stored in a 15 ml vial containing silica gel at a 4 g silica/g faeces ratio (Wasser et al. 1997). All samples were stored at −20°C prior to DNA extraction. Genomic DNA was extracted from blood and tissue samples using a standard Proteinase-K digestion and phenol-chloroform-isoamyl alcohol protocol (Sambrook et al. 1989). Extractions from the hair samples were performed with the Puregene DNA Purification Kit (GENTRA), and those of scat samples used the QIAamp DNA Stool Mini Kit (QIAGEN), following the manufacturers' instructions. Scat DNA extractions were carried out in a separate laboratory area, in a UV-sterilized laminar flow hood dedicated to the analysis of DNA from noninvasive samples. Each batch of extractions ($n = 10$) included one negative extraction control to monitor the occurrence of contamination with extrinsic DNA.

In order to avoid amplification of the *Panthera numt* and to aim for high amplification success and sequence variability, we targeted a short segment of the mtDNA *ATP synthase subunit 6* (*ATP6*) gene, including its overlapping portion with the *ATP8* gene. Previous studies indicated that this segment was quite variable in carnivores (Trigo et al. 2008; E. Eizirik et al., unpublished), and that it lay outside of the *numt* (Kim et al. 2006). We employed the reverse primer ATP6-DR1 (5′-CCAGTATTTGTTTTGATGTTAG TTG-3′), originally reported by Trigo et al. (2008), and designed two new forward primers: ATP6-DF2 (5′-ATGA ACGAAAATCTATTCGC-3′) and ATP6-DF3 (5′-AACG AAAATCTATTCGCCTCT-3′). These forward primers anneal at very similar positions (PCR product size in combination with ATP6-DR1 is 175 bp for DF2 and 172 bp for DF3), and were designed to maximize the probability of successful amplification in carnivores, given a preliminary alignment including sequences drawn from GenBank that represented the major lineages of this mammalian order (i.e. Feliformia, Arctoidea, and Cynoidea). On the basis of initial tests, both forward primers appeared to perform well in carnivores (not shown), and primer ATP6-DF2 was employed throughout this study.

Polymerase chain reactions were performed in a final volume of 20 µl, containing 1× PCR buffer (Invitrogen), 2.0–2.5 mM $MgCl_2$, 200 µM dNTPs, 0.2 µM of each primer, 0.5 unit of regular *Taq* DNA polymerase (Invitrogen) or Platinum *Taq* DNA polymerase (Invitrogen) and 1–6 µl of empirically diluted template DNA. The reaction profile was as follows: 10 cycles (Touchdown) of 94°C for 45 s, 60–51°C for 45 s, 72°C for 1.5 min, followed by 30 cycles of 94°C for 45 s, 50°C for 45 s, 72°C for 1.5 min, and a final extension at 72°C for 3 min. Products were visualized on a 1% agarose gel stained with GelRed (Biotium), purified with >PEG8000, sequenced using the DYEnamic ET Dye

Table 1 Samples utilized as reference in the present study

ID	Sample	Geographic origin	Institution/Contact
Panthera onca			
bPon-01	Blood	Paraná state, Brazil	Proj. Carnívoros-Ibama—P. G. Crawshaw Jr.
bPon-03	Blood	Mato Grosso do Sul state, Brazil	Proj. Porto Primavera—P. G. Crawshaw Jr.
bPon-13	Blood	Amazonas state, Brazil	CIGS, Manaus
bPon-15[a], bPon-27[a], bPon-32[a]	Blood and faeces	Mato Grosso do Sul state, Brazil	CENAP/ICMBio; I. Pró-Carnívoros; Ilha Solteira Zoo; V. Queirós
bPon-16, bPon-35	Blood	Mato Grosso do Sul state, Brazil	CENAP/ICMBio; Pró-Carnívoros
bPon-18[a]	Blood and faeces	São Paulo state, Brazil	CENAP/ICMBio; I. Pró-Carnívoros; Ilha Solteira Zoo; V. Queirós
bPon-24, bPon-51	Blood	São Paulo state, Brazil	Inst. Pesquisas Ecológicas (IPE)/L. Cullen and A. Nava
bPon-34	Muscle	French Guiana	Benoit de Thoisy
bPon-55[a], bPon-56[a], bPon-102[a], bPon-103[a], bPon-104[a]	Faeces	Captivity, Brazil	Sapucaia do Sul Zoo/R. von Hohendorff
bPon-59[a]	Faeces	Paraná state, Brazil	CASIB/W. de Moraes
bPon-66	Blood	Mato Grosso do Sul state, Brazil	Embrapa-Pantanal/G Mourão
bPon-81[a], bPon-82[a]	Faeces	Captivity, Brazil	Americana Zoo/M. Falcade; Limeira Zoo/A. C. A. Sorg
bPon-83[a]	Faeces	Amazonas state, Brazil	Limeira Zoo/A. C. A. Sorg
bPon-90[a], bPon-91[a], bPon-93[a], bPon-94[a]	Faeces	Captivity, Brazil	Parque Municipal "Danilo Galafassi"/L. E. S. Delgado
bPon-95[a], bPon-96[a], bPon-97[a]	Faeces	Captivity, Brazil	CEBUS/C. D. P. Coelho and L. C. Silva
bPon-98[a], bPon-99[a]	Faeces	Captivity, Brazil	Goiania Zoo/R. F. de Carvalho and D. Nogueira
bPon-100[a]	Faeces	Captivity, Brazil	Campinas Zoo/E. F. Santos
bPon-101[a]	Faeces	Acre state, Brazil	Campinas Zoo/E. F. Santos
bPon-105[a], bPon-112[a]	Faeces	Captivity, Brazil	Guarulhos Zoo/C. E. Bolochio
bPon-107[a]	Faeces	Acre state, Brazil	Parque Ambiental Chico Mendes/J. O. Guimarães
bPon-108[a]	Faeces	Rondonia state, Brazil	Parque Ambiental Chico Mendes/J. O. Guimarães
bPon-114[a], bPon-115[a]	Faeces	Captivity, Brazil	Parque Cyro Gevaerd/M. R. Achutti
bPon-116[a]	Faeces	Amazonas state, Brazil	Curitiba Zoo/M. L. Javorouski
bPon-117[a]	Faeces	Santa Catarina state, Brazil	Curitiba Zoo/M. L. Javorouski
bPon-120[a]	Faeces	Amazonas state, Brazil	Pomerode Zoo (Fund. Hermann Weege)/C. H. Maas
bPon-123[a]	Faeces	Mato Grosso do Sul state, Brazil	Pomerode Zoo (Fund. Hermann Weege)/C. H. Maas
bPon-126 (Pon-31)	Blood	San Luis Potosí, Mexico	Leon Zoo
bPon-127 (Pon-50)	Blood	Chaco, Paraguay	Itaipu, Paraguay
bPon-128 (Pon-54)	Blood	Amazonas, Venezuela	Las Delicias
P31-1	Hair	Misiones Province, Argentina	Proyecto Yaguareté, CeIBA/Carlos De Angelo
P3-2	Hair	Misiones Province, Argentina	Proyecto Yaguareté, CeIBA./Carlos De Angelo
Puma concolor			
bPco-72 (Pco-356)	Blood	Texas, USA	Laboratory of Genomic Diversity (LGD), USA
bPco-73 (Pco-541)	Blood	Panama	Laboratory of Genomic Diversity (LGD), USA
bPco-74 (Pco-556)	Blood	Guatemala	Laboratory of Genomic Diversity (LGD), USA
bPco-75 (Pco-560)	Blood	Argentina	Laboratory of Genomic Diversity (LGD), USA
bPco-76 (Pco-700)	Blood	Paraiba state, Brazil	Laboratory of Genomic Diversity (LGD), USA
bPco-77 (Pco-704)	Blood	Venezuela	Laboratory of Genomic Diversity (LGD), USA
bPco-78 (Pco-707)	Blood	Bolivia	Laboratory of Genomic Diversity (LGD), USA

Table 1 continued

ID	Sample	Geographic origin	Institution/Contact
bPco-79 (Pco-7)	Blood	Oregon, USA	Laboratory of Genomic Diversity (LGD), USA
bPco-014	Tissue	São Paulo state, Brazil	F. Olmos
Felis catus			
F3-120[a]	Faeces	Buenos Aires, Argentina	Proyecto Yaguareté, CeIBA/Carlos De Angelo

[a] Faeces collected in captivity

Terminator Sequencing Kit (GE Healthcare), and analyzed in a MegaBACE 1000 automated sequencer (GE Healthcare). To assess the performance of a fast, straightforward and lower-cost identification strategy, PCR products were routinely sequenced for only one strand (using the forward primer ATP6-DF2), and only bases that could be reliably scored were kept in the data set. To verify sequence accuracy and to ascertain the validity of only using a single strand for identification purposes, we sequenced the reverse strand for one jaguar and one puma sample (bPon-127 and bPco-014, respectively), in both cases confirming the original result. Sequences were visually checked and manually corrected using CHROMAS 2.0 (http://www.technelysium.com.au/chromas.html). All haplotypes identified here have been deposited in GenBank (Accession numbers FJ596283-FJ596287).

DNA sequences were aligned with the CLUSTALW algorithm implemented in MEGA 3.1 (Kumar et al. 2004). MEGA was also used to identify identical haplotypes, to assess the presence and consistency of diagnostic sites between the two species, and to perform phylogenetic analyses. In most cases the identification of samples could be performed with a direct, character-based approach, as field-collected scats often contained identical haplotypes to those observed in reference samples. To test whether different haplotypes observed in each species could be reliably grouped, so as to provide an easy and consistent identification tool even in cases where haplotypes were not identical, we performed several phylogenetic analyses. These included maximum parsimony and distance-based approaches using the Neighbor-Joining algorithm (Saitou and Nei 1987) with various types of genetic distances. The reliability of inferred nodes was assessed using 1,000 bootstrap replications. In addition to the data generated here, phylogenetic analyses also included sequences from other felid species available in GenBank, especially domestic cat (U20753) and cheetah (*Acinonyx jubatus;* AY463959).

Results and discussion

The use of PCR primers ATP6-DF2 and ATP6-DR1 led to excellent amplification success (100%) using blood, tissue or hair as DNA sources. High quality DNA sequences could thus be obtained from all reference samples. PCR products were 175 bp long in both species, yielding a 130 bp long analyzable segment after removal of primer sequences. When employing only forward sequences for streamlined and lower-cost sample identification, 96 sites could be reliably used after additional removal of bases that did not present high quality scores with this DNA strand alone. However, if the reverse strand was also used, all 130 sites could be reliably scored. Even considering only the 96 sites scored with the forward strand, species-level identification was found to be highly accurate, with a minimum of 15 diagnostic sites identified between any jaguar and puma sequence (Table 2).

Two different haplotypes, differing by a single nucleotide, were observed in the *P. onca* samples. Among pumas, two variable sites were identified, leading to three different haplotypes (Table 2). In addition to the character-based analysis that clearly diagnosed pumas versus jaguars, phylogenetic analyses also demonstrated that the two species could be easily differentiated using this mtDNA segment (Fig. 1).

PCR amplification of this segment from faecal samples also led to promising results. Thirty-four out of 39 fresh scat samples collected from captive jaguars were successfully amplified and sequenced, corresponding to an 87% success rate. Field-collected scats yielded variable success rates, likely due to heterogeneity in environmental conditions as well as sample age (when collected) and storage time. A success rate of 78% (seven out of nine samples) was obtained with scats from the RV location (see Materials and methods), in contrast to only 50% (21 out of 42 scats) for the samples from Misiones (MP). We examined the underlying causes of this lower success rate with the MP samples by partitioning these scats into different categories of field-assigned quality ("freshness") and storage time. If only samples categorized as "fresh" were considered, the success rate became 89% (8/9 scats) for scats from MP. Within the group categorized in the field as "intermediate" in freshness, a 59% success rate (10/17) was obtained for samples that had been stored for <1 year prior to extraction, and only 22% (2/9) for those stored for ≥1 year. Finally, the group of samples categorized as "low

Table 2 Mitochondrial DNA *ATP6* haplotypes identified from jaguar (PonH1, PonH2) and puma (PcoH1, PcoH2, PcoH3) samples

Haplotype ID	Variable sites	Known samples	Field samples[b]
	112344556667889		
	369067025013692171		
PonH1	TCACACTCGGTCTTACGT	bPon01, 03, 13, 15[a], 16, 18[a], 24, 27[a], 32[a], 34, 35, 51, 55, 56, 59, 66, 82, 83, 90, 91, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 107, 108, 112, 114, 115, 116, 117, 120, 123, 126, 127, 128; P3-2, 31-1	RV04, 06, 07, 08, 09, 19, 31; PI32, 40; MP5-9, 5-10, 5-12, 4-120, 23-18, 43-200
PonH2	..........T.....	bPon81	
PcoH1	CTCTGACAAACTCCGGAC	bPco14, 72, 73, 74, 75, 76, 77, 78, 79	RD01; MP18-31, 38-55, 56-26, 57-1, 2-635, 2-636, 2-711, 2-659
PcoH2	C.NTGACAAACTCCGGAC		MP48-2, 61-3, 12-101
PcoH3	C.CT.ACAAACTCCGGAC		MP4-13

Only variable sites are shown. Site numbers (vertical notation) refer to the aligned position in our 96 bp data set. Known samples (see Table1) and field-collected scats bearing each haplotype are also indicated

[a] Individuals for which paired blood and faecal samples were analyzed, in every case leading to identical results

[b] Field samples consist of scats collected in the following locations: Reserva Natural Vale, Espírito Santo state, Brazil (RV); Parque Estadual do Rio Doce, Minas Gerais state, Brazil (RD); Parque Estadual das Várzeas do Rio Ivinhema, Mato Grosso do Sul state, Brazil (PI); and Misiones Province, Argentina (MP)

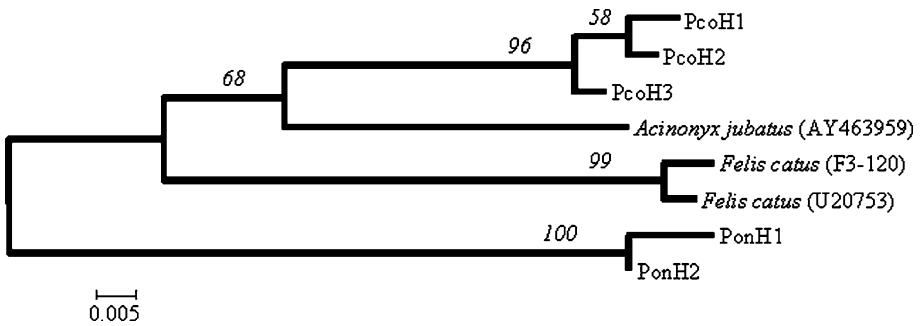

Fig. 1 Phylogenetic tree depicting the evolutionary relationships among mtDNA haplotypes sampled in jaguars (PonH), pumas (PcoH) and other carnivores (see text and Table 2). The tree is based on 96 bp of the mtDNA *ATP6* gene, and was constructed using the Neighbor-joining algorithm on the basis of a p-distance matrix. *Numbers* above branches represent bootstrap support values generated with 1,000 replicates

quality" in the field did exhibit the lowest success rate (1/7 scats, i.e. 14%), indicating that this initial assessment at the collection stage did predict to some extent the success rate of PCR and sequencing. Scats from other locations presented satisfactory success rates (1/1 for RD; 2/3 for PI), though their smaller sample size precludes a more detailed assessment of local variables.

Throughout all analyses of this mtDNA segment from scat samples, no evidence of prey DNA amplification was observed. If affirmed by further sampling and analyses of other carnivore communities, this result would indicate that the markers proposed here might have advantages in terms of identification performance relative to others that have been published previously (e.g. see Adams et al. 2003 and Onorato et al. 2006). In addition, we also saw no evidence of *numt* amplification or co-amplification (e.g. double peaks, high sequence background) in the jaguar samples, supporting the prediction that this segment excludes this nuclear insertion. This would represent an additional advantage of employing this marker relative to the *cyt b* gene, targeted by other studies (e.g. Farrell et al. 2000).

Since very little variation was observed within each species (see Fig. 1), most field-collected scats bore identical haplotypes to those observed in reference samples (Table 2), leading to straightforward identification. In the few cases where a unique haplotype was identified in scat samples (e.g. PcoH2 and PcoH3, see Table 2), the presence of multiple diagnostic sites made species-level identification conclusive with either a character-based or a phylogenetic approach (see Fig. 1). Employing this assay, we where thus able to confidently detect the presence of one or both species in all surveyed field locations (see

Table 2). For MP, which presented the largest available sample size, 12 scats were identified as originating from pumas and six from jaguars, allowing the use of these samples in downstream analyses addressing fragment occupation, diet and population genetics. Interestingly, all seven scats that could be sequenced from the RV site were identified as jaguars, providing the first genetic samples of *P. onca* collected from a Coastal Atlantic Forest location. Further sampling from this area will be extremely important to allow for an initial survey of the genetic diversity and evolutionary distinctiveness of this isolated and critically endangered remnant population.

Finally, in addition to its diagnostic power for pumas and jaguars, the informative content of this mtDNA segment holds promise to provide reliable identification for other carnivore species. For example, as a control in this study, we amplified and sequenced DNA from a domestic cat faecal sample, which was easily grouped with the reference sequence for this species available in GenBank (see Fig. 1). In the context of the inherent uncertainty of field-based scat identification, especially in ecosystems harboring multiple sympatric species, the usefulness of a sequence-based assay is noteworthy. One graphic example was observed among the samples investigated in this study. Of the 21 sequenced samples from the MP location, all of which had been identified in the field as originating from a "large felid", 18 were identified as jaguar or puma (see above and Table 2). Three others were found to bear considerably different *ATP6* haplotypes, and were subsequently identified as originating from ocelots (*L. pardalis*) by comparison with a multi-species data base (P. B. Chaves et al. unpublished data). The finding that ocelot samples were identified as belonging to a "large felid" by experienced field researchers highlights the urgent issue of procuring accurate and standardized faecal identification methods, so as to provide a reliable basis for the development of non-invasive ecological and genetic investigations on jaguars and pumas, and on carnivore species in general.

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CAPÍTULO IV

2° ARTIGO

MOLECULAR TRACKING OF JAGUAR MELANISM USING FAECAL DNA

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TECHNICAL NOTE

Molecular tracking of jaguar melanism using faecal DNA

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Abstract Major evolutionary questions remain elusive due to persistent difficulties in directly studying the genetics of variable phenotypes in natural populations. Many phenotypic variants may be of adaptive relevance, and thus important to consider in the context of conservation genetics. However, since the dynamics of these traits is usually poorly understood in the wild, their incorporation in conservation strategies is difficult to accomplish. For animals which exhibit intriguing phenotypic variation but are difficult to track in the wild, innovative approaches are required to investigate such issues. Here we demonstrate that non-invasive DNA sampling can be used to study the genetics and ecology of melanism in the jaguar, by directly genotyping the molecular polymorphism underlying this coloration trait. These results open new prospects for the in-depth investigation of this polymorphism, and highlight the broader potential of non-invasive DNA-based phenotype tracking for wildlife in general.

Keywords Phenotypic polymorphism · *Panthera onca* · *MC1R* · Non-invasive sampling

Electronic supplementary material The online version of this article (doi:10.1007/s10592-009-9933-x) contains supplementary material, which is available to authorized users.

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Introduction

The use of non-invasive sampling from free-ranging animals has revolutionized the study of ecological, behavioral, genetic and epidemiological aspects of many taxa (Smith and Wayne 1996; Beebee and Rowe 2008). In the last two decades, the fields of conservation genetics and molecular ecology have grown dramatically, largely due to the increased ability to analyze natural populations using molecular markers applied to samples such as scats. Many research groups routinely employ faecal DNA to identify field-collected samples at the species or individual level, as well as to determine gender, kinship, genetic diversity, or diet (e.g. Hedmark et al. 2004; Livia et al. 2007; Deagle and Tollit 2007). However, up to now we are unaware of any study that has applied faecal DNA to identify a morphological phenotype. This is largely due to the fact that the molecular basis of such traits remains unknown for most naturally occurring polymorphisms, precluding the development of assays to survey the dynamics of

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morphological variants and their underlying alleles. If this impediment is overcome, the population genetics of loci influencing natural phenotypic polymorphisms could be studied directly using non-invasive sampling, thus opening up tremendous prospects for the investigation of their evolutionary and ecological significance.

The jaguar (*Panthera onca*) is the largest felid in the Americas and has been the focus of considerable scientific and conservationist attention in the last decades (e.g. Medellín et al. 2002). Melanism (dark background coloration) has been long known to occur in this species, representing a dramatically visible coat color polymorphism whose ecological impact and adaptive significance remain a mystery. Although melanistic jaguars are common in zoos and often seen in the wild, no study has so far addressed the ecological role of this variant, and even its frequency in natural populations has not been surveyed systematically. Therefore, the conservation relevance of this phenotype cannot currently be assessed, and may be an issue in the context of dwindling natural populations.

Studying this trait in the wild using conventional methods poses major challenges given low densities and high costs of capture-based operations, especially when compared to the effectiveness of scat collection in the field. We have previously identified a 15-bp deletion in the jaguar *MC1R* gene (the "*MC1R*-Δ15" allele) that was perfectly associated with melanism (Eizirik et al. 2003). These analyses were based on blood or tissue samples,

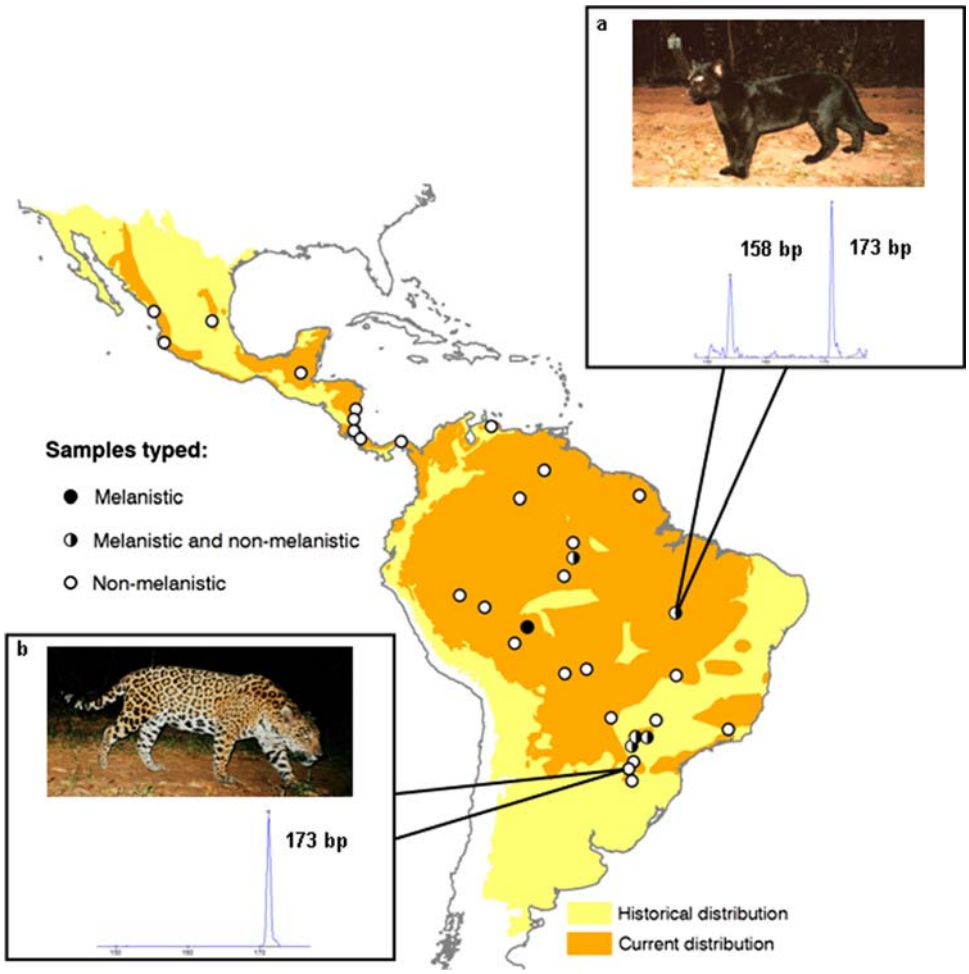

Fig. 1 Range map of the jaguar in the Americas, depicting its historical and current distribution—Modified from Sanderson et al. (2002) and Zeller (2007). *Circles* indicate sample collection locales for our association study between melanism and *MC1R* variation (*white*: one or more non-melanistic samples from a given site; *black*: one or more melanistic samples from a site). *Insets*: **a** Heterozygous *MC1R* genotype (containing the melanism-related *MC1R*-Δ15 allele that yields a 158-bp PCR product, and the 173-bp wild-type allele) obtained from a scat sample collected at an Amazonian field site where camera traps also recorded the presence of melanistic animals (camera-trap photo of melanistic individual taken in Morro do Diabo State Park, SP, Brazil; credit: Instituto de Pesquisas Ecológicas); **b** Homozygous wild-type *MC1R* genotype (containing only the 173-bp wild-type allele) obtained in six independent PCR trials from a scat sample collected at an Argentinean field site where only non-melanistic animals were detected by camera-trapping (camera-trap photo of wild-type jaguar from Iguazú National Park, Argentina; credit: A. Paviolo, C. De Angelo and M. Di Bitetti—Proyecto Yaguareté, Argentina)

Table 1 Genotyping results for the melanism-related *MC1R* polymorphism in field-collected jaguar scats

Collection site	N^e	Genotypes identified[f]			Predicted phenotype
		158/158	158/173	173/173	
Ivinhema State Park[a]	2	–	–	6	Wild-type
Iguaçu National Park[b]	2	–	–	6	Wild-type
Misiones Province[c]	5	–	–	6	Wild-type
Cantão State Park[d]	14	–	–	6	Wild-type
Cantão State Park[d]	1	3	1	4	Melanistic

Genotypes are given as all possible combinations of alleles 158 (*MC1R*-Δ15) and 173 (wild-type allele)

[a] Mato Grosso do Sul state, southwestern Brazil

[b] Paraná state, southern Brazil

[c] Multiple Atlantic Forest sites in northeastern Argentina (adjacent to Iguaçu National Park)

[d] Tocantins state, southern Amazonian region, northern Brazil

[e] Only samples that could be reliably and reproducibly genotyped are included. Five additional samples from Cantão State Park yielded *MC1R* genotypes, but our replication threshold for phenotype prediction could not be reached after 12 genotyping attempts: two of them yielded four replicates each of the 173/173 genotype; two others yielded a single replicate each of 173/173; and one sample was typed once as 158/158 and three times as 173/173

[f] Values indicate the number of times each *MC1R* genotype was independently observed for each faecal sample

prompting the question of whether this variant could be genotyped in natural populations using scats. To pursue this possibility, it is important to ascertain that the association between genotype and phenotype is affirmed by additional sampling, and to test whether this molecular variant can be effectively and reliably typed in faecal samples.

In the present study we addressed these issues by performing three sequential analytical steps: (1) an expanded association study testing the ability of our molecular assay to predict the melanistic phenotype; (2) a blind test using faecal samples from known animals to assess whether scat DNA-based phenotype prediction is reliable and reproducible; and (3) field tests assessing whether the quality of samples found in wild settings is sufficient to interrogate this nuclear genetic locus. Details of materials and methods are provided as online Supplementary Information.

Results and discussion

The association study included 95 broadly sampled jaguar individuals (Fig. 1 and Supplementary Information) whose color was known, and resulted in 100% correspondence between the melanistic phenotype and presence of the *MC1R*-Δ15 allele, thus strongly implicating the latter in this morphological variant. The blind test was conducted with 34 faecal samples collected from captive jaguars whose identity and coloration were hidden from the geneticist performing the molecular assay, and only revealed after reaching a pre-established threshold of reliable genotypes (three for heterozygotes [or at least two independent scores for each allele], and six for homozygotes). In 32 out of 34 samples this threshold could be reached, in every case correctly predicting the individual's phenotype (see Supplementary Information). In the two additional samples the phenotype was also predicted correctly, with four and five homozygote genotypes, respectively. This experiment demonstrated that it is possible to reliably predict a jaguar's coat color on the basis of scat DNA.

Finally, we applied this approach to survey field-collected scats identified as having been deposited by jaguars based on mtDNA sequencing (Haag et al. 2009). Twenty-three jaguar scats from the southern Amazon region (P.E. Cantão, TO, Brazil) and ten from the Atlantic Forest biome (Argentina and southwestern Brazil) were analyzed; 15 among the former and 9 among the latter could be reproducibly genotyped by reaching our threshold (Table 1). No evidence of melanism was detected in the Argentinean Atlantic Forest sites or in adjacent Iguaçu Park (Brazil) in agreement with current camera-trapping data (Fig. 1; Table 1). In contrast, at least one melanistic animal was detected by scat-DNA genotyping of the *MC1R* polymorphism in the Amazonian site, where this phenotypic variant has been observed. These results demonstrate that it is possible to survey the presence and frequency of melanism in natural jaguar populations using non-invasive DNA, and create an opportunity for in-depth investigation of the evolutionary forces driving the dynamics of this polymorphism in the wild.

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Online Supplemental Information - Supplementary Materials and Methods

We initially used blood, tissue, pelt, hair and serum samples (see Supplementary Table 1) to expand the genotype-phenotype association study performed by Eizirik et al. (2003), aiming to test the presence of the *MC1R*-Δ15 allele in animals of known coloration. DNA extraction from blood and tissue was performed with a standard phenol-chloroform protocol (Sambrook et al. 1989), while that of pelt and hair samples used the Puregene DNA Purification Kit (GENTRA) or the ChargeSwitch® Forensic DNA Purification Kit (Invitrogen).

Subsequent analyses used faecal samples collected from known captive animals (blind test), and then field-collected scats collected in different areas of Argentina and Brazil (Table 1). Faecal samples were stored in silica gel or in ethanol 96%, and their DNA was extracted using the the QIAamp DNA Stool Mini Kit (QIAGEN). Scat DNA extractions were carried out in a separate laboratory area, in a UV-sterilized laminar flow hood dedicated to the analysis of DNA from noninvasive samples. Each batch of extractions (n=10) included one negative extraction control to monitor the occurrence of contamination with extrinsic DNA. We also used the QIAamp DNA Stool Mini Kit (QIAGEN), with slight protocol modifications, to extract DNA from serum samples.

All faecal samples were initially identified at species level using a sequence-based assay targeting a short segment of the mtDNA *ATP6* gene (Haag et al. *in press*). Only samples that were reliably identified as originating from a jaguar were included in this study. Known jaguar faecal samples obtained from zoos for the melanism blind study were also subjected to this DNA-based species-identification procedure, to exactly mimic the protocol performed for field-collected scats. In addition to the species-level identification, this mtDNA-based assay served as a preliminary assessment of DNA quality and quantity for PCR amplification, prior to the performance of experiments targeting the *MC1R* gene.

To genotype the melanism-related *MC1R* polymorphism we utilized the fluorescent assay described by Eizirik et al. (2003), employing PCR primers that flank the implicated deletion. PCRs were performed in a final volume of 10ul, containing 1x PCR buffer (Invitrogen), 2.0-2.5 mM $MgCl_2$, 0.2% Triton X-100 or 3% DMSO as PCR additives, 0.2 mM dNTPs, 0.5 uM of each primer, 0.5U of Platinum *Taq* DNA polymerase (Invitrogen) and 1-3 ul of empirically diluted template DNA, following the cycle conditions described by Eizirik et al. (2003). PCR product sizes were scored in a MegaBACE 1000 automated sequencer (GE Healthcare) and analyzed using the Genetic Profiler 2.2 software (Amersham Biosciences). To improve and verify the reliability of genotypes from faecal samples, we employed a multiple-tube approach (Taberlet et al. 1996), by performing several independent PCR amplifications for each DNA extract (see Table 1).

Supplementary References

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Supplementary Table 1. Samples of known jaguars utilized in this present study, with genotypes for the melanism-related *MC1R* polymorphism (+ represents the wild-type allele [173-bp PCR product]; Δ15 is the *MC1R*-Δ15 deletion allele [158-bp PCR product]).

ID	Sample type	Geographic Origin/Captive Institution	Contact	Color	Genotype
PON-03[a]	Blood	Ceri Zoo, USA	Laboratory of Genomic Diversity	Melanistic	+/Δ15
PON-13[a]	Muscle	Philadelphia Zoo, USA	Laboratory of Genomic Diversity	Melanistic	+/Δ15
PON-16[a]	Blood	El Peten, Guatemala	ARCAS	wild-type	+/+
PON-18[a]	Fibroblast	San Diego Zoo, USA	Laboratory of Genomic Diversity	Melanistic	+/Δ15
PON-20[a]	Blood	Chepo, Panama Prov., Panama	Summit Zoo	wild-type	+/+
PON-21[a]	Blood	Upala, Alajuela Prov., Costa Rica	Las Pumas	wild-type	+/+
PON-22[a]	Blood	Puerto Limon Prov., Costa Rica	Simon Bolivar Zoo	wild-type	+/+
PON-23[a]	Blood	Atlantico Sur Department, Nicaragua	Juigalpa Zoo	wild-type	+/+
PON-25[a]	Blood	Rio San Juan Department, Nicaragua	Managua Zoo	wild-type	+/+
PON-28[a]	Blood	Peru (Probably)	Mendoza Zoo	wild-type	+/+
PON-31[a]	Blood	San Luis Potosi state, Mexico	Leon Zoo	wild-type	+/+
PON-43[a,b]	Blood	P.N. do Iguaçu, PR state, S Brazil	Proj. Carnívoros; CENAP/ICMBio	wild-type	+/+
PON-44[a,b]	Blood	Bataguassu, MS state, SW Brazil	Proj. Porto Primavera; CENAP/ICMBio	wild-type	+/+
PON-46[a]	Blood	Goiás state, CW Brazil	Goiania Zoo	wild-type	+/+
PON-48[a]	Blood	Brazil, unknown state	Goiania Zoo	Melanistic	Δ15/Δ15
PON-52[a]	Blood	Jalisco state, Mexico	Idaho State University	wild-type	+/+
PON-54[a]	Blood	Amazonas state, Venezuela	Las Delicias	wild-type	+/+
PON-56[a]	Blood	Falcon state, Venezuela	Las Delicias	wild-type	+/+
PON-57[a]	Blood	Venezuela (Probably)	Las Delicias	wild-type	+/+
PON-58[a]	Blood	Falcon state, Venezuela	Las Delicias	wild-type	+/+
PON-62[a]	Blood	Bolivar state, Venezuela	Valencia Castellito	wild-type	+/+
PON-63[a]	Blood	Bolivar state, Venezuela	Valencia Castellito	wild-type	+/+
PON-64[a]	Blood	Sta. Cruz Department, Bolivia	Santa Cruz Zoo	wild-type	+/+
PON-66[a]	Blood	N. Bolívia	Santa Cruz Zoo	wild-type	+/+
PON-67[a]	Pelt	Amazonas state, Venezuela	PROFAUNA	wild-type	+/+
PON-68[a]	Blood	French Guiana	J.C. Vie	wild-type	+/+
PON-72[a]	Blood	Brazil, unknown state	CENAP/ICMBio	wild-type	+/+
PON-75[a]	unknown	Sinaloa state, México	B. Miller	wild-type	+/+
PON-76[a]	Blood	Amazonas state, Brazil	CIGS, Manaus	wild-type	+/+
PON-91[a]	Blood	Montgomery Zoo, AL, USA	C. Hilton	Melanistic	+/Δ15
PON-92[a]	Blood	P.E. Morro do Diabo, SP state, SW Brazil	Inst. Pesquisas Ecológicas	Melanistic	+/Δ15

PON-146[a,c]	Blood/Faeces	Anaurilândia, MS state, SW Brazil	I. Pró-Carnívoros; Ilha Solteira Zoo; V. Queirós	wild-type	+/+
PON-147[a]	Blood	Bataguassu, MS state, SW Brazil	CENAP/ICMBio; I. Pró-Carnívoros	wild-type	+/+
PON-148[a,b]	Blood	Anaurilândia, MS state, SW Brazil	CENAP/ICMBio; I. Pró-Carnívoros	wild-type	+/+
PON-149[a,b,c]	Blood/Faeces	Marabá Paulista, SP state, SW Brazil	I. Pró-Carnívoros; Ilha Solteira Zoo; V. Queirós	wild-type	+/+
PON-150[a]	Blood	Marabá Paulista, SP state, SW Brazil	CENAP/ICMBio; I. Pró-Carnívoros	Melanistic	+/Δ15
PON-151[a]	Blood	Anaurilândia, MS state, SW Brazil	CENAP/ICMBio; I. Pró-Carnívoros	wild-type	+/+
PON-152[a]	Blood	Bataguassu, MS state, SW Brazil	CENAP/ICMBio; I. Pró-Carnívoros	wild-type	+/+
PON-154[a]	Blood	Anaurilândia, MS state, SW Brazil	CENAP/ICMBio; I. Pró-Carnívoros	wild-type	+/+
PON-155[a]	Blood	Alto Paraná, PR state, S Brazil	Inst. Pesquisas Ecológicas	Melanistic	+/Δ15
PON-156[a,b]	Tissue	Sapucaia do Sul Zoo, Brazil	T. Freitas; T. Trigo	Melanistic	+/Δ15
PON-157[a]	Blood	Anaurilândia, MS state, SW Brazil	Proj. Porto Primavera; CENAP/ICMBio	Melanistic	+/Δ15
PON-CRSB23[a]	Blood	Costa Rica	R. Spindler; S. Bolivar Zoo	wild-type	+/+
PON-CRSB24[a]	Blood	Costa Rica	R. Spindler; S. Bolivar Zoo	wild-type	+/+
PON-CRSB61[a]	Blood	Costa Rica	R. Spindler; S. Bolivar Zoo	wild-type	+/+
PON-CRSB145[a]	Blood	Costa Rica	R. Spindler; S. Bolivar Zoo	wild-type	+/+
bPon-02	Blood	P.N. do Iguaçu, PR state, S Brazil	Proj. Carnívoros; CENAP/ICMBio	wild-type	+/+
bPon-04	Tissue	P.E. do Turvo, RS state, S Brazil	Proj. Felinos (RS)	wild-type	+/+
bPon-12	Blood	P.N. Iguazu, Misiones Prov., Argentina	Proj. Carnívoros; CENAP/ICMBio	wild-type	+/+
bPon-22	Blood	Anaurilândia, MS state, SW Brazil	CENAP/ICMBio; I. Pró-Carnívoros	wild-type	+/+
bPon-27[c]	Blood/Faeces	Anaurilândia, MS state, SW Brazil	I. Pró-Carnívoros; Ilha Solteira Zoo; V. Queirós	wild-type	+/+
bPon-28	Blood	Ilha Solteira Zoo, Brazil	CENAP/ICMBio; I. Pró-Carnívoros	Melanistic	+/Δ15
bPon-29	Muscle	Anaurilândia, MS state, SW Brazil	CENAP/ICMBio; Pró-Carnívoros	wild-type	+/+
bPon-30	Blood	Anaurilândia, MS state, SW Brazil	CENAP/ICMBio; I. Pró-Carnívoros	wild-type	+/+
bPon-31	Blood	Anaurilândia, MS state, SW Brazil	CENAP/ICMBio; I. Pró-Carnívoros	wild-type	+/+
bPon-32[c]	Blood/Faeces	Anaurilândia, MS state, SW Brazil	I. Pró-Carnívoros; Ilha Solteira Zoo; V. Queirós	wild-type	+/+
bPon-33	Blood	Unknown (Probably Brazil)	CENAP/ICMBio; P.Crawshaw Jr.	wild-type	+/+
bPon-34	Muscle	French Guiana	Benoit de Thoisy	wild-type	+/+
bPon-35	Blood	P.E.V. do Rio Ivinhema, MS state, SW Brazil	CENAP/ICMBio; Pró-Carnívoros	wild-type	+/+
bPon-36	Muscle	Taquarussu, MS state, SW Brazil	CENAP/ICMBio; I. Pró-Carnívoros	Melanistic	+/Δ15
bPon-38	Pelt	Nova Andradina, MS state, SW Brazil	CENAP/ICMBio; I. Pró-Carnívoros	Melanistic	+/Δ15
bPon-40	Blood	P.E.V. do Rio Ivinhema, MS state, SW Brazil	CENAP/ICMBio; I. Pró-Carnívoros	wild-type	+/+
bPon-41	Blood	P.E.V. do Rio Ivinhema, MS state, SW Brazil	CENAP/ICMBio; I. Pró-Carnívoros	wild-type	+/+
bPon-45	Blood	Anaurilândia, MS state, SW Brazil	CENAP/ICMBio; I. Pró-Carnívoros	wild-type	+/+
bPon-46	Blood	P.E.V. do Rio Ivinhema, MS state, SW Brazil	CENAP/ICMBio; I. Pró-Carnívoros	wild-type	+/+
bPon-47	Blood	P.E.V. do Rio Ivinhema, MS state, SW Brazil	CENAP/ICMBio; I. Pró-Carnívoros	Melanistic	+/Δ15
bPon-48	Blood	P.E. Morro do Diabo, SP state, SW Brazil	Inst. Pesquisas Ecológicas	wild-type	+/+

bPon-49	Blood	P.E. Morro do Diabo, SP state, SW Brazil	Inst. Pesquisas Ecológicas	Melanistic	+/Δ15
bPon-50	Blood	P.E. Morro do Diabo, SP state, SW Brazil	Inst. Pesquisas Ecológicas	wild-type	+/+
bPon-51	Blood	P.E. Morro do Diabo, SP state, SW Brazil	Inst. Pesquisas Ecológicas	wild-type	+/+
bPon-52	Blood	P.E. Morro do Diabo, SP state, SW Brazil	Inst. Pesquisas Ecológicas	wild-type	+/+
bPon-53	Blood	P.E. Morro do Diabo, SP state, SW Brazil	Inst. Pesquisas Ecológicas	Melanistic	+/Δ15
bPon-54	Blood	P.E. Morro do Diabo, SP state, SW Brazil	Inst. Pesquisas Ecológicas	wild-type	+/+
bPon-55[c]	Faeces	Sapucaia do Sul Zoo, Brazil	R. von Hohendorff	Melanistic	+/Δ15
bPon-56[c]	Faeces	Sapucaia do Sul Zoo, Brazil	R. von Hohendorff	wild-type	+/+
bPon-57	Pelt	Pouso Alegre, MG state, SE Brazil	CENAP/ICMBio; F. Bonillo	wild-type	+/+
bPon-58	Pelt	Mato Grosso do Sul state, SW Brazil	CENAP/ICMBio	Melanistic	+/Δ15
bPon-59[c]	Faeces	P.N. do Iguaçu, PR state, S Brazil	CASIB; W. de Moraes	wild-type	+/+
bPon-78	Blood	P.E.V. do Rio Ivinhema, MS state, SW Brazil	I. Pró-Carnívoros	wild-type	+/+
bPon-81[c]	Faeces	Americana Zoo, Brazil	M. Falcade	wild-type	+/+
bPon-82[c]	Faeces	Limeira Zoo, Brazil	A. C. A. Sorg	Melanistic	+/Δ15
bPon-83[c]	Faeces	Manaus, AM state, N Brazil	Limeira Zoo; A. C. A. Sorg	wild-type	+/+
bPon-90[c]	Faeces	Parque Municipal "Danilo Galafassi", Brazil	L. S. Delgado	wild-type	+/+
bPon-91[c]	Faeces	Vicinity of P.N. do Iguaçu, PR state, S Brazil	Parque Municipal "Danilo Galafassi; L.S Delgado	wild-type	+/+
bPon-93[c]	Faeces	Parque Municipal "Danilo Galafassi", Brazil	L. S. Delgado	wild-type	+/+
bPon-94[c]	Faeces	Parque Municipal "Danilo Galafassi", Brazil	L. S. Delgado	Melanistic	+/Δ15
bPon-95[c]	Faeces	CEBUS, Brazil	C. D. P. Coelho and L. C. Silva	wild-type	+/+
bPon-96[c]	Faeces	CEBUS, Brazil	C. D. P. Coelho and L. C. Silva	wild-type	+/+
bPon-97[c]	Faeces	CEBUS, Brazil	C. D. P. Coelho and L. C. Silva	Melanistic	+/Δ15
bPon-98[c]	Faeces	Goiania Zoo, Brazil	R. F. de Carvalho and D. Nogueira	wild-type	+/+
bPon-99[c]	Faeces	Goiania Zoo, Brazil	R. F. de Carvalho and D. Nogueira	Melanistic	+/Δ15
bPon-100[c]	Faeces	Campinas Zoo, Brazil	E. F. Santos	Melanistic	+/Δ15
bPon-101[c]	Faeces	Cruzeiro do sul, AC state, N Brazil	Campinas Zoo; E. F. Santos	wild-type	+/+
bPon-102[c]	Faeces	Sapucaia do Sul Zoo, Brazil	R. von Hohendorff	wild-type	+/+
bPon-103[c]	Faeces	Sapucaia do Sul Zoo, Brazil	R. von Hohendorff	wild-type	+/+
bPon-104[c]	Faeces	Sapucaia do Sul Zoo, Brazil	R. von Hohendorff	wild-type	+/+
bPon-105[c]	Faeces	Guarulhos Zoo, Brazil	C. E. Bolochio	Melanistic	+/Δ15
bPon-107[c]	Faeces	Feijó, AC state, N Brazil	Parque Ambiental Chico Mendes; J. O. Guimarães	wild-type	+/+
bPon-108[c]	Faeces	Guajará-mirim, RO state, N Brazil	Parque Ambiental Chico Mendes; J. O. Guimarães	Melanistic	+/Δ15
bPon-112[c]	Faeces	Guarulhos Zoo, Brazil	C. E. Bolochio	wild-type	+/+
bPon-114[c]	Faeces	Parque Cyro Gevaerd, Brazil	M. R. Achutti	wild-type	+/+
bPon-115[c]	Faeces	Parque Cyro Gevaerd, Brazi	M. R. Achutti	Melanistic	+/Δ15

bPon-116[c]	Faeces	Amazonas state, Brazil	Curitiba Zoo; M. L. Javorouski	wild-type	+/+
bPon-117[c]	Faeces	Unknown (Probably Brazil)	Curitiba Zoo; M. L. Javorouski	Melanistic	+/Δ15
bPon-120[c]	Faeces	Manaus, AM state, N Brazil	Pomerode Zoo (Fund. Hermann Weege);C. Maas	Melanistic	+/Δ15
bPon-123[c]	Faeces	Campo Grande, MS state, CW Brazil	Pomerode Zoo (Fund. Hermann Weege);C. Maas	wild-type	+/+
bPon-124	Serum	P.N. do Iguaçu, PR state, S Brazil	CENAP/ICMBio; I. Pró-Carnívoros	wild-type	+/+
bPon-125	Serum	P.N. do Iguaçu, PR state, S Brazil	CENAP/ICMBio; I. Pró-Carnívoros	wild-type	+/+
bPon-129	Pelt	Santana do Araguaia, PA state, N Brazil	Jaguar Conservation Fund	Melanistic	+/Δ15
bPon-130	Blood	Pereira Barreto, SP state, SW Brazil	Proj. Porto Primavera; CENAP/ICMBio	wild-type	+/+
bPon-131	Blood	Bataguassu, MS state, SW Brazil	Proj. Porto Primavera; CENAP/ICMBio	wild-type	+/+
bPon-132	Blood	Bataguassu, MS state, SW Brazil	Proj. Porto Primavera; CENAP/ICMBio	wild-type	+/+
bPon-133	Hair	Puerto Libertad, Misiones Prov., Argentina	Proyecto Yaguareté, CeIBA	wild-type	+/+
bPon-134	Pelt	Misiones-Alto Paraná; Argentina-Paraguay border	Proyecto Yaguareté, CeIBA	wild-type	+/+
bPon-135	Blood	Ruiz de Montoya, Misiones Prov., Argentina	Proyecto Yaguareté, CeIBA	wild-type	+/+
bPon-136	Hair	Colonia La Flor, Misiones Prov., Argentina	Proyecto Yaguareté, CeIBA	wild-type	+/+
bPon-137	Pelt	San Vicente, Misiones Prov., Argentina	Proyecto Yaguareté, CeIBA	wild-type	+/+
bPon-334	Blood	Lambari d`Oeste, MT state, CW Brazil	CENAP/ICMBio; R. Jorge	wild-type	+/+
bPon-335	Blood	Presidente Figueiredo, AM state, N Brazil	CENAP/ICMBio; IBAMA (AM)	wild-type	+/+
bPon-336	Blood	Manicoré, AM state, N Brazil	CENAP/ICMBio; IBAMA (AM)	wild-type	+/+
bPon-353	Blood	P.E. do Cantão, TO state, N Brazil	Jaguar Conservation Fund	wild-type	+/+
bPon-354	Blood	P.E. do Cantão, TO state, N Brazil	Jaguar Conservation Fund	wild-type	+/+
bPon-355	Blood	P.E. do Cantão, TO state, N Brazil	Jaguar Conservation Fund	wild-type	+/+
bPon-359	Blood	P.E. do Cantão, TO state, N Brazil	CRAS; CENAP/ICMBio; I. Pró-Carnívoros	wild-type	+/+
bPon-366	Serum	P.N. do Iguaçu, PR state, S Brazil	CENAP/ICMBio; I. Pró-Carnívoros	wild-type	+/+

[a] Data from Eizirik et al. (2003).

[b] Data from Eizirik et al. (2003) with genotyping repeated in this study.

[c] Faecal samples from captive individuals utilized in this study only for the blind test of melanism prediction.

CAPÍTULO V

3° ARTIGO

THE EFFECT OF HABITAT FRAGMENTATION ON THE GENETIC STRUCTURE OF A TOP PREDATOR: LOSS OF DIVERSITY AND HIGH DIFFERENTIAION AMONG REMNANT POPULATIONS OF ATLANTIC FOREST JAGUARS (*PANTHERA ONCA*)

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The effect of habitat fragmentation on the genetic structure of a top predator: loss of diversity and high differentiation among remnant populations of Atlantic Forest jaguars (*Panthera onca*)

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Abstract

Habitat fragmentation may disrupt original patterns of gene flow and lead to drift-induced differentiation among local population units. Top predators such as the jaguars may be particularly susceptible to this effect given their low population densities, leading to small effective sizes in local fragments. On the other hand, the jaguar's high dispersal capabilities and relatively long generation time might counteract this process, slowing the effect of drift on local populations over the time frame of decades or centuries. In this study we have addressed this issue by investigating the genetic structure of jaguars in a recently fragmented Atlantic Forest region, aiming to test whether loss of diversity and differentiation among local populations are detectable, and whether they can be attributed to the recent effect of drift. We used 13 microsatellite loci to characterize the genetic diversity present in four remnant populations, and observed marked differentiation among them, with evidence of recent allelic loss in local areas. Although some migrant and admixed individuals could be identified, our results indicate that the recent habitat fragmentation among these areas has been sufficiently strong to promote differentiation induced by drift, and loss of alleles at each site. Low estimated effective sizes supported the inference that genetic drift could have caused this effect in this short time frame. These results indicate that jaguars are unable to effectively disperse across the human-dominated landscapes that separate the fragments, and that each fragment contains a small, isolated population that is already suffering the effects of genetic drift.

Introduction

The jaguar (*Panthera onca*) is the largest wild felid in the Americas and the only living representative of the genus *Panthera* in the New World (Nowell & Jackson 1996). This species historically ranged from the southwestern United States to the Argentinean Patagonia (Guggisberg 1975). However, during the 20th century the species lost almost half of its original range and is currently distributed from northern Mexico to northern Argentina (Misiones Province) and southern Brazil (Turvo State Park, Rio Grande do Sul state [RS]) (see Fig. 1) (Swank & Teer 1989; Sanderson *et al.* 2002; Zeller 2007). This present distribution comprises a mosaic of remnant populations of variable size, whose increasing isolation is a consequence of severe habitat loss and fragmentation, associated with a declining prey base and direct human persecution.

In South America, the species past distribution included a diverse array of biomes, including all of the Atlantic Forest and its associated ecosystems. With the Atlantic Forest now severly fragmented and most of its previous realm occupied by human-dominated landscapes, the species has lost almost all of its range in this portion of the continent. In the Upper Paraná Atlantic Forest (UPAF) Ecoregion (Fig. 1), spanning southwestern Brazil, northeastern Argentina and eastern Paraguay, resident jaguars are essentially restricted to semiconnected protected areas that possibly form a metapopulation structure (Cullen *et al*. 2005; Cullen 2007). Conservation Units in the UPAF have been categorized as bearing highest priority because they correspond to the last viable populations of jaguars left in such a type of ecosystem (Zeller 2007). However, recent estimates of these populations indicated that there are only 25 - 53 adult jaguars in the so-called “Green Corridor” of Argentina (Misiones Province) and Brazil (Iguaçu National Park, Paraná state [PR]) (Paviolo *et al*. 2008), in addition to 9 - 15 adults in the Morro do Diabo State Park (São

Paulo state [SP], Brazil) (Cullen 2007), 10 in the Ivinhema State Park (Mato Grosso do Sul state [MS], Brazil) (Sana *et al*. 2006), and 10 - 20 in the riverine region that has now been flooded by the Porto Primavera dam (MS/SP states) (Crawshaw *et al*. 1993). Moreover, no core area in the UPAF is sufficiently large to sustain viable populations of this species, which need extensive areas to survive (Galindo-Leal & Câmara 2003; Di Bitetti *et al*. 2003).

Several field studies have been developed in the UPAF aiming to assess aspects of the ecology, distribution and conservation of this threatened felid (Crawshaw *et al*. 1993; Crawshaw 1995; Sana 2003; Cullen *et al*. 2005; Paviolo *et al*. 2008). In spite of these efforts, many aspects of the biology and dynamics of these population fragments remain unknown, and there is a growing need for genetic analyses that complement these ecological approaches, by addressing issues such as current and historical population connectivity as well the potential effects of genetic drift and inbreeding on these small demographic units. In fact, in the context of a regional conservation strategy for jaguars proposed by Argentinean and Brazilian governments as well as NGOs (Chalukian 2006), one of the identified priorities is the study of the population genetic structure of this felid in the area (K. Schiaffino *et al*. in preparation).

So far, few genetic studies have focused on this species (Eizirik *et al.* 2001; Ruiz-Garcia *et al*. 2006; Moreno *et al*. 2006; Soares *et al*. 2006) and analyses addressing the connectivity of natural populations on a local scale have not yet been performed. This is mostly due to the great difficulty of sufficiently sampling biological materials from this felid in the wild. To overcome this challenge, long-term sampling through continued, intensive field efforts is required, if possible combined with alternative strategies such as the use of non-invasive approaches.

In the present study we investigated the magnitude and spatial distribution of genetic diversity in remnant jaguar populations of the UPAF ecoregion. We assessed the occurrence of genetic differentiation among local population fragments, and measured demographic connectivity by identifying migrants and inferring patterns of historical and current gene flow. Our results indicate that these populations are losing diversity and undergoing rapid genetic differentiation induced by genetic drift, as a consequence of anthropogenic isolation and very small population sizes in individual fragments.

Materials and Methods

Study sites

The study area included a network of large protected areas located in the Paraná River Basin (see Fig. 1) including Morro do Diabo State Park (37,000 ha; SP state), Ivinhema State Park (73,300 ha; MS state), Iguaçu National Park (185,262 ha; PR state), and Turvo State Park (17,491 ha; RS state) in Brazil. In addition, there are smaller forest fragments that may be able to sustain jaguar individuals in connection to the core, protected areas. Another surveyed field site included the area of influence of the Porto Primavera dam (MS/SP states; Fig. 1) where jaguar monitoring was performed on its upstream side before, during and after the filling of the reservoir. This region suffered a strong environmental impact in 1998, when the filling of the dam flooded an area of *ca.* 220,000 ha, obliterating the riverine marshes that were the jaguar's strongholds in that location. The last radio-collared jaguar in that area was found dead in 2003 (Sana *et al*. 2006), and there are currently only occasional reports of scattered animals roaming this region (D. A. Sana, pers. obs.), suggesting that the species is essentially extinct locally. Further south, another large dam (Itaipu Binacional, on the border between Paraguay and

119 southern Brazil; Fig. 1) in 1982 flooded an area of *ca.* 135,000 ha containing primary
120 forests, likely leading to partial or complete demographic isolation between the jaguar
121 populations located in the northern and southern sectors of the UPAF ecoregion.
122 In the Misiones Province in Argentina, the largest and most continuous remnant of
123 the UPAF, the "Green Corridor" (Fig. 1), encompasses 1,100,000 ha and spans 200 km
124 linking Iguaçu National Park to Turvo State Park in Brazil through a patchwork of
125 intervening protected and unprotected areas in the Argentinean province of Misiones (Di
126 Bitetti *et al*. 2003). In adjoining eastern Paraguay, deforestation has occurred at a high rate
127 in recent years and most protected areas are now isolated and cover less than 10,000 ha (Di
128 Bitetti *et al*. 2003).
129
130 *Sample collection and laboratory procedures*
131 Biological samples were obtained from remnant areas in the Upper Paraná Atlantic
132 Forest Ecoregion where field projects addressing jaguar ecology and conservation have
133 been carried out in the last two decades. Samples were subdivided on the basis of their
134 origin and proximities to four pre-defined geographical groups (referred to as
135 "populations"; Table 1; Fig. 1) which have been suggested by radio-telemetry and camera-
136 trapping data for jaguars in this region (L. Cullen Jr., D. A. Sana and C. De Angelo, pers.
137 obs.). Eight blood samples were obtained from Morro do Diabo State Park and its
138 surroundings (SP state) between 1998 and 2004; 23 samples including blood, tissue and
139 pelts were obtained from the area affected by the Porto Primavera dam (MS/SP states)
140 between 1993 and 2004; eight blood and tissue samples were collected between 2002 and
141 2007 from Ivinhema State Park and its surroundings (MS state); and 11 samples including
142 blood, tissue, serum, pelt and hair were obtained between 1992 and 2007 from the "Green

143 Corridor" (encompassing areas in Brazil [PR and RS states] and several forested locations
144 in the Misiones Province, Argentina). Blood and serum samples were collected from
145 captured individuals while pelts, hairs and tissue samples were obtained from animals
146 found dead or maintained in local zoological collections.

147 In addition to the samples mentioned above, faeces were collected opportunistically
148 in the field, and two scats were collected from captive animals whose geographic origin
149 was known (both from the Green Corridor region; Table 1). All faecal samples, as well as
150 those consisting of pelts and hairs, were subjected to a rigorous procedure to confirm the
151 species source using a short segment of the mtDNA *ATP synthase subunit 6* (*ATP6*) gene
152 (Haag *et al.* 2009). Only samples identified reliably as jaguar were utilized in this study.
153 Four faecal samples were obtained from the Ivinhema State Park, two of which were
154 confirmed as originating from jaguars. We also included six faecal samples collected in the
155 Green Corridor region that had been identified in a previous study (Haag *et al*. 2009) as
156 belonging to jaguar. More recently, 12 additional scats were collected in the Green
157 Corridor region, five of which could be identified as originating from jaguar, so that a total
158 of 11 scats from the Green Corridor were included in this study.

159 Blood samples were preserved with EDTA, and in some cases with an equal
160 volume of a salt saturated solution (100mM Tris, 100mM EDTA, 2% SDS). Pelts, tissues
161 and hairs, as well as some scats, were preserved in 96% ethanol. Most faecal samples were
162 stored in sterile vials containing silica gel at a ratio of 4 g silica/g faeces (Wasser *et al*.
163 1997). All samples were stored at -20°C prior to DNA extraction. Genomic DNA was
164 extracted from blood and tissue samples using a standard phenol-chloroform protocol
165 (Sambrook *et al*. 1989). Extractions from pelt and hair samples were performed with the
166 Puregene DNA Purification Kit (GENTRA) or using the ChargeSwitch® Forensic DNA

Purification Kit (Invitrogen). DNA from scats was extracted using the QIAamp DNA Stool Mini Kit (QIAGEN), following the manufacturer's instructions. Each batch of extractions (n = 10) included one negative extraction control to monitor the occurrence of contamination with extrinsic DNA. To avoid contamination of samples containing poor DNA quality (e.g. scats), extractions and other pre-PCR procedures were carried out in a separate laboratory area, within a UV-sterilized laminar flow hood and employing dedicated pipettes with aerosol-resistant tips.

All jaguar DNA extracts were screened for 13 microsatellite loci: one containing a dinucleotide repeat (FCA742), two with trinucleotide repeats (F146 and F98), and 10 with tetranucleotide repeats (FCA741, FCA740, FCA723, FCA453, FCA441, FCA391, F124, F85, F53 and F42). These primers were originally developed for the domestic cat (*Felis silvestris catus*) by Menotti-Raymond *et al*. (1999, 2005) and have been optimized and standardized for use with jaguar samples (Eizirik *et al.* 2001, 2008). Every forward primer was 5'-tailed with an M13 sequence (5'- CACGACGTTGTAAAACGAC- 3') (Boutin-Ganache *et al*. 2001) and used in combination with an M13 primer that had the same sequence but was dye-labeled (with FAM, HEX or NED) on its 5' end. PCR reactions were performed in a 10 ul volume containing 0.5 - 3 ul of empirically diluted DNA, 1x PCR Buffer (Invitrogen), 1.5 - 2.5 mM $MgCl_2$, 200 uM of each dNTP, 0.2 uM of the reverse and M13-fluorescent primers, 0.0133 uM of the M13-tailed forward primer, and 0.25 or 0.5U of *Taq* DNA Polymerase or Platinum *Taq* DNA polymerase (Invitrogen). The reaction profile was as follows: 10 cycles (Touchdown) of 94°C for 45s, 60 - 51°C for 45s, 72°C for 1.5 min, followed by 30 cycles of 94°C for 45s, 50°C for 45s, 72°C for 1.5 min, and a final extension at 72°C for 30 min. PCR reactions were carried out for each locus separately, and products from 1 to 3 loci were diluted and pooled together based on yield,

size range and fluorescent dye. In the case of faecal samples, we empirically improved the PCR yield by including additives such as 0.2% Triton X-100, 3% DMSO and/or 0.5 x PCR enhancer solutions (Invitrogen). Microsatellite genotyping was performed using a MegaBACE 1000 automated sequencer and the ET-ROX 550 size standard (GE Healthcare), and then analyzed utilizing the accompanying Genetic Profiler 2.2 software. Negative controls were run for each batch of PCR reactions, and genotyped to monitor the presence of any exogenous DNA at this step.

Since the use of non-invasive samples (containing low DNA quality and quantity) may induce genotyping problems such as allelic dropout and the presence of false alleles, we adopted the multiple-tube approach (Taberlet *et al.* 1996) for materials such as faeces and hairs. In such cases, we only considered genotypes that had been sufficiently replicated, by establishing an *a priori* threshold for inclusion (heterozygotes were identified by a minimum of two independent scores of each allele, and homozygotes were considered to be corrrect if the same allele was detected by at least five independent PCR experiments). In addition, to assess our confidence on results generated from scat samples, we genotyped paired faecal and blood samples obtained from five different individuals to verify the congruence between the two types of material (Table 1).

The pre-established threshold of the multiple-tube approach was reached for both hair samples, as well as for the two scats collected from captive animals from the Green Corridor region, and the two field scats collected in Ivinhema State Park. However, for the 11 field-collected jaguar scats from the Green Corridor region, only six could be reliably genotyped.

Data analysis

We used the program MICRO-CHECKER 2.2.3 (Van Oosterhout *et al.* 2004) to identify possible nonamplified alleles (null alleles), large allele dropout and scoring errors due to stutter peaks, which might lead to erroneous estimates of population genetic parameters.

Searches for identical genotypes were performed using the program GENECAP (Wilberg & Dreher 2004), which employs an executable macro within Microsoft EXCEL to compare each multilocus genotype with all others within the dataset. GENECAP was utilized to investigate whether different non-invasive DNA samples could have originated from the same individual, but also to assess results from standard samples (e.g. tissue, blood or pelt), so as to monitor potential errors stemming from repeat collections from the same animal (e.g. by different field teams) or mislabeled tubes. In addition, GENECAP identified composite genotypes that differed by only one or two alleles, thus allowing us to scrutinize samples with similar genotypes for possible genotyping errors.

To quantify the discriminatory power of our microsatellite dataset, we calculated the probability of identity ($P_{(ID)}$) index, *i.e.* the probability of two individuals in a population randomly sharing identical genotypes for all the analysed loci; this was perfomed using two different approaches implemented in GENECAP. One of them assumed Hardy-Weinberg equilibrium (HW $P_{(ID)}$), and the other assumed that the individuals are siblings (Sib $P_{(ID)}$). It has been suggested that HW $P_{(ID)}$ may be biased, so that the more conservative Sib $P_{(ID)}$ index may be a more appropriate measure (Waits *et al*. 2001).

Genetic diversity was measured by the number of alleles per locus (K), mean number of alleles per locus (A), presence of private alleles, observed heterozygosity (H_O) and expected heterozygosity (H_E) under Hardy-Weinberg assumptions (Nei 1978). These

239 analyses were performed using FSTAT 2.9.3.2 (Goudet 2002) and GENEPOP 3.4
240 (Raymond & Rousset 1995). FSTAT was also used to calculate allelic richness, a measure
241 of the observed number of alleles per locus independent of sample size, hence allowing the
242 comparison of this quantity between different sample sizes using a rarefaction approach,
243 which standardizes estimates to the smallest included sample size (Petit *et al*. 1998).
244 Global and population-specific tests for deviations from the Hardy-Weinberg
245 equilibrium (HWE) were performed with ARLEQUIN 3.11 using an exact test based on
246 the procedure described by Guo & Thompson (1992) with 10,000 dememorization steps
247 (Excoffier *et al*. 2005). An assessment of linkage disequilibrium (LD) among loci was
248 conducted using FSTAT. Significance levels ($\alpha = 0.05$) for inferred LD or departures from
249 HWE were corrected for multiple simultaneous comparisons with the sequential
250 Bonferroni approach (Rice 1989).
251 The degree of genetic differentiation among the pre-defined geographic groups was
252 investigated with pairwise F_{ST} measures as implemented in ARLEQUIN (Weir &
253 Cockerham 1984), as well as the related R_{ST} index which incorporates a stepwise mutation
254 model (Slatkin 1995). The statistical significance of F_{ST} and R_{ST} values was tested using
255 10,000 permutations. We also calculated the F_{ST} and R_{ST} between different time periods in
256 the Green Corridor and Porto Primavera populations, to test for temporal changes in allele
257 frequencies, since these populations were sampled over a longer time frame than the
258 remaining localities.
259 Recently, Bayesian clustering algorithms emerged as an important computational
260 tool to infer the spatial structure of genetic diversity, and to assess if *a priori* geographic
261 groups indeed represent genetically distinct units. We choose STRUCTURE 2.2 (Pritchard
262 *et al.* 2000; Pritchard & Wen 2004) and BAPS 5.2 (Corander *et al*. 2003, 2004, 2006,

263 2008) for these assessments because of their versatility and proven use in uncovering
264 cryptic spatial structure (Latch *et al*. 2006). STRUCTURE 2.2 estimates the optimal
265 number of genetic clusters present in a sample as it strives to minimize deviations from
266 Hardy-Weinberg proportions and from linkage equilibrium by dividing the individuals into
267 a number of *K* clusters. The program uses a Markov chain Monte Carlo (MCMC)
268 procedure to estimate the posterior probability that the data fit the hypothesis of *K* clusters
269 [Pr(*X/K)*]. In the first step of this analysis, we estimated the number of genetic clusters by
270 performing 10 independent runs for each *K* between 1 and 10 using 1,000,000 MCMC
271 iterations and a burn-in period of 500,000 steps. We ran the program without supplying any
272 prior information on the sampling locations, so that the most likely number of clusters was
273 determined only from the multilocus genotypic data. We used the correlated allele
274 frequencies model and assumed the admixture model, which allows individuals to have
275 mixed ancestry, and outputs the fractional membership (*q*) of each individual to each
276 cluster. The optimal value of *K* was selected as the one that maximized the probability of
277 the data (averaged across differents runs).
278 In the second step of the analysis, we utilized STRUCTURE incorporating prior
279 population information (assuming $K = 4$ based on the geographic populations) to identify
280 which individuals were not residents of their sampled location (i.e. were migrants) and
281 those that had admixed ancestry. Individuals were considered residents if $q > 0.8$ for the
282 area where they were sampled. Individuals with *q*-values from 0.2 to 0.8 were considered
283 to be potentially admixed, as they could not be readily assigned as residents or migrants
284 (Lecis *et al*. 2006; Bergl & Vigilant 2007). Burn-in and run length were the same as
285 described above.

We also used BAPS 5.2 which uses stochastic optimization to infer the posterior mode of the number of populations. BAPS was used to cluster individuals and groups of individuals (defined on base of geographical sampling) using non-spatial and spatial mixture options. We ran the software with a predefined maximum of $K = 2 - 10$ and repeated the runs five times in order to check the stability of the results. For each run, the program reports the probabilities for different numbers of subpopulations, $K \leq$maximum K, and we averaged probabilities over the five runs.

Assignment/exclusion of individuals using predefined subpopulations was performed using the program GENECLASS 2 (Piry *et al*. 2004). In assignment tests, biased results can be obtained if the true source is not among the sampled populations, thus we used GENECLASS which does not assume that all potential source populations have been sampled. The program was also used to detect first-generation migrants. To assign or exclude individuals, we choose to employ the Bayesian criterion (Rannala & Mountain 1997) applying the Monte Carlo resampling method with 10,000 simulated individuals and an alpha of 0.01 (Paetkau *et al*. 2004). We computed a likelihood ratio test comparing the population where the individual was sampled over the highest likelihood value among all available populations (L = L_home/L_max).

A nonequilibrium Bayesian method was also applied to estimate recent migration rates among all populations using BAYESASS 1.2 (Wilson & Rannala 2003). We ran a total of 3×10^7 MCMC iterations with a burn-in of 10^7 steps and a thinning interval of 2000 iterations. Various delta values for migration rates (m), allele frequencies (P), and inbreeding coefficients (F) were tested. A realistic output (when the accepted numbers of proposed changes were between 40% and 60%) was obtained with delta values of 0.2 for all parameters.

The subprogram ISOLDE within GENEPOP was used to test for a relationship between geographic and genetic (F_{ST}) distances among populations, with the statistical significance assessed using a Mantel test with 10,000 permutations. Geographic distance among populations were measured as the euclidean distance between the "mean center" of each population, constructed from the average x and y coordinates of each population sample using ArcMap 9.1 software (ESRI Inc., Redlands, CA, USA).

Two methods were utilized to estimate effective population size, which require only a single sample. We initially used the program LDNE (Waples & Do 2008) to estimate N_e from genotypic data based on the LD method and implementing the bias correction of Waples (2006). We used jackknife methods for obtaining confidence intervals for N_e, and assumed a random mating model. The program calculates separate estimates using different criteria for excluding rare alleles, and we tested the following critical values (P_{crit}): 0.05; 0.02; 0.01. An evaluation of the effects of sample size on estimates of N_e was performed by limiting the number of individuals analyzed to six for each population.

In addition, N_e was also estimated with the program ONESAMP 1.1 (Tallmon *et al.* 2008), incorporating a novel approach that uses summary statistics and approximate Bayesian computation. The upper and lower bounds of the prior distribution for N_e were 2 and 100, respectively. In addition, priors for N_e of 2-50 e 2-200 were tested to verify if the results were robust to changes in these assumed values. Tests were also carried out changing the number of individuals analyzed for each population to assess the effects of sample size on our final estimates.

Results

Discrimination of individuals and genetic variability

Probability of identity calculations showed that our panel of microsatellites had considerable power to discriminate among individuals. The HW $P_{(ID)}$ was 1.86×10^{-13} and the more conservative measure Sib $P_{(ID)}$ was 1.06×10^{-5}, indicating that even related individuals would have a very low probability of bearing identical genotypes. Thus, different samples that yielded identical multilocus genotypes could be assumed with high confidence to originate from the same individual.

All samples were subjected to a search for genotype identity using GENECAP, and only two produced identical genotypes. These samples were collected in Uruguaí Provincial Park (Green Corridor region), 2 km apart from each other (individual bPon140 in Table 1). The remaining samples presented unique multilocus genotypes (Table 1). In addition, to evaluate the degree of confidence on data generated from faecal samples, we compared faecal and blood samples obtained from five individuals (Table 1) for nine microsatellite loci. Blood and faecal samples from the same individual produced identical genotypes, lending confidence to the reliability of genotypes generated from scat samples.

Global evaluation of the microsatellite data set utilizing MICROCHECKER indicated that null alleles may be present at loci FCA441, FCA723, and FCA741. However, no evidence of nonamplifying alleles was detected when local populations were analyzed separately. The results suggested that the inference of null alleles based on the global sample was more likely due to genetic structure among sites (see below). Further tests for errors in the data showed no evidence of stuttering or large allele dropout. Moreover, significant deviations from HWE were observed at two loci (F42 and F85) after the Bonferroni correction ($\alpha = 0.05$) when all samples were tested as a single population, in both cases induced by heterozygote deficiency. Nevertheless, no evidence was found for deviations from HWE within each population after Bonferroni correction. These results are

358 consistent with a Wahlund effect (Wahlund 1928). All pairwise locus combinations were
359 in linkage equilibrium for global and population-specific analyses (α= 0.05, after
360 Bonferroni correction for 78 comparisons).

361 Whole-sample analysis of genetic variability in the UPAF showed a mean of 0.682
362 and 0.731 for the observed (H_O) and expected (H_E) heterozygosity, respectively. The
363 number of alleles per locus ranged from 3 (FCA741) to 14 alleles (FCA742), with a mean
364 of 7.23. All loci were polymorphic in all populations except locus FCA741 in the Morro do
365 Diabo State Park. Individual analysis of each population separately (Table 2) showed
366 expected heterozygosity ranging from 0.497 (Morro do Diabo State Park) to 0.737 (Green
367 Corridor region) whereas observed heterozygosity ranged from 0.548 (Morro do Diabo
368 State Park) to 0.782 (Porto Primavera region). Mean number of alleles per locus ranged
369 between 3.2 (Morro do Diabo State Park) and 6.0 (Green Corridor region). In much the
370 same way, allelic richness, calculated using minimum sample size of eight diploid
371 individuals, was lowest in Morro do Diabo State Park (3.2) and highest in Green Corridor
372 region (5.05). Unique alleles could be observed in all populations (Table 3 and Fig. 2)
373 except Morro do Diabo State Park, and the largest fragment (Green Corridor) had the
374 largest number of unique alleles (18), followed by the Porto Primavera region (3) and
375 Ivinhema State Park (2).
376
377 *Population structure*
378 In the genetic clustering analysis computed with STRUCTURE, the lowest
379 likelihood value of the data was observed with K = 1 [Ln P (D) = -2318.18], indicating the
380 presence of population subdivision in the UPAF (Fig. 3). Two modes were observed, one
381 at K = 4 [Ln P (D) = -2106.02] and another at K = 6 [Ln P (D) = -2075.63]. At K = 4,

382 genetic clusters corresponded to the four pre-defined geographic groups (Cluster 1 = Morro
383 do Diabo State Park; Cluster 2 = Ivinhema State Park; Cluster 3 = Porto Primavera Region;
384 Cluster 4 = Green Corridor Region). We observed that 75% of the individuals sampled at a
385 given locale were assigned to the corresponding genetic cluster.

386 At $K = 6$, genetic clusters also corresponded to the geographically-defined groups
387 and most of the individuals from a given site were also assigned to that particular cluster.
388 However, with $K = 6$, STRUCTURE partitioned samples from the Porto Primavera region
389 (cluster 3 at $K = 4$) into two different groups with a similar number of individuals (cluster
390 3a, $n = 8$ [only Porto Primavera samples]; and cluster 3b, $n = 10$ [including also one
391 individual from Ivinhema and one from the Green Corridor]). Additionally, this analysis
392 also divided samples from the Green Corridor (cluster 4 at $K = 4$) into two clusters (cluster
393 4a, $n = 14$ [including four jaguars from Porto Primavera]; and cluster 4b, $n = 5$ [only Green
394 Corridor samples]). There was no clear biological interpretation for these subdivisions
395 within the Porto Primavera and Green Corridor groups. The subdivisions did not
396 correspond to different geographic locales within each region, nor were individuals
397 grouped by time of collection. At the same time, different factors could lead to
398 overestimating *K*, such as genotyping errors, inbreeding or the presence of related
399 individuals in the sample (Pritchard & Wen 2004). In addition, the model of correlated
400 allele frequencies employed in the analysis is more likely to overestimate *K* than other
401 models. It was used in this study to realistically analyze the differentiation of closely
402 related populations (Pritchard & Wen 2004). To investigate whether the presence of related
403 individuals in our sample could underlie this result, we analyzed the data using the
404 KINSHIP 1.2 program (Goodnight *et al*. 1998). We examined the mean genetic relatedness
405 of pairs of individuals within each cluster from Porto Primavera and Green Corridor

406 regions, as well as that between the two clusters from each region. Within each of the two
407 Porto Primavera clusters (cluster 3a and 3b) the mean relatedness coefficient (*R*) was 0.21
408 and 0.18, respectively, while that between these clusters was 0.10. In addition, almost all
409 pairs of individuals from Porto Primavera showing $R > 0.25$ were grouped in the same
410 STRUCTURE cluster (22 of 24 pairs). Similarly, the cluster 4b from the Green Corridor
411 grouped individuals that were more closely related to each other (mean $R = 0.19$) than to
412 the remaining individuals in this area (the mean *R* between clusters 4a and 4b was only
413 0.08). Also, most pairs of individuals from the Green Corridor with an $R > 0.25$ belonged to
414 the same genetic cluster (14 of 19 pairs). Therefore, the number of clusters seems to have
415 been overestimated by STRUCTURE due to presence of related individuals in the
416 sampling of Porto Primavera and Green Corridor regions. Based in these results, along
417 with the recommendations by Pritchard & Wen (2004), we concluded that $K = 4$ seems to
418 best represent the genetic structure of jaguars in this region, largely corresponding to the
419 four pre-defined geographic groups that can be circumscribed in the UPAF.
420 In BAPS, clustering of individuals using non-spatial information provided the
421 highest posterior probability for $K = 5$ [mean log (marginal likelihood) of optimal partition
422 = -2259.8932], considering the minimum threshold of three individuals per cluster
423 suggested by Corander & Marttinen (2006) and Latch *et al.* (2006). The analysis
424 partitioned individuals in four clusters that corresponded to the geographic groups (Cluster
425 1 = Morro do Diabo State Park; Cluster 2 = Ivinhema State Park; Cluster 3 = Porto
426 Primavera; Cluster 4 = Green Corridor Region) and the majority of the individuals (88%)
427 was assigned to their sampling population. However, as observed in STRUCTURE, the
428 BAPS analysis also divided the Green Corridor into two different clusters ($n = 11$ [similar
429 to cluster 4a in STRUCTURE]; and $n = 5$ [similar to cluster 4b in STRUCTURE).

Particularly, in both programs, samples grouped in the smaller cluster formed in the Green Corridor (cluster 4b in STRUCTURE) had a considerable amount of missing data (15-23%) which may have affected the analysis.

Clustering of individuals using spatial information in BAPS determined four clusters as the best partition of the samples [mean log (marginal likelihood) of optimal partition = -2330.3512]. In this analysis, BAPS formed practically the same groups as in the analysis without spatial information, also dividing the Green Corridor into two clusters, but grouping samples from Porto Primavera and Ivinhema in the same cluster (Cluster 1 = Morro do Diabo State Park; Cluster 2 = Ivinhema + Porto Primavera; Cluster 3 = 11 samples from the Green Corridor; Cluster 4 = five other samples from the Green Corridor).

At the same time, we also performed spatial and non-spatial clustering of groups of individuals in BAPS (groups utilized were the four pre-defined geographical populations). Both analyses indicated that the optimal number of partitions among groups was $K = 3$ [mean log (marginal likelihood) of optimal partition = -2320.3176 and -2318.5258 for spatial and non-spatial clustering, respectively]. The best partition of the groups separated the Green Corridor and Morro do Diabo State Park as isolated clusters, and joined the samples from Porto Primavera and Ivinhema in another isolated group.

Using the GENECLASS assignment/exclusion test, 43/59 (72.9%) of the jaguars were assigned with the highest probability to the location at which they had been sampled. No individual was excluded from all populations, indicating that all potential source populations had been sampled. This result suggests that individuals forming separate clusters in some of the STRUCTURE or BAPS analyses are likely not derived from unsampled populations. Almost all the individuals that were ‘misassigned’ with respect to their sampling locale were the same that had been assigned to a different genetic cluster in

454 the STRUCTURE and/or BAPS analyses. Finally, it is noteworthy that four of the 16
455 misassignments in GENECLASS were individuals from Ivinhema State Park assigned to
456 Porto Primavera region, since both localities were grouped together by most of the BAPS
457 analyses, and exhibited a low pairwise F_{ST} value (see below).
458 An initial analysis of pairwise F_{ST} and R_{ST} comparing samples collected at different
459 time periods supported the temporal stability of allele frequencies in both the Green
460 Corridor (F_{ST} = 0.025; P = 0.067) and Porto Primavera (F_{ST} = -0.005; P = 0.296) indicating
461 that it was valid to pool our full data set for each locality. The F_{ST} among all locations was
462 0.089 (P = 0.000) and the R_{ST} was 0.075 (P = 0.003) indicating the existence of significant
463 differences among the populations. Pairwise F_{ST} values were significant for all
464 comparisons (Table 4). The highest differentiation was between the Green Corridor and
465 Morro do Diabo State Park (F_{ST} = 0.198; P = 0.000) and the lowest was between Porto
466 Primavera and Green Corridor regions (F_{ST} = 0.048; P = 0.000). On the other hand, R_{ST}
467 values were significant only when populations were compared with Green Corridor region
468 (Table 4). The highest differentiation obtained was also between Green Corridor and
469 Morro do Diabo State Park (R_{ST} = 0.112; P =0.001). Differentiation between the Green
470 Corridor and Porto Primavera regions was low but significant (R_{ST} = 0.036; P = 0.030).
471 The association between populational geographic distance (measured in kilometers) and
472 F_{ST} values was non-significant (P = 0.77; Fig. 4).
473
474 *Identification of migrants and admixed individuals in the UPAF populations*
475 To better investigate the genetic composition of our data set and to detect migrants
476 as well as admixed individuals, we performed a second set of analyses with STRUCTURE
477 incorporating geographic sampling as prior population information. We considered the

four geographic groups as separate populations and observed that 88% (52/59) of the individuals had high probability of being residents, with $q > 0.8$ for the locality in which they had been sampled (Table 5; Fig. 5). The analysis identified seven individuals (bPon21, bPon25, bPon31, bPon35, bPon47, bPon132 and bPon359; Tables 5 and 6) as potential migrants or bearers of admixed ancestry ($q< 0.8$ for the sampling locale). These samples were assigned to a different cluster relative to their sampling site by one or more of the assignment tests (STRUCTURE, BAPS, or GENECLASS). Two of those individuals (bPon21 and bPon35) were strongly assigned ($q > 0.9$) to a different cluster, and were thus considered to be migrants (Tables 5 and 6). The sample bPon21, from Porto Primavera, was estimated to have a 98.4% probability of belonging to Morro do Diabo State Park. Likewise, the individual bPon35, captured in Ivinhema State Park, had a 90.6% probability of originating in the Porto Primavera region. Both results imply some level of recent gene flow between Porto Primavera and the nearby populations of Morro do Diabo State Park and Ivinhema State Park. All other individuals (bPon25, bPon31, bPon47, bPon132 and bPon359; Tables 5 and 6) had q-values ranging from 0.4 to 0.8, and were defined as potentially admixed, because they could not be classified as migrants, but not clearly assigned as residents either. In particular, the sample bPon47, collected in Ivinhema State Park, had a 75.3% probability of belonging to Morro do Diabo State Park (Tables 5 and 6). In the GENECLASS analysis designed to detect first-generation migrants (below), bPon47 showed the highest probability of belonging to Morro do Diabo State Park, but could not be considered a migrant because it failed to reach the established threshold ($P = 0.028$; Table 6). Overall, these results suggest that this individual should be considered to be a product of admixture between Morro do Diabo and Ivinhema State Parks.

In the analysis aimed at detecting first-generation migrants, GENECLASS identified five individuals with a probability below the threshold (bPon02; bPon21; bPon31; bPon35; bPon136; $P < 0.01$; Table 6). In the same way as STRUCTURE, GENECLASS strongly assigned bPon21 and bPon35 to Morro do Diabo State Park and Porto Primavera region, respectively. The sample bPon31 classified as migrant by GENECLASS was considered an admixed individual in the STRUCTURE analysis (q = 0.698). The other two individuals, bPon02 and bPon136, from the Green Corridor, were assigned to Porto Primavera, but showed similar probabilities for both localities. In STRUCTURE, both individuals were not considered migrants or admixed (q = 0.85 and q = 0.90 for the Green Corridor, respectively). In addition to STRUCTURE, the GENECLASS assignment/exclusion test also assigned bPon02 and bPon136 to the Green Corridor region. Therefore, GENECLASS could not accurately assign bPon02 and bPon136, probably due to the low F_{ST} value between the two areas (F_{ST} = 0.048). Empirical and simulated data sets have suggested that the GENECLASS assignment test is less accurate than the STRUCTURE assignment test, especially when F_{ST} is low (F_{ST} <0.15-0.2; Manel *et al.* 2002). We thus used a conservative approach and classified only bPon21 and bPon35 as migrants (Table 6).

The BAYESASS analysis showed that the current migration rate between the Green Corridor and all other populations in the UPAF is very low (m = 0.01 - 0.02; Table 7). Markedly low migration was also suggested from Porto Primavera to Ivinhema State Park (m = 0.04) and in the opposite direction it was even lower (m = 0.01). High migration rate was only estimated to occur from Morro do Diabo State Park to Porto Primavera (m = 0.28) and to Ivinhema State Park (m = 0.21). Migration in the opposite direction, from these two populations to Morro do Diabo State Park, was considerably low (m = 0.01).

525

526 *Effective population size*

527 The two methods used to determine N_e provided rather congruent estimates (Table
528 8). The results obtained with ONESAMP were robust to changes in the prior (data not
529 shown). At the same time, separate estimates using different criteria for excluding rare
530 alleles, which may overestimate N_e values in LDNE (Waples & Do 2008), also produced
531 similar results (data not shown). The highest value of N_e was obtained for the Green
532 Corridor region utilizing both LDNE (N_e = 51.4) and ONESAMP (N_e = 30.3), with the two
533 methods yielding broadly overlapping confidence limits (Table 8). The lowest effective
534 size was estimated for Morro do Diabo State Park (N_e = 7.8 with ONESAMP and 4.6 with
535 LDNE). Estimates for Ivinhema State Park and Porto Primavera yielded intermediate
536 values. We observed that the N_e estimates produced with ONESAMP were affected by
537 sample size, showing a strong correlation with it. For example, ONESAMP estimated an
538 *Ne* of 21.7 individuals for Porto Primavera region (n = 23); but this value decreased to only
539 11.2 when the sample was reduced twofold (n = 11). Likewise, when 10 individuals were
540 analysed for Ivinhema State Park the estimated N_e was 10.3, while an *Ne* of 5.6 was
541 estimated utilizing a sample of 6 individuals.

542

543 **Discussion**

544 G*enetic variability*

545 The overall genetic variation of jaguars in the UPAF is still high (H_E = 0.731 and a
546 mean of 7.23 alleles per locus) and comparable to that estimated for the species throughout
547 its geographic distribution (H_E = 0.739 and a mean of 8.31 alleles per locus; Eizirik *et al*.
548 2001). But the genetic diversity found here was somewhat lower than that obtained by

549 Ruiz-Garcia *et al.* (2006) using mainly samples from Colombia, with some individuals
550 from Guatemala, Paraguay, Peru, Bolivia, Venezuela, and Brazil (H_E = 0.846 and a mean
551 of 11.33 alleles per locus). However, no strict comparison among these studies should be
552 attempted given that they employed different markers (no locus is shared by all three).
553 Even though the genetic variation observed in the UPAF jaguar populations may be
554 considered to be high, our results indicate that a relevant portion of this diversity has been
555 locally lost and is now spatially subdivided. Indices of genetic diversity and private alleles
556 are lower in the populations with a smaller population size (Morro do Diabo and Ivinhema
557 State Parks). Particularly, Morro do Diabo State Park exhibited reduced genetic variation
558 relative to the other locations. This population was the only one that showed an allele fixed
559 for a microsatellite locus. So far, Morro do Diabo State Park has the lowest level of genetic
560 diversity reported for the species (Eizirik *et al*. 2001; Ruiz-Garcia *et al.* 2006). At the same
561 time, although the Green Corridor, Porto Primavera, and Ivinhema State Park exhibited
562 high to moderate levels of expected heterozygosity, this diversity is likely lower than what
563 was present in the original population (as can be inferred if we assume that the pool of
564 local populations harbors the original allelic diversity in the UPAF).
565 The presence of private alleles in almost all populations, except in Morro do Diabo
566 State Park, suggests that gene flow has been reduced among them and that genetic drift has
567 caused the loss of alleles within each population. As expected in the presence of recent
568 historical gene flow among these populations, we can observe that alleles that have been
569 lost within each population (e.g. those of intermediate size within the sampled range) are
570 present in another (Table 3 and Fig. 2). Moreover, the Green Corridor and Porto Primavera
571 regions are those that shared the greatest number of alleles, even though they are more
572 distant and geographically isolated (*ca*. 500 km) than the other pairs. This could be

573 explained by the fact that both populations are considerably larger than the others, thus
574 retaining greater allelic diversity in the face of fragmentation.
575
576 *Population structure*
577 Our results clearly indicated that jaguars in the UPAF are currently not a panmictic
578 population. Non-spatial analyses in BAPS and STRUCTURE indicated that the four
579 geographic groups are differentiated and form distinct genetic clusters. However, BAPS
580 spatial and group-based analyses suggested that Porto Primavera and Ivinhema State Park
581 might be considered a single population. A recent debate has addressed the issue of
582 whether clusters identified by non-spatial Bayesian algorithms were artificially defined due
583 to uneven sampling along clines, or were in fact real genetic units (Serre & Pääbo 2004;
584 François *et al*. 2006; Corander *et al.* 2008). However, studies that assess the performance
585 of spatial algorithms and compare them to non-spatial approaches are scarce. Chen *et al.*
586 (2007) compared the STRUCTURE (non-spatial) results and those of three spatially-
587 oriented clustering programs, verifying that STRUCTURE performs very well even along
588 a cline of variation, countering previous claims (Serre & Pääbo 2004). Field data indicate
589 that there was likely demographic continuity between the Porto Primavera and Ivinhema
590 State Park populations until recently, which was almost certainly interrupted by the
591 flooding of Porto Primavera hydroelectric dam in 1998 (D.A. Sana, pers. obs.). The
592 flooding itself may have induced the movement of individuals downstream to the Ivinhema
593 region, increasing the genetic contribution of Porto Primavera animals in this latter area.
594 However, after the flooding, the Porto Primavera population has essentially gone extinct,
595 with few animals sighted in the area in the last few years (D. A. Sana pers. obs.), making it
596 very unlikely that any current gene flow with Ivinhema remains.

In agreement with the clustering analyses, traditional F_{ST} assessments detected population structure in the UPAF. All F_{ST} values were significant among all populations. However, R_{ST} values were lower than F_{ST}'s, suggesting that genetic drift has been more important than mutation in creating differences between these populations. R_{ST} values were significant only when other populations were compared to the Green Corridor region. This population is located further south in this ecoregion (*ca*. 380 km from the nearest population, Ivinhema State Park) and probably was the first to be partially or completely isolated from the others. This separation process might have been ongoing for decades in the second half of the 20th century due to agricultural changes to the landscape, but may have become complete due to the flooding of the huge Itaipu Binacional hydroelectric dam in 1982, which submerged thousands of hectares of riverine habitat, likely severing the connectivity between the northern and southern UPAF ecoregion.

The observed genetic differentiation among populations (F_{ST} values) was remarkably high given the geographical proximity of the areas (their pairwise distances ranging from *ca.* 69 km to 500 km), the ability of this species to disperse over broad areas (Oliveira 1994), as well as the short time frame (30-40 years, *ca*. 6-8 generations) in which these populations have been isolated or semi-isolated (Di Bitetti *et al*. 2003; Galindo-Leal & Câmara 2003; C. De Angelo *et al.* 2009, in preparation). These levels of genetic differentiation imply a strong impact of local genetic drift, indicating that the effective population sizes are very small in each fragment, and that current gene flow among them is likely very low. The highest F_{ST} values were observed between the most distant population (Green Corridor) and the smallest fragments (Morro do Diabo and Ivinhema State Park). At the same time, high levels of differentiation were observed between Morro do Diabo and Ivinhema State Parks, while both of these populations showed lower differentiation

relative to Porto Primavera. These results are not surprising given the field-based knowledge on the current and historical landscape connectivity between these areas, and jaguar dispersal patterns in this region (D.A. Sana, pers. obs.). However, the Green Corridor and Porto Primavera populations, which are quite distant from one another (*ca*. 500 km), exhibited the lowest F_{ST}. Probably, these populations exhibited low differentiation not due to recent gene flow between them, but because they have lost fewer alleles via genetic drift (given their larger *Ne*) during this ongoing fragmentation process. In addition, the relationship between geographic and genetic (F_{ST}) distances among populations was not significant, suggesting that the observed subdivision could not be explained by geographical distances between populations only, but that genetic drift may be the primary force affecting differentiation among them.

Identification of migrants and admixed individuals among UPAF populations

Assignment tests indicated that most jaguars had originated from the populations where they had been sampled, so that migration among populations is low, according to the observation of high differentiation among areas. At the same time, extensive radio-telemetry data indicated that dispersal between sampling sites was not observed by any of the GPS collared individuals. Although some adult jaguars show long distance movements within their home range, of up to 30 km in 3-4 days through the fragmented landscape, they significantly avoided the use of areas modified by agriculture, pasture and human settlement (Cullen 2007).

Although ongoing field work has not revealed any migrants among these populations, our genetic analyses did identify some individuals that indicate recent connectivity among them. Using conservative criteria, two individuals were inferred to be

645 migrants, both of which were young/adult females (3-4 years old). One individual from
646 Porto Primavera (bPon21), captured in 1998 during the wildlife rescue before the filling of
647 the Porto Primavera dam, was identified as a migrant from Morro do Diabo State Park.
648 Demographic connectivity between the two areas was likely widespread in previous times
649 and this animal in particular was captured at a site that was close (albeit on the opposite
650 side of the Paraná river) to the northern end of Morro do Diabo State Park. The other
651 migrant was captured in Ivinhema State Park in 2004, but was genetically assigned to the
652 Porto Primavera population. This female may have dispersed southward due to the loss of
653 habitat that took place in the Porto Primavera region after the flooding of its reservoir, so
654 that it would have left the area and eventually established a home range in Ivinhema.
655 Another jaguar sampled in Ivinhema (bPon47) in 2002 showed more probability of
656 belonging to Morro do Diabo State Park, and was ultimately inferred to be of admixed
657 ancestry, supporting the conclusion that there was recent gene flow between the two
658 populations. In addition, an adult male (bPon24) captured in 1999 in the municipality of
659 Alto Paraná (PR state, see Fig. 1) was translocated to Morro do Diabo State Park and a few
660 days later returned to an area near the capture site. Our genetic assignment indicated that
661 this individual originated in the Porto Primavera region. Although it is difficult to know the
662 exact travel route of this individual, we may conclude that it was able to cross long
663 distances across severely disturbed areas. It is possible that this animal left the Porto
664 Primavera region due to the filling of the reservoir in 1998 and wandered through the
665 landscape without reaching any suitable habitat fragment (L.Cullen Jr., pers. obs.).
666 The presence of admixed individuals in these areas suggests that jaguars have been
667 able to move across the landscape and reproduce in their new area, at least in the recent
668 past. Dispersal with subsequent reproduction is vital for the long-term viability of small

669 fragmented populations (Lacy 1997). However, these episodes of inferred gene flow seem
670 to have been insufficient to avoid differentiation among areas due to intense genetic drift,
671 and their frequency has likely decreased in recent years due to increasing isolation of
672 fragments and extermination of jaguars remaining in intervening forest patches. It has been
673 proposed that one successful migrant per generation (OMPG) would be sufficient to
674 prevent population differentiation due to genetic drift (Wright 1931; Ohta & Kimura 1973;
675 Franklin 1980). Nevertheless, more recent studies have suggested that 1-10 migrants per
676 generation may be necessary (Millis & Allendorf 1996) or even more than 10 migrants
677 may be required (Vucetich & Waite 2000).
678 In stark contrast to this requirement, Bayesian estimation of migration rates
679 indicated quite low ($m \leq 0.04$) migration among the majority of the sampled locations. An
680 extremely low migration rate ($m \leq 0.02$) was estimated between the Green Corridor region
681 and those of the other populations, suggesting (along with the assignment tests and *F*-
682 statistics) isolation between the northern and southern sectors of the UPAF ecoregion.
683 Additionally, Porto Primavera and Ivinhema exhibited a very low migration rate ($m \leq 0.04$)
684 whereas the F_{ST} value (0.060) indicated moderate gene flow between them and the BAPS
685 spatial clustering analysis considered them to be a single population.
686 Two cases with relatively high proportions of migrants between sampling locations
687 (as assessed by BAYESASS) were observed. This flux appeared to be asymmetric with
688 jaguars from Morro do Diabo State Park migrating into the Porto Primavera region (m =
689 0.28) and Ivinhema State Park (m = 0.21), but with essentially no movement (m = 0.01) in
690 the opposite direction. The assignment test detected just one migrant between the two
691 populations in the first case, as well as one admixture individual between populations in
692 the second case. The estimated high rates may not reflect current movement of jaguars

693 between these areas, but movement of animals some generations ago, when connectivity is
694 known to have been higher (Wilson & Rannala 2003). Care should also be taken with these
695 estimates due to the relatively small number of individuals sampled in Ivinhema and Morro
696 do Diabo State Park (n = 10 and 8, respectively) (Faubet *et al*. 2007). Consistent results
697 could not be achieved in other studies using samples of less than 20 individuals (Hansen *et*
698 *al*. 2007; Schmidt *et al.* 2008).

699 N_e estimates based on a single sample have been problematic because methods
700 based on linkage disequilibrium (LD) and heterozygote excess have proven to be imprecise
701 or biased (Waples 1991; England *et al*. 2006; Tallmon *et al*. 2008). However, we used a
702 new program (LDNE) that employs a bias correction developed by Waples (2006) for
703 estimates of N_e based on the LD method. This author concluded that this new approach
704 could provide unbiased estimates of N_e under a wide range of sample sizes. We also used
705 another new program (ONESAMP) which employs summary statistics and approximate
706 Bayesian computation to estimate N_e. In our analyses, ONESAMP appeared to be biased
707 by the sample size, as the resulting N_e estimate was correlated with it, a restriction already
708 pointed out by Sotelo *et al.* (2008). Conversely, robust results were obtained by LDNE
709 with different sample sizes. At any rate, our results indicated a very low effective size for
710 all populations. This inference is plausible and biologically realistic given their very small
711 estimated census sizes. LDNE effective sizes ranged from 51.4 in the Green Corridor to
712 4.6 in the Morro do Diabo State Park. Thus, the minimum of 50 effective breeders that has
713 been suggested as needed to prevent inbreeding depression in the short term (Franklin
714 1980) was only reached by the Green Corridor population. The target effective population
715 sizes of 500 - 5000 recommended for securing long-term viability (Franklin 1980; Lande
716 1995; Franklin & Frankham 1998) is of course several times larger than those observed. In

717 particular, the Morro do Diabo population exhibited extremely small effective size and
718 results indicated low genetic variability. Thus, Morro do Diabo State Park may be
719 seriously compromised if management measures are not taken in the short term.
720
721 *Conclusions*
722 Our results, in combination with field data and satellite image analyses, indicate
723 that loss and fragmentation of once contiguous habitat has caused the reduction of genetic
724 diversity in the UPAF jaguar populations, as well as drift-induced differentiation among
725 local fragments. It is therefore important, in the context of avoiding negative demographic
726 and genetic consequences of small population size, as well as to ensure the long-term
727 viability of these groups, or restore gene flow among the analyzed areas.
728 Ecological data indicated that jaguar persistence can be achieved in this ecoregion
729 if these populations are managed as a metapopulation (Cullen 2007). Our genetic data
730 strongly support this view, and the restoration of the connectivity between these jaguar
731 populations should be viewed as a management priority. Habitat selection analyses have
732 indicated that jaguars in this region (where most of the original forest cover has already
733 been lost) exhibit a preference for riverine marshes (Cullen 2007). These are currently the
734 only types of habitat that potentially connect the remaining protected areas along the
735 Paraná River basin, and can serve as corridors or stepping-stones facilitating natural
736 dispersal and allowing genetic exchange among populations (Cullen 2007). Forests have
737 also been restored on the edges of the Itaipu Binacional dam, an action that may have
738 positive effects in the future, connecting populations located in the northern and southern
739 sectors of the UPAF ecoregion (L. Cullen Jr., C. De Angelo, pers. obs.). Additionally,
740 direct intervention via translocations or assisted reproduction could be considered as

741 additional management strategies for jaguars in the region. Techniques of assisted
742 reproduction have been recognized as important tools in the genetic management of this
743 species (Morato & Barnabe 2002). *In vivo* (captive individuals) and *in vitro* (semen and
744 embryos) materials from jaguars of this ecoregion are available for this type of
745 methodology, and may be considered as a promising alternative for the future, in case
746 habitat-oriented measures fail to achieve the targeted conservation goals in the short term.
747 Overall, effective dispersal of jaguars through of this human-dominated landscape,
748 ultimately resulting in an increased probability of its persistence in the region, will only be
749 successful with the mitigation of the present threats (Cullen 2007) and will require a
750 comprehensive and effective integration of efforts from multiple disciplines.

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955 **Figure Legends**

956

957 **Fig. 1** Detailed map of the Upper Paraná Atlantic Forest representing the studied
958 populations. Each circle represents the geographic origin of one or more samples (numbers
959 indicated in Table 1). The smallest map shows the historical and current distribution of
960 jaguar (modified from Sanderson *et al.* 2002 and Zeller 2007) and the UPAF ecoregion.

961

962 **Fig. 2** Histograms showing the distribution of the allele frequencies in "Green Corridor"
963 Region (white bars), Morro do Diabo State Park (light grey bars), Ivinhema State Park
964 (dark grey bars) and Porto Primavera Region (black bars). Each graph depicts alleles from
965 a single microsatellite locus, identified at the top.

966

967 **Fig. 3** Results of Bayesian clustering analysis performed in STRUCTURE. For each
968 number of population clusters tested (*K*), Ln P (D) is the estimated mean of the log
969 probability of the data among different runs for each *K*.

970

971 **Fig. 4** Pairwise population comparison of genetic (F_{ST}) and geographical distance (in
972 kilometers) analysed with GENEPOP. Population names are abbreviated as GC = Green
973 Corridor, MD = Morro do Diabo, IV = Ivinhema and PP = Porto Primavera.

974

975 **Fig. 5** Proportional membership (*q*) of each jaguar in the four populations inferred by
976 STRUCTURE utilizing prior population information. Each individual is represented by a
977 vertical bar, and the length of each bar indicates the probability of membership in each
978 cluster ["Green Corridor" Region (red); Morro do Diabo State Park (green); Ivinhema

979 State Park (blue); Porto Primavera Region (yellow)]. Numbers below the horizontal axis
980 are sample identification (number after “bPon” in Table 1) of *Panthera onca* individuals in
981 each area. Locality of origin is indicated in parentheses: (1) “Green Corridor” Region; (2)
982 Morro do Diabo State Park; (3) Ivinhema Sate Park; (4) Porto Primavera Region.

Table 1. Samples of jaguars analyzed in the present study. Numbers in parentheses next to the geographic origin represent each sample locality in Fig. 1.

Population Name[1]	**Individual**	**Sample**	**Sex**	**Geographic origin**	**Coordinated geographical**	**Contact**
"Green Corridor" Trinational Region (Brazil, Argentina, Paraguay)	bPon-01	Blood	M	P. N. do Iguaçu - PR state (12)	25°37'40"S; 54°27'46"W	Proj. Carnívoros; CENAP/ICMBio
	bPon-02	Blood	M	P. N. do Iguaçu - PR state (12)	25°37'40"S; 54°27'46"W	Proj. Carnívoros; CENAP/ICMBio
	bPon-04	Tissue	?	P. E. do Turvo - RS state (21)	27°12'02"S; 53°53'58"W	Proj. Felinos (RS)
	bPon-12	Blood Faeces[2]	M	P. N. Iguazú - Misiones Prov. (13)	25°43'33"S; 54°28'21"W	Proj. Carnívoros; CENAP/ICMBio; Proj. Yaguareté, CeIBA; Zoo Sáenz
	bPon-91	Faeces[3]	F	Vicinity of P.N. do Iguaçu - PR state	?	Parque Municipal "Danilo Galafassi"; L. E. S. Delgado
	bPon-124	Serum	F	P. N. do Iguaçu - PR state (12)	25°36'37"S; 54°25'50"W	CENAP/ICMBio; I. Pró-Carnívoros
	bPon-133	Hair	M	Puerto Libertad - Misiones Prov. (16)	25°55'21"S; 54°34'47"W	Proj.Yaguareté; CeIBA
	bPon-134	Pelt	F	Misiones-Alto Paraná; Dep. Argentina-Paraguay border (15)	25°49'57"S; 54°49'40"W	Proj. Yaguareté; CeIBA
	bPon-135	Blood	F	Ruiz Montoya- Misiones Prov. (19)	26°59'05"S; 54°56'09"W	Proj. Yaguareté; CeIBA
	bPon-136	Hair	?	Colonia La Flor - Misiones Prov. (20)	26°59'42"S; 54°07'34"W	Proj. Yaguareté; CeIBA
	bPon-137	Pelt	?	San Vicente - Misiones Prov. (18)	26°51'38"S; 54°32'58"W	Proj. Yaguareté; CeIBA
	bPon-138	Faeces[4]	?	P. P. Uruguaí - Misiones Prov. (14)	25°54'21"S; 54°13'32"W	Proj. Yaguareté; CeIBA
	bPon-139	Faeces[4]	?	P. P. Uruguaí - Misiones Prov. (14)	25°49'19"S; 54°07'13"W	Proj. Yaguareté; CeIBA
	bPon-140	Two faeces[5]	?	P. P. Uruguaí - Misiones Prov. (14)	25°54'32"S; 54°15'06"W	Proj. Yaguareté; CeIBA
	bPon-141	Faeces[3]	M	Vicinity of Montecarlo- Misiones Prov. (17)	26°39'19"S; 54°34'49"W	Proj. Yaguareté; CeiBA; Zoo Sr. Waidelich
	bPon-142	Faeces[4]	?	P. N. do Iguaçú - PR state (11)	25°07'34"S; 53°38'50"W	P. N. do Iguaçú; M. X. Silva; A. Rodrigues
	bPon-143	Faeces[4]	?	P. N. do Iguaçú - PR state (12)	25°39'21"S; 54°26'45"W	P. N. do Iguaçú; M. X. Silva; A. Rodrigues
	bPon-366	Serum	M	P. N. Iguazú - Misiones Prov. (13)	25°44'49"S; 54°25'36"W	Proj. Carnívoros; CENAP/ICMBio
Morro do Diabo State Park (Brazil)	bPon-25	Blood	F	P. E. Morro do Diabo - SP state (7)	22°33'07"S; 52°20'26"W	I. Pesquisas Ecológicas

Table 1. Continued.

Population Name[1]	Individual	Sample	Sex	Geographic origin	Coordinated geographical	Contact
Morro do Diabo State Park (Brazil)	bPon-48	Blood	M	P. E. Morro do Diabo - SP state (7)	22°37'40"S; 52°10'04"W	I. Pesquisas Ecológicas
	bPon-49	Blood	M	P. E. Morro do Diabo - SP state (7)	22°37'40"S; 52°10'04"W	I. Pesquisas Ecológicas
	bPon-50	Blood	F	P. E. Morro do Diabo - SP state (7)	22°35'37"S; 52°15'59"W	I. Pesquisas Ecológicas
	bPon-51	Blood	F	P. E. Morro do Diabo - SP state (7)	22°37'40"S; 52°10'04"W	I. Pesquisas Ecológicas
	bPon-52	Blood	F	P. E. Morro do Diabo - SP state (7)	22°35'37"S; 52°15'59"W	I. Pesquisas Ecológicas
	bPon-53	Blood	F	P. E. Morro do Diabo - SP state (7)	22°37'57"S; 52°16'06"W	I. Pesquisas Ecológicas
	bPon-54	Blood	F	P. E. Morro do Diabo - SP state (7)	22°37'57"S; 52°16'06"W	I. Pesquisas Ecológicas
Ivinhema State Park (Brazil)	bPon-35	Blood	F	P. E. V.Rio Ivinhema - MS state (9)	22°51'33"S; 53°38'00"W	CENAP/ICMBio; I. Pró-Carnívoros
	bPon-36	Muscle	?	Taquarussu - MS state (8)	22°44'41"S; 53°28'57"W	CENAP/ICMBio; I. Pró-Carnívoros
	bPon-40	Blood	M	P. E.V. Rio Ivinhema - MS state (9)	22°47'27"S; 53°40'20"W	CENAP/ICMBio; I. Pró-Carnívoros
	bPon-41	Blood	F	P. E.V. Rio Ivinhema - MS state (9)	22°47'27"S; 53°40'20"W	CENAP/ICMBio; I. Pró-Carnívoros
	bPon-44	Faeces[4]	?	P. E.V. Rio Ivinhema - MS state (9)	22°56'15"S; 53°41'08"W	CENAP/ICMBio; I. Pró-Carnívoros
	bPon-46	Blood	F	P. E.V. Rio Ivinhema - MS state (9)	22°52'43"S; 53°39'20"W	CENAP/ICMBio; I. Pró-Carnívoros
	bPon-47	Blood	M	P. E.V. Rio Ivinhema - MS state (9)	22°53'34"S; 53°44'00"W	CENAP/ICMBio; I. Pró-Carnívoros
	bPon-78	Blood	F	P. E.V. Rio Ivinhema - MS state (9)	22°46'26"S; 53°39'59"W	CENAP/ICMBio; I. Pró-Carnívoros
	bPon-80	Faeces[4]	?	P. E.V. Rio Ivinhema - MS state (9)	22°47'10"S; 53°40'02"W	CENAP/ICMBio; I. Pró-Carnívoros
	bPon-359	Blood	M	Taquarussu - MS state (8)	22°43'36"S; 53°31'25"W	CRAS; CENAP/ICMBio; I. Pró-Carnívoros
Porto Primavera Region (Brazil)	bPon-03	Blood	F	Bataguassu - MS state (3)	21°59'10"S; 52°22'37"W	Proj. Porto Primavera; CENAP/ICMBio
	bPon-11	Blood	F	Anaurilândia - MS state (6)	22°23'38"S; 52°56'42"W	Proj. Porto Primavera; CENAP/ICMBio
	bPon-15	Blood Faeces[2]	F	Anaurilândia - MS state (5)	22°14'31"S; 52°45'14"W	CENAP/ICMBio; I. Pró-Carnívoros; Ilha Solteira Zoo; V. Queirós
	bPon-16	Blood	M	Bataguassu - MS state (3)	21°57'48"S; 52°28'55"W	CENAP/ICMBio; I. Pró-Carnívoros
	bPon-17	Blood	M	Anaurilândia - MS state (5)	22°06'47"S; 52°38'34"W	CENAP/ICMBio; I. Pró-Carnívoros
	bPon-18	Blood Faeces[2]	M	Marabá Paulista - SP state (4)	22°05'14"S; 52°07'08"W	CENAP/ICMBio; I. Pró-Carnívoros; Ilha Solteira Zoo; V. Queirós
	bPon-19	Blood	F	Marabá Paulista - SP state (4)	22°05'14"S; 52°07'08"W	CENAP/ICMBio; I. Pró-Carnívoros
	bPon-20	Blood	F	Anaurilândia - MS state (5)	22°04'11"S; 52°24'51"W	CENAP/ICMBio; I. Pró-Carnívoros
	bPon-21	Blood	F	Anaurilândia - MS state (5)	22°06'47"S; 52°38'34"W	CENAP/ICMBio; I. Pró-Carnívoros
	bPon-22	Blood	F	Anaurilândia - MS state (5)	22°04'11"S; 52°24'51"W	CENAP/ICMBio; I. Pró-Carnívoros
	bPon-23	Blood	M	Anaurilândia - MS state (5)	22°06'52"S; 53°01'22"W	CENAP/ICMBio; I. Pró-Carnívoros

Table 1. Continued.

Population Name[1]	Individual	Sample	Sex	Geographic origin	Coordinated geographical	Contact
	bPon-24	Blood[6]	M	Alto Paraná - PR state (10)	22°57'22"S; 52°53'02"W	I. Pesquisas Ecológicas
	bPon-27	Blood Faeces[2]	M	Anaurilândia - MS state (5)	22°05'14"S; 52°07'08"W	CENAP/ICMBio; I. Pró-Carnívoros; Ilha Solteira Zoo; V. Queirós
	bPon-29	Muscle	M	Anaurilândia - MS state (5)	22°05'41"S; 52°52'57"W	CENAP/ICMBio; Pró-Carnívoros
	bPon-30	Blood	F	Anaurilândia - MS state (5)	22°10'12"S; 52°38'23"W	CENAP/ICMBio; Pró-Carnívoros
	bPon-31	Blood	M	Anaurilândia - MS state (5)	22°07'17"S; 52°31'31"W	CENAP/ICMBio; Pró-Carnívoros
	bPon-32	Blood Faeces[2]	F	Anaurilândia - MS state (5)	22°05'14"S; 52°07'08"W	CENAP/ICMBio; I. Pró-Carnívoros; Ilha Solteira Zoo; V. Queirós
	bPon-38	Pelt	?	Nova Andradina - MS state (2)	21°53'20"S; 53°25'29"W	CENAP/ICMBio; Pró-Carnívoros
	bPon-45	Blood	F	Anaurilândia - MS state (5)	22°07'49"S; 52°34'03"W	CENAP/ICMBio; Pró-Carnívoros
	bPon-58	Pelt	?	Bataguassu - MS state (3)	21°59'10"S; 52°22'37"W	CENAP/ICMBio; Pró-Carnívoros
	bPon-130	Blood	M	Pereira Barreto - MS state (1)	20°38'20"S; 51°06'35"W	Proj. Porto Primavera; CENAP/ICMBio
	bPon-131	Blood	F	Bataguassu - MS state (3)	22°10'47"S; 52°36'59"W	Proj. Porto Primavera; CENAP/ICMBio
	bPon-132	Blood	F	Bataguassu - MS state (3)	21°59'17"S; 52°23'36"W	Proj. Porto Primavera; CENAP/ICMBio

[1]Including areas in the mosaic surrounding the current protected areas; [2]Blood and faecal samples from the same individual that produced identical genotypes; [3]Scats collected from captive animals whose geographic origin was known; [4]Scats collected in field and identified as from different individuals based on DNA; [5]Two scats collected in field that produced identical genotypes (same individual); [6]Analysis in STRUCTURE indicated this individual as belonging to Porto Primavera Region (q = 0.980).

Table 2. Measures of diversity at 13 microsatellite loci in the four jaguar populations of the UPAF investigated in this study. Sample size (*N*), observed number of alleles (*A*), allelic richness (*AR*), observed (*H*o) and expected (*H*e) heterozygosities.

	Green Corridor (*n*= 18)					**Morro do Diabo (*n*= 8)**					**Ivinhema (*n*= 10)**					**Porto Primavera (*n*= 23)**				
Locus	*N*	*A*	AR^1	H_o	H_e^2	*N*	*A*	AR^1	H_o	H_e^2	*N*	*A*	AR^1	H_o	H_e^2	*N*	*A*	AR^1	H_o	H_e^2
FCA742	15	8	6.97	0.733	0.831	8	4	4.00	0.625	0.589	10	8	7.31	0.800	0.844	23	10	7.64	0.957	0.881
FCA723	16	5	4.00	0.500	0.708	8	2	2.00	0.250	0.232	10	2	2.00	0.400	0.511	23	6	4.71	0.696	0.707
FCA740	17	5	4.32	0.765	0.730	8	3	3.00	0.250	0.348	9	3	2.99	0.667	0.514	23	4	3.81	0.913	0.696
FCA441	18	4	3.79	0.444	0.639	8	2	2.00	0.125	0.125	10	4	3.77	0.700	0.656	23	4	3.79	0.696	0.660
FCA391	16	7	5.98	0.750	0.808	8	4	4.00	0.750	0.723	10	5	4.74	0.800	0.728	23	6	4.93	0.826	0.728
F98	17	3	2.86	0.588	0.517	8	3	3.00	0.625	0.482	10	4	3.97	0.800	0.717	23	3	2.83	0.609	0.552
F53	16	9	6.95	0.688	0.856	8	4	4.00	1.000	0.732	10	7	6.53	0.900	0.839	23	8	5.73	0.913	0.798
F124	17	7	5.50	0.824	0.761	8	4	4.00	0.750	0.741	10	6	6.53	0.800	0.811	23	7	5.72	0.826	0.803
F146	18	4	3.66	0.444	0.629	8	3	3.00	0.500	0.509	10	3	2.99	0.800	0.639	23	4	3.34	0.739	0.658
F85	14	10	7.55	0.643	0.860	8	3	3.00	0.625	0.607	9	5	4.88	0.778	0.708	22	6	4.99	0.818	0.769
F42	17	8	6.38	0.647	0.838	8	5	5.00	0.875	0.714	10	3	2.97	0.600	0.478	23	6	4.42	0.696	0.725
FCA453	13	5	4.81	0.538	0.792	8	3	3.00	0.750	0.661	10	3	2.80	0.400	0.350	23	5	4.25	0.783	0.723
FCA741	15	3	2.99	0.333	0.610	8	1	1.00	—	0.000	10	3	2.80	0.400	0.550	23	3	2.90	0.696	0.602
Overall		6.0	5.05	0.607	0.737		3.2	3.2	0.548	0.497		4.3	4.2	0.680	0.642		5.5	4.54	0.782	0.716

[1]Allelic richness is calculated using minimum sample size of eight diploid individuals; [2] Unbiased gene diversity (corrected for sampling bias in FSTAT).

Table 3. Observed allele frequencies at each locus in each population. The number of individuals typed is shown in parentheses. Private alleles are double underlined.

		Green Corridor	Morro do Diabo	Ivinhema	Porto Primavera
Locus	**Allele**	**(15)**	**(8)**	**(10)**	**(23)**
FCA742	**142**	—	0.062	0.100	0.087
	146	—	—	0.050	0.130
	150	—	—	0.050	—
	152	0.067	—	—	0.022
	154	—	—	0.050	0.109
	156	0.100	—	—	—
	158	0.067	0.625	0.350	0.217
	160	0.366	—	0.100	0.087
	162	0.100	—	—	—
	164	—	—	0.100	0.109
	166	—	0.188	0.200	0.174
	170	0.166	—	—	0.022
	172	0.067	—	—	—
	188	0.067	0.125	—	0.043
Locus	**Allele**	**(16)**	**(8)**	**(10)**	**(23)**
FCA723	**220**	—	—	—	0.152
	232	0.281	—	—	0.022
	236	0.219	0.875	0.400	0.478
	240	0.438	0.125	0.600	0.217
	244	0.031	—	—	0.087
	256	0.031	—	—	0.044
Locus	**Allele**	**(17)**	**(8)**	**(9)**	**(23)**
FCA740	**300**	0.176	—	—	0.087
	304	0.088	0.812	0.667	0.413
	308	0.324	0.063	0.222	0.174
	312	0.382	0.125	0.111	0.326
	316	0.029	—	—	—
Locus	**Allele**	**(18)**	**(8)**	**(10)**	**(23)**
FCA441	**157**	0.139	—	0.100	0.109
	165	0.556	—	0.050	0.282
	169	0.222	0.938	0.400	0.500
	173	—	0.062	0.450	0.109
	177	0.083	—	—	—
Locus	**Allele**	**(16)**	**(8)**	**(10)**	**(23)**
FCA391	**215**	0.031	0.125	0.100	0.109
	219	0.125	—	—	—
	223	0.094	—	0.050	0.174
	227	—	0.312	—	0.022
	231	0.094	—	0.400	0.065
	235	0.344	0.438	0.350	0.457
	239	0.250	0.125	0.100	0.173
	243	0.062	—	—	—
Locus	**Allele**	**(17)**	**(8)**	**(10)**	**(23)**
F98	**189**	0.265	0.062	0.150	0.326
	192	0.647	0.688	0.350	0.587
	195	0.088	0.250	0.400	0.087
	198	—	—	0.100	—
Locus	**Allele**	**(16)**	**(8)**	**(10)**	**(23)**
F53	**160**	0.188	—	—	—

Table 3. Continued.

Locus	**Allele**	**(16)**	**(8)**	**(10)**	**(23)**
F53	**164**	0.031	—	—	0.022
	176	0.125	—	0.100	—
	180	0.031	—	0.050	0.065
	184	0.063	0.312	0.300	0.261
	188	0.281	0.062	0.250	0.239
	192	0.188	0.313	0.150	0.043
	196	0.031	—	0.050	0.065
	200	0.062	0.313	0.100	0.283
	204	—	—	—	0.022
Locus	**Allele**	**(17)**	**(8)**	**(10)**	**(23)**
F124	**199**	—	—	0.200	0.022
	203	0.235	0.375	0.150	0.326
	207	0.088	0.062	0.050	0.087
	211	0.147	0.250	0.200	0.174
	215	0.413	—	0.350	0.239
	219	0.029	0.313	0.050	0.087
	223	0.059	—	—	0.065
	227	0.029	—	—	—
Locus	**Allele**	**(18)**	**(8)**	**(10)**	**(23)**
F146	**170**	0.055	0.187	0.450	0.261
	173	0.556	0.688	0.400	0.478
	176	0.250	0.125	0.150	0.239
	179	—	—	—	0.022
	182	0.139	—	—	—
Locus	**Allele**	**(14)**	**(8)**	**(9)**	**(22)**
F85	**143**	0.036	—	—	—
	147	0.250	—	0.111	0.341
	151	0.071	0.562	0.500	0.295
	155	0.107	—	0.222	0.159
	159	0.036	0.125	—	—
	163	—	—	0.056	0.068
	175	0.036	—	—	—
	179	0.036	—	—	0.023
	183	0.286	—	—	—
	187	0.106	—	—	—
	211	0.036	—	—	—
	215	—	0.313	0.111	0.114
Locus	**Allele**	**(17)**	**(8)**	**(10)**	**(23)**
F42	**247**	0.059	—	—	—
	251	0.089	0.125	—	0.065
	255	0.029	0.063	0.100	0.217
	259	0.029	—	—	0.022
	263	0.088	—	—	—
	267	0.265	0.125	0.200	0.413
	271	0.176	0.500	0.700	0.261
	275	—	0.188	—	0.022
	279	0.265	—	—	—
Locus	**Allele**	**(13)**	**(8)**	**(10)**	**(23)**
FCA453	**192**	0.346	0.250	0.800	0.283
	196	0.077	0.500	0.050	0.130
	208	0.192	—	—	0.152
	212	0.269	—	—	0.022
	216	0.115	0.250	0.150	0.413

Table 3. Continued.

Locus	Allele	(15)	(8)	(10)	(23)
FCA741	**175**	0.267	—	0.600	0.456
	179	0.567	1.000	0.350	0.435
	183	0.167	—	0.050	0.109

Table 4. Pairwise F_{ST} (above the diagonal) and R_{ST} values (below the diagonal) for the four jaguar populations of the UPAF.

	Green Corridor	**Morro do Diabo**	**Ivinhema**	**Porto Primavera**
Green Corridor	—	0.198***	0.122***	0.048***
Morro do Diabo	0.112**	—	0.120***	0.073***
Ivinhema	0.081**	-0.010	—	0.060***
Porto Primavera	0.036*	-0.007	0.024	—

Significant values *P < 0.05, **P < 0.01 and ***P < 0.001.

Table 5. Population assignment and inferred ancestry of jaguar individuals using STRUCTURE analysis with geographical information (Pop 1 = Green Corridor; Pop 2 = Morro do Diabo; Pop 3 = Ivinhema; Pop 4 = Porto Primavera). The membership proportion for each individual is expressed as a *q*. Individuals considered as potentially admixed are shown in bold and immigrant individuals are shaded.

Individual	Geographical population assumed	Probability in assumed population	Probability in other population										
bPon-01	Pop 1	0.976	Pop 2:	0.000	0.000	0.005	Pop 3:	0.000	0.000	0.002	Pop 4:	0.000	0.005	0.012
bPon-02	Pop 1	0.853	Pop 2:	0.000	0.001	0.029	Pop 3:	0.000	0.000	0.001	Pop 4:	0.020	0.045	0.051
bPon-133	Pop 1	0.986	Pop 2:	0.000	0.000	0.005	Pop 3:	0.000	0.000	0.001	Pop 4:	0.000	0.000	0.008
bPon-134	Pop 1	0.997	Pop 2:	0.000	0.000	0.000	Pop 3:	0.000	0.000	0.002	Pop 4:	0.000	0.000	0.001
bPon-135	Pop 1	0.992	Pop 2:	0.000	0.000	0.001	Pop 3:	0.000	0.000	0.002	Pop 4:	0.000	0.000	0.004
bPon-140	Pop 1	0.999	Pop 2:	0.000	0.000	0.000	Pop 3:	0.000	0.000	0.000	Pop 4:	0.000	0.000	0.001
bPon-137	Pop 1	0.988	Pop 2:	0.000	0.000	0.005	Pop 3:	0.000	0.000	0.003	Pop 4:	0.000	0.000	0.003
bPon-139	Pop 1	0.989	Pop 2:	0.000	0.000	0.000	Pop 3:	0.000	0.000	0.002	Pop 4:	0.000	0.002	0.008
bPon-138	Pop 1	0.992	Pop 2:	0.000	0.000	0.000	Pop 3:	0.000	0.000	0.002	Pop 4:	0.000	0.000	0.006
bPon-136	Pop 1	0.897	Pop 2:	0.000	0.000	0.026	Pop 3:	0.000	0.001	0.024	Pop 4:	0.010	0.014	0.028
bPon-141	Pop 1	0.994	Pop 2:	0.000	0.000	0.001	Pop 3:	0.000	0.000	0.002	Pop 4:	0.000	0.000	0.003
bPon-143	Pop 1	0.993	Pop 2:	0.000	0.000	0.001	Pop 3:	0.000	0.000	0.003	Pop 4:	0.000	0.000	0.003
bPon-142	Pop 1	0.992	Pop 2:	0.000	0.000	0.001	Pop 3:	0.000	0.000	0.002	Pop 4:	0.000	0.001	0.004
bPon-124	Pop 1	0.975	Pop 2:	0.000	0.000	0.012	Pop 3:	0.000	0.000	0.008	Pop 4:	0.000	0.000	0.004
bPon-366	Pop 1	0.993	Pop 2:	0.000	0.000	0.002	Pop 3:	0.000	0.000	0.001	Pop 4:	0.000	0.000	0.004
bPon-12	Pop 1	0.972	Pop 2:	0.000	0.000	0.006	Pop 3:	0.000	0.003	0.014	Pop 4:	0.000	0.000	0.004
bPon-91	Pop 1	0.948	Pop 2:	0.000	0.000	0.002	Pop 3:	0.000	0.000	0.005	Pop 4:	0.011	0.007	0.026
bPon-04	Pop 1	0.992	Pop 2:	0.000	0.000	0.001	Pop 3:	0.000	0.000	0.001	Pop 4:	0.000	0.001	0.005
bPon-25	**Pop 2**	**0.678**	**Pop 1:**	**0.000**	**0.013**	**0.066**	**Pop 3:**	**0.000**	**0.020**	**0.047**	**Pop 4:**	**0.000**	**0.055**	**0.120**
bPon-48	Pop 2	0.998	Pop 1:	0.000	0.000	0.000	Pop 3:	0.000	0.000	0.001	Pop 4:	0.000	0.000	0.001
bPon-49	Pop 2	0.847	Pop 1:	0.000	0.000	0.003	Pop 3:	0.000	0.051	0.085	Pop 4:	0.000	0.003	0.011
bPon-50	Pop 2	0.998	Pop 1:	0.000	0.000	0.000	Pop 3:	0.000	0.000	0.001	Pop 4:	0.000	0.000	0.001
bPon-51	Pop 2	0.998	Pop 1:	0.000	0.000	0.000	Pop 3:	0.000	0.000	0.001	Pop 4:	0.000	0.000	0.001
bPon-52	Pop 2	0.990	Pop 1:	0.000	0.000	0.001	Pop 3:	0.000	0.000	0.005	Pop 4:	0.000	0.000	0.004
bPon-53	Pop 2	0.979	Pop 1:	0.000	0.000	0.003	Pop 3:	0.000	0.000	0.006	Pop 4:	0.000	0.001	0.011
bPon-54	Pop 2	0.998	Pop 1:	0.000	0.000	0.000	Pop 3:	0.000	0.000	0.000	Pop 4:	0.000	0.000	0.001
bPon-35	Pop 3	0.004	Pop 1:	0.000	0.001	0.000	Pop 2:	0.000	0.000	0.000	Pop 4:	0.906	0.082	0.006
bPon-46	Pop 3	0.967	Pop 1:	0.000	0.000	0.001	Pop 2:	0.000	0.001	0.022	Pop 4:	0.000	0.001	0.007
bPon-40	Pop 3	0.968	Pop 1:	0.000	0.000	0.008	Pop 2:	0.000	0.000	0.005	Pop 4:	0.000	0.003	0.016
bPon-41	Pop 3	0.990	Pop 1:	0.000	0.000	0.001	Pop 2:	0.000	0.000	0.006	Pop 4:	0.000	0.000	0.002

Table 5. Continued.

Individual	Geographical population assumed	Probability in assumed population	Probability in other population										
bPon-47	**Pop 3**	**0.085**	**Pop 1:**	**0.000**	**0.000**	**0.001**	**Pop 2:**	**0.753**	**0.120**	**0.033**	**Pop 4:**	**0.001**	**0.003**	**0.004**
bPon-78	Pop 3	0.988	Pop 1:	0.000	0.000	0.002	Pop 2:	0.000	0.001	0.008	Pop 4:	0.000	0.000	0.002
bPon-36	Pop 3	0.987	Pop 1:	0.000	0.000	0.001	Pop 2:	0.000	0.000	0.007	Pop 4:	0.000	0.000	0.004
bPon-359	**Pop 3**	**0.422**	**Pop 1:**	**0.000**	**0.003**	**0.006**	**Pop 2:**	**0.000**	**0.028**	**0.042**	**Pop 4:**	**0.022**	**0.338**	**0.139**
bPon-44	Pop 3	0.978	Pop 1:	0.000	0.000	0.003	Pop 2:	0.000	0.000	0.009	Pop 4:	0.000	0.001	0.008
bPon-80	Pop 3	0.973	Pop 1:	0.000	0.000	0.004	Pop 2:	0.000	0.000	0.001	Pop 4:	0.003	0.001	0.017
bPon-03	Pop 4	0.962	Pop 1:	0.000	0.011	0.021	Pop 2:	0.000	0.000	0.004	Pop 3:	0.000	0.000	0.002
bPon-11	Pop 4	0.877	Pop 1:	0.000	0.002	0.007	Pop 2:	0.000	0.037	0.045	Pop 3:	0.000	0.012	0.019
bPon-15	Pop 4	0.976	Pop 1:	0.000	0.001	0.006	Pop 2:	0.000	0.000	0.007	Pop 3:	0.000	0.002	0.007
bPon-16	Pop 4	0.863	Pop 1:	0.000	0.000	0.003	Pop 2:	0.000	0.044	0.086	Pop 3:	0.000	0.000	0.003
bPon-17	Pop 4	0.996	Pop 1:	0.000	0.000	0.002	Pop 2:	0.000	0.000	0.001	Pop 3:	0.000	0.000	0.001
bPon-18	Pop 4	0.989	Pop 1:	0.000	0.001	0.006	Pop 2:	0.000	0.000	0.001	Pop 3:	0.000	0.000	0.003
bPon-19	Pop 4	0.986	Pop 1:	0.000	0.000	0.003	Pop 2:	0.000	0.001	0.008	Pop 3:	0.000	0.000	0.003
bPon-20	Pop 4	0.986	Pop 1:	0.000	0.000	0.002	Pop 2:	0.000	0.000	0.002	Pop 3:	0.000	0.001	0.009
bPon-21	Pop 4	0.000	Pop 1:	0.000	0.000	0.000	Pop 2:	0.984	0.015	0.001	Pop 3:	0.000	0.000	0.000
bPon-22	Pop 4	0.946	Pop 1:	0.000	0.001	0.004	Pop 2:	0.000	0.002	0.011	Pop 3:	0.003	0.012	0.022
bPon-23	Pop 4	0.963	Pop 1:	0.000	0.000	0.002	Pop 2:	0.000	0.001	0.005	Pop 3:	0.000	0.010	0.019
bPon-27	Pop 4	0.981	Pop 1:	0.000	0.000	0.003	Pop 2:	0.000	0.000	0.005	Pop 3:	0.000	0.002	0.008
bPon-29	Pop 4	0.975	Pop 1:	0.000	0.000	0.002	Pop 2:	0.000	0.000	0.005	Pop 3:	0.000	0.004	0.014
bPon-30	Pop 4	0.978	Pop 1:	0.000	0.000	0.003	Pop 2:	0.000	0.000	0.008	Pop 3:	0.000	0.001	0.009
bPon-31	**Pop 4**	**0.698**	**Pop 1:**	**0.002**	**0.057**	**0.064**	**Pop 2:**	**0.000**	**0.000**	**0.003**	**Pop 3:**	**0.009**	**0.108**	**0.058**
bPon-32	Pop 4	0.957	Pop 1:	0.000	0.002	0.008	Pop 2:	0.000	0.004	0.011	Pop 3:	0.000	0.006	0.013
bPon-38	Pop 4	0.801	Pop 1:	0.000	0.001	0.005	Pop 2:	0.000	0.001	0.013	Pop 3:	0.029	0.059	0.090
bPon-45	Pop 4	0.991	Pop 1:	0.000	0.000	0.003	Pop 2:	0.000	0.000	0.003	Pop 3:	0.000	0.000	0.002
bPon-130	Pop 4	0.953	Pop 1:	0.001	0.004	0.018	Pop 2:	0.000	0.001	0.007	Pop 3:	0.000	0.003	0.012
bPon-131	Pop 4	0.971	Pop 1:	0.000	0.001	0.006	Pop 2:	0.000	0.002	0.013	Pop 3:	0.000	0.001	0.006
bPon-132	**Pop 4**	**0.795**	**Pop 1:**	**0.000**	**0.000**	**0.002**	**Pop 2:**	**0.087**	**0.063**	**0.050**	**Pop 3:**	**0.000**	**0.000**	**0.003**
bPon-58	Pop 4	0.960	Pop 1:	0.000	0.000	0.003	Pop 2:	0.000	0.007	0.020	Pop 3:	0.000	0.002	0.008
bPon-24	Pop 4	0.980	Pop 1:	0.000	0.001	0.005	Pop 2:	0.000	0.000	0.005	Pop 3:	0.000	0.002	0.008

Table 6. Results of migrant detection analyses. Individuals marked by ** were identified as a migrant by both analyses. Individuals marked by * were identified as admixed by STRUCTURE. The most likely source population for each individual is shown in bold.

Sample	Sex	Geographic origin	Year of collection	STRUCTURE (*q*-values) (Green Corridor / Morro do Diabo / Ivinhema / Porto Primavera clusters)	GENECLASS F_0 migrant: LOD value[1]	GENECLASS (*P*- value)[2]	GENECLASS [-log (L)] (Green Corridor/ Morro do Diabo/ Ivinhema / Porto Primavera clusters)
bPon-02	M	Green Corridor	1993	**0.853** / 0.000 / 0.000 / 0.020	2.260	0.005	18.57 / 24.10 / 23.75 / **16.31**
bPon-21**	F	Porto Primavera	1998	0.000 / **0.984** / 0.000 / 0.000	6.263	0.000	19.64 / **8.23** / 14.23 / 14.50
bPon-25*	F	Morro do Diabo	1998	0.000 / **0.678** / 0.000 / 0.000	0.000	0.506	18.05 / **15.06** / 17.16 / 15.42
bPon-31*	M	Porto Primavera	2000	0.002 / 0.000 / 0.009 / **0.698**	2.603	0.008	19.02 / 26.12 / **15.81** / 18.42
bPon-35**	F	Ivinhema	2005	0.000 / 0.000 / 0.004 / **0.906**	8.430	0.000	23.80 / 28.48 / 24.03 / **15.60**
bPon-47*	M	Ivinhema	2002	0.000 / **0.753** / 0.085 / 0.001	3.060	0.028	18.60 / **10.44** / 13.50 / 11.86
bPon-132*	F	Porto Primavera	1993	0.000 / 0.087 / 0.000 / **0.795**	0.282	0.050	20.23 / 12.95 / 15.81 / **12.90**
bPon-136	?	Green Corridor	1992	**0.897** / 0.000 / 0.000 / 0.010	1.764	0.007	15.93 / 18.19 / 16.05 / **14.16**
bPon-359*	M	Ivinhema	2007	0.000 / 0.000 / **0.422** / 0.022	0.261	0.132	19.62 / 18.23 / 13.50 / **13.24**

[1]LOD = -log (L_home/L_max); [2]$P < 0.01$ (underlined) is potential F0 migrant.

Table 7. Pairwise contemporary migration rates among jaguar populations as estimated with BAYESASS. Numbers are migration rates from the population listed in the first column to the population listed in the first row. Standard deviations are given in parentheses.

	Green Corridor	**Morro do Diabo**	**Ivinhema**	**Porto Primavera**
Green Corridor	—	0.01 (0.02)	0.02 (0.02)	0.01 (0.01)
Morro do Diabo	0.01 (0.02)	—	0.21 (0.05)	0.28 (0.03)
Ivinhema	0.01 (0.01)	0.01 (0.02)	—	0.01 (0.01)
Porto Primavera	0.01 (0.02)	0.01 (0.02)	0.04 (0.03)	—

Table 8. Effective population size estimates and their approximate confidence limits for each UPAF jaguar population based on two different methods (LDNE and ONESAMP; see text for details).

	LDNE (P_{crit} = 0.05)		**ONESAMP** (Prior = 2 - 100)	
	N_e	Confidence limits (95%)	N_e	Confidence limits (95%)
Green Corridor	51.4	23.5 - 11004.5	30.3	23.4 - 45.8
Morro do Diabo	4.6	2.3 - 12.8	7.8	6.8 - 9.8
Ivinhema	12.3	6.9 - 27.1	10.3	9.2 - 12.5
Porto Primavera	13.5	10.4 - 18.0	21.7	19.4 - 26.6

Fig. 1

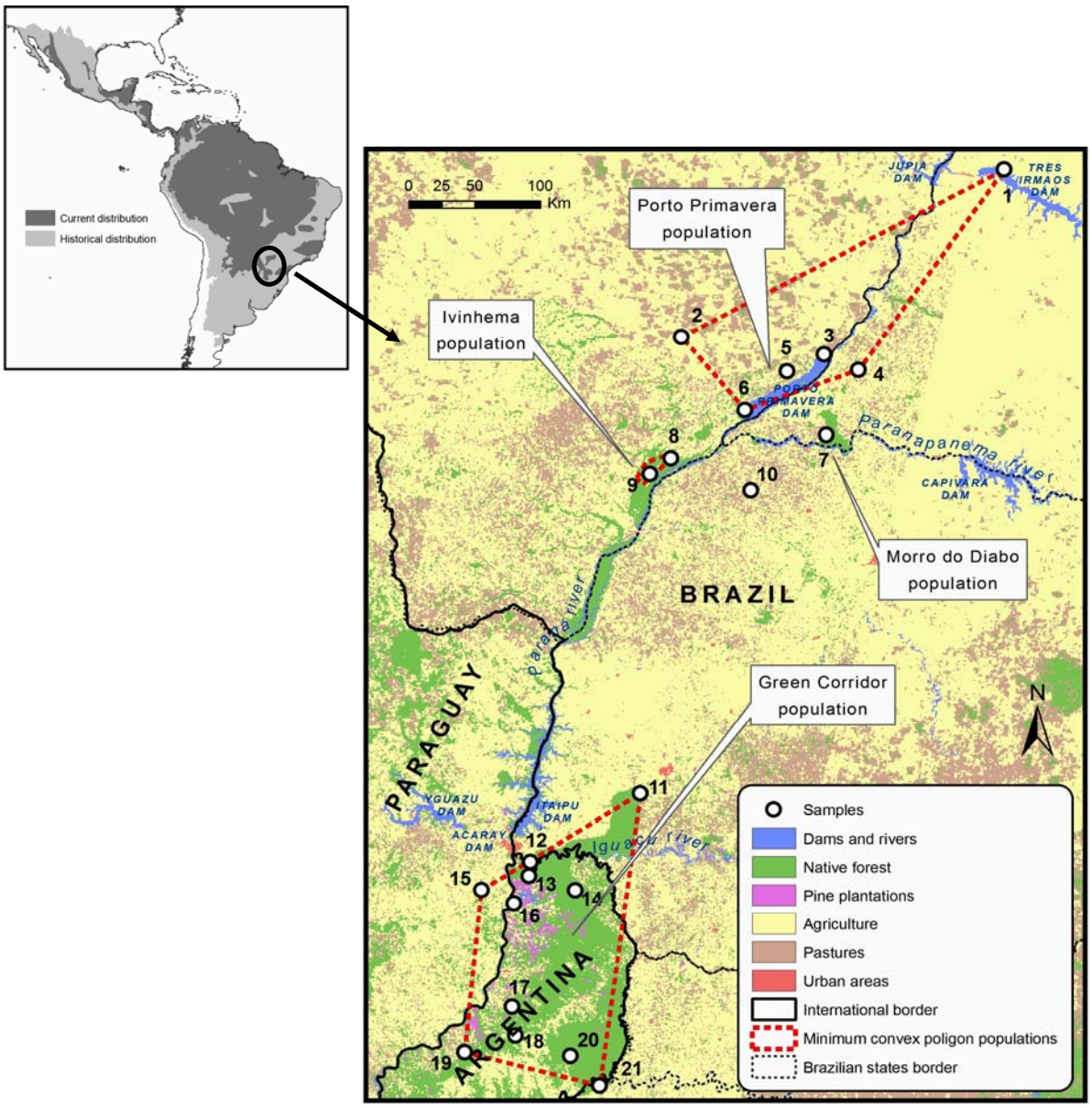

Current distribution
Historical distribution
0 25 50 100 Km
Porto Primavera population
Ivinhema population
Morro do Diabo population
Green Corridor population
BRAZIL
PARAGUAY
ARGENTINA
Paraná river
Paranapanema river
Iguaçu river
JUPIA DAM
TRES IRMAOS DAM
PORTO PRIMAVERA DAM
CAPIVARA DAM
YGUAZU DAM
ITAIPU DAM
ACARAY DAM
N
Samples
Dams and rivers
Native forest
Pine plantations
Agriculture
Pastures
Urban areas
International border
Minimum convex poligon populations
Brazilian states border

Fig. 2

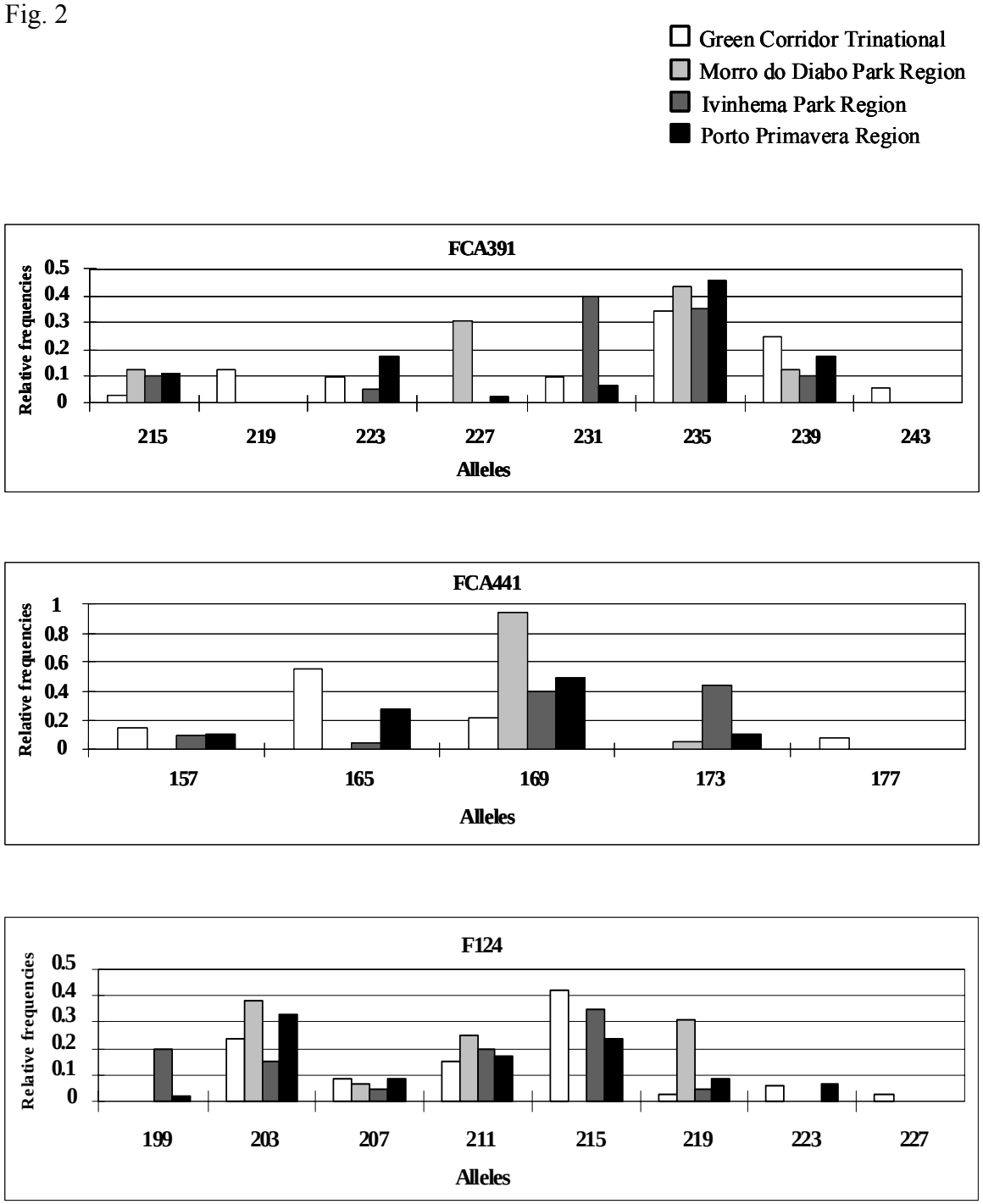

Green Corridor Trinational
Morro do Diabo Park Region
Ivinhema Park Region
Porto Primavera Region
FCA391
Relative frequencies
0.5
0.4
0.3
0.2
0.1
0
215
219
223
227
231
235
239
243
Alleles
FCA441
Relative frequencies
1
0.8
0.6
0.4
0.2
0
157
165
169
173
177
Alleles
F124
Relative frequencies
0.5
0.4
0.3
0.2
0.1
0
199
203
207
211
215
219
223
227
Alleles

Fig. 2 Continued.

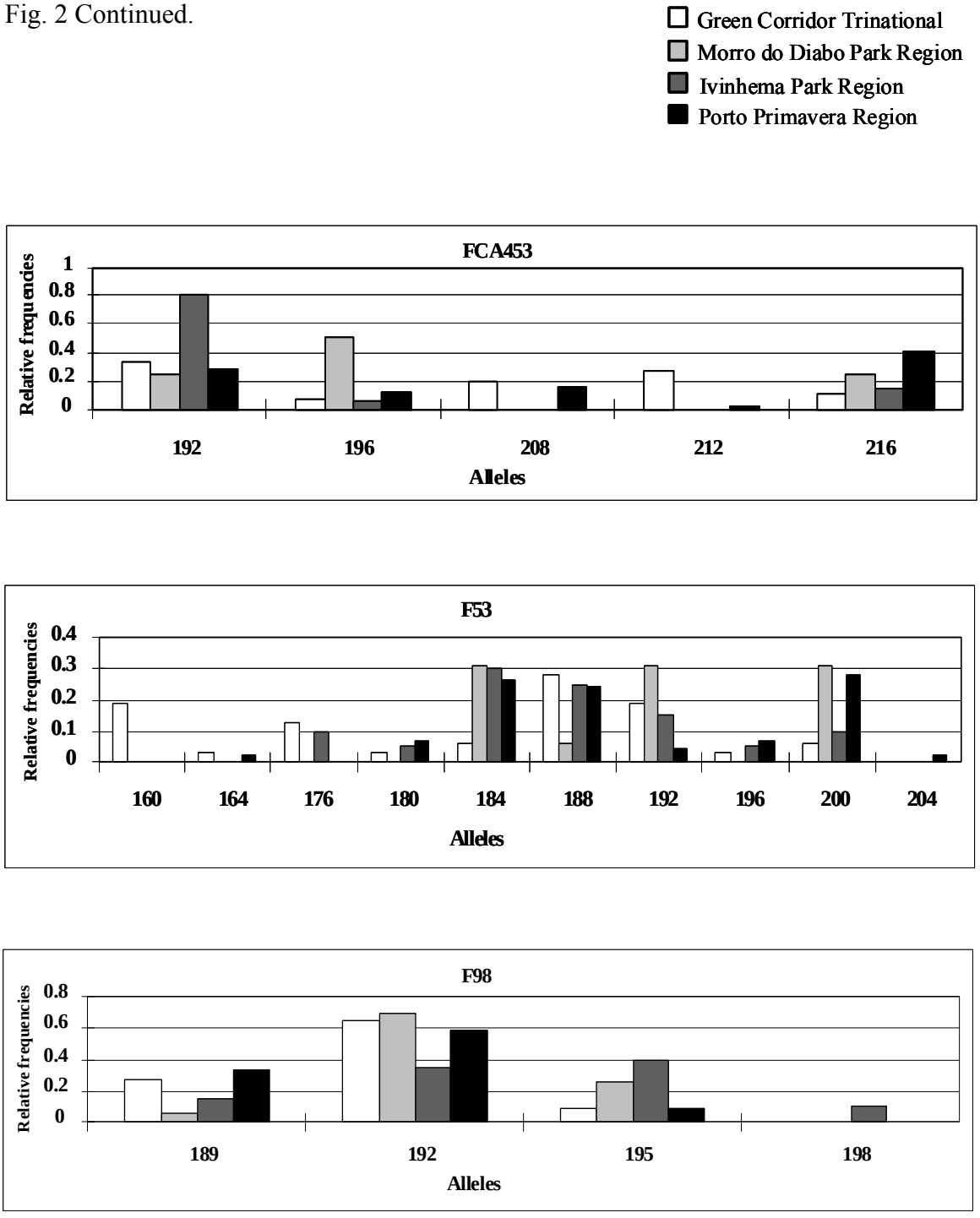


Fig. 2 Continued.

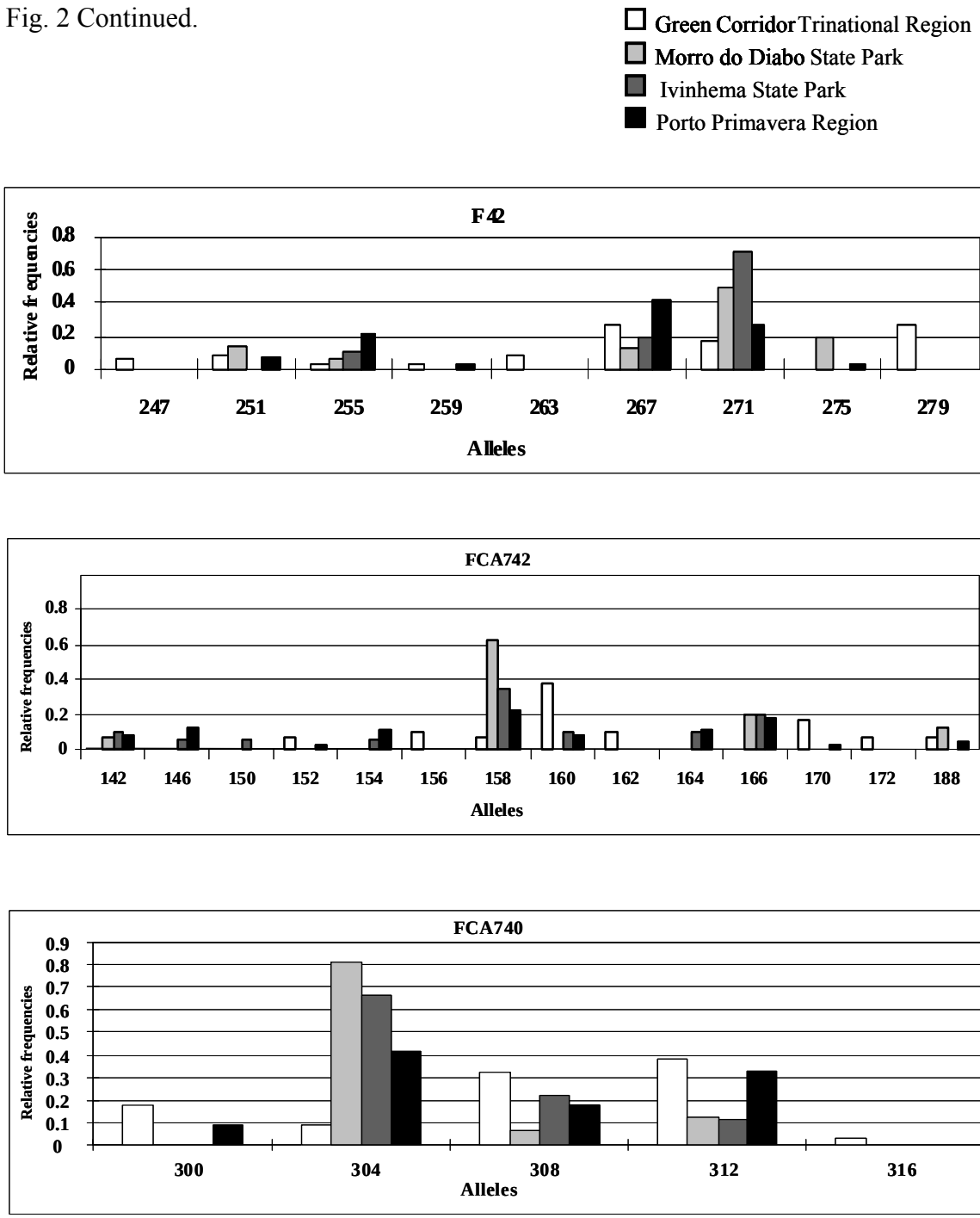


Fig. 2 Continued.

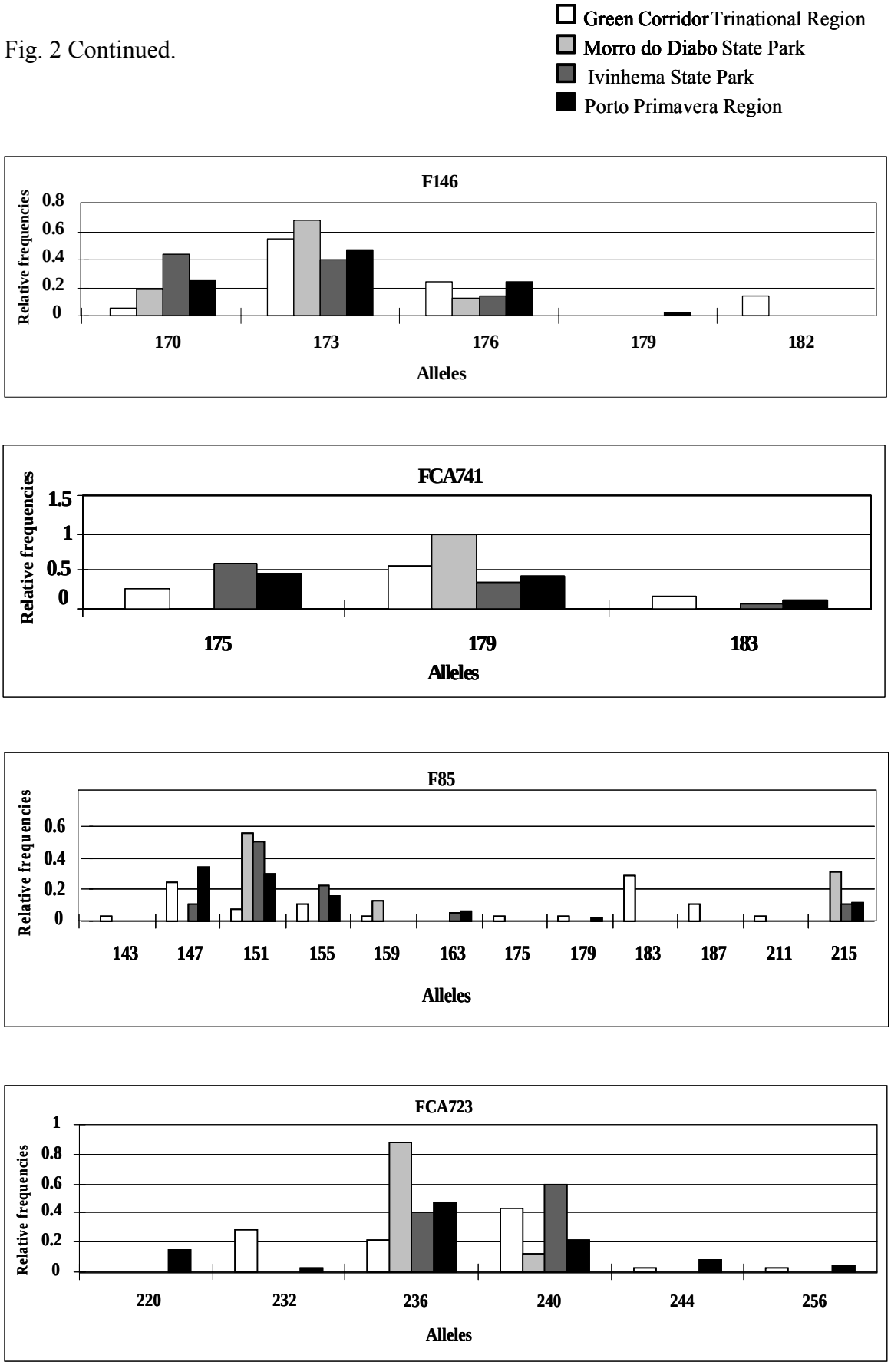


Fig. 3

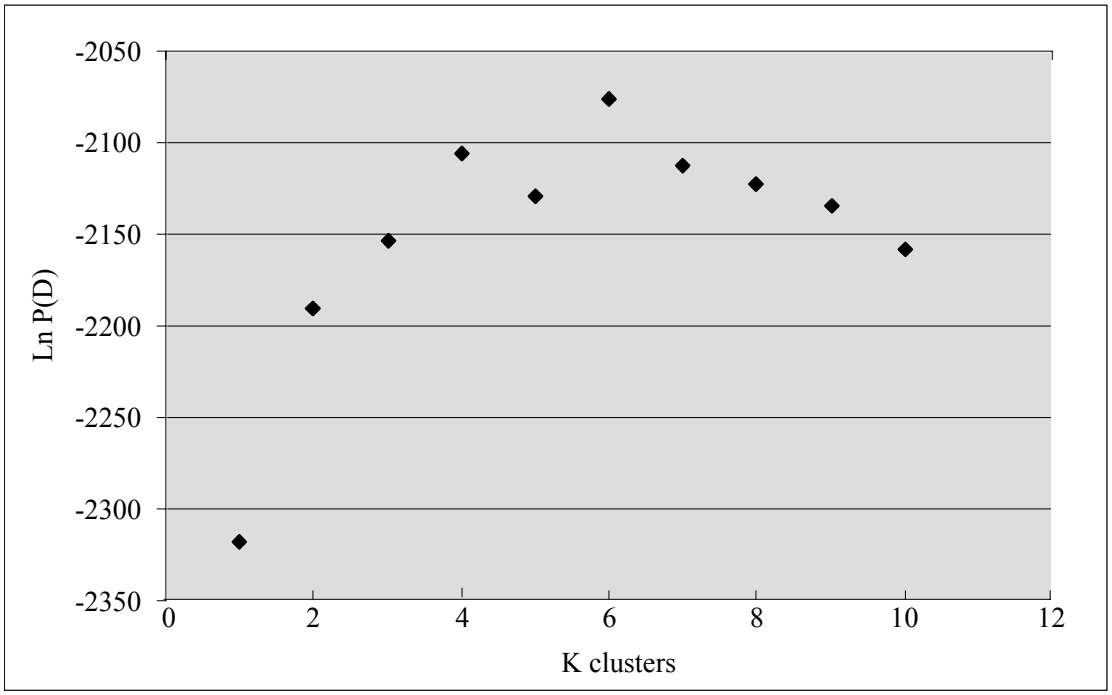

-2050
-2100
-2150
-2200
-2250
-2300
-2350
Ln P(D)
0
2
4
6
8
10
12
K clusters

Fig. 4

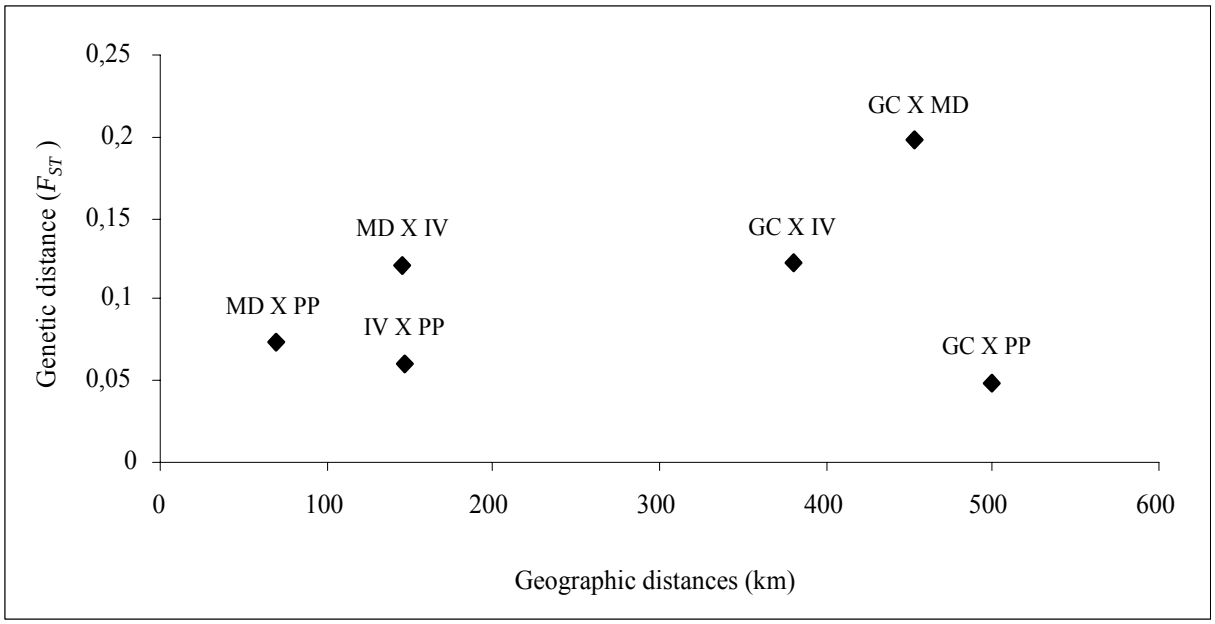
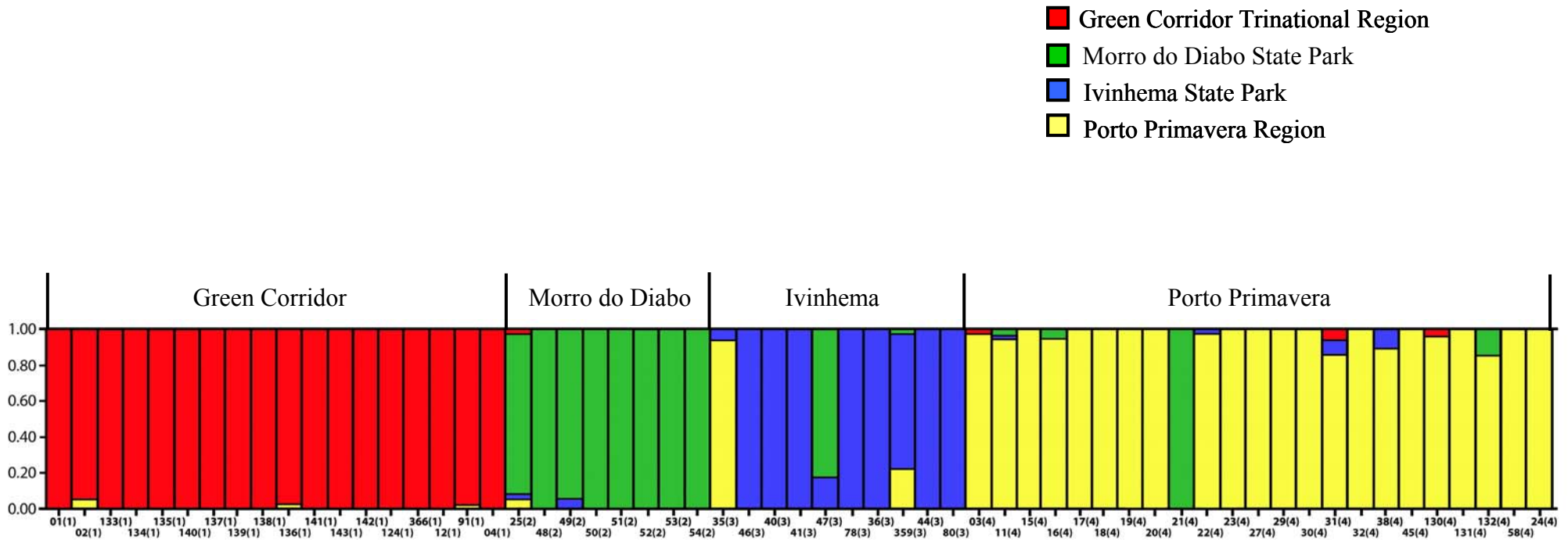

0,25
0,2
0,15
0,1
0,05
0
Genetic distance (F_{ST})
GC X MD
MD X IV
GC X IV
MD X PP
IV X PP
GC X PP
0
100
200
300
400
500
600
Geographic distances (km)

Fig. 5


Green Corridor Trinational Region
Morro do Diabo State Park
Ivinhema State Park
Porto Primavera Region
Green Corridor
Morro do Diabo
Ivinhema
Porto Primavera
1.00
0.80
0.60
0.40
0.20
0.00
01(1) 02(1) 133(1) 134(1) 135(1) 140(1) 137(1) 139(1) 138(1) 136(1) 141(1) 143(1) 142(1) 124(1) 366(1) 12(1) 91(1) 04(1)
25(2) 48(2) 49(2) 50(2) 51(2) 52(2) 53(2) 54(2)
35(3) 46(3) 40(3) 41(3) 47(3) 78(3) 36(3) 359(3) 44(3) 80(3)
03(4) 11(4) 15(4) 16(4) 17(4) 18(4) 19(4) 20(4) 21(4) 22(4) 23(4) 27(4) 29(4) 30(4) 31(4) 32(4) 38(4) 45(4) 130(4) 131(4) 132(4) 58(4) 24(4)

CAPÍTULO VI

DISCUSSÃO GERAL

O presente trabalho teve por objetivo desenvolver metodologias para embasar futuros estudos genéticos e ecológicos extremamente necessários para a conservação da onça-pintada, bem como dar início a estudos moleculares que visem estimar os níveis de diversidade genética e conectividade de populações naturais desta espécie. Aspectos detalhados de cada tema foram abordados na discussão dos artigos precedentes. Neste espaço será feita uma breve revisão dos mesmos, salientando alguns aspectos e contribuições destes estudos ao conhecimento da espécie.

Primeiramente, devido à grande dificuldade de se obter amostras representativas de populações naturais de onça-pintada, é muito importante a utilização de amostras não-invasivas em estudos genéticos, evolutivos e ecológicos com esta espécie. Tradicionalmente, amostras fecais coletadas em campo têm sido identificadas com base exclusivamente em critérios morfológicos. No entanto, diferentes estudos têm mostrado que esta abordagem não é totalmente confiável, pois em muitos casos, a morfologia das fezes de diferentes espécies pode ser similar (Foran *et al*. 1997; Farrell *et al*. 2000; Davison *et al*. 2002; Chame 2003). Este fato, por sua vez, é particularmente relevante quando espécies focais de determinado estudo ocorrem em simpatria com táxons relacionados, como é o caso da onça-pintada e puma. Desta maneira, visando identificar amostras fecais de onça-pintada coletadas em campo de maneira mais rigorosa e segura, é apresentada no artigo do capítulo III uma abordagem baseada no sequenciamento de um segmento curto de um gene (*ATP6*) do DNA mitocondrial.

Os resultados deste estudo revelaram a importância da utilização de um método molecular para a identificação correta de amostras fecais, embasando a realização de estudos ecológicos e genéticos com resultados mais confiáveis. Além de permitir a distinção correta entre amostras fecais de onça-pintada e puma, o método proposto permite

a identificação de amostras fecais de outras espécies de carnívoros com distribuição simpátrica. Através desta abordagem, é possível ampliar de maneira significativa a amostragem de diferentes espécies, o que possibilita o acesso a informações básicas como ocorrência e distribuição das mesmas em determinadas regiões. Do mesmo modo, o uso de amostras não-invasivas é de grande importância no âmbito da conservação, pois análises moleculares de espécies elusivas, raras, e/ou ameaçadas serão possíveis sem que os indivíduos tenham que ser capturados ou até mesmo observados. A utilização de DNA fecal tem permitido, por exemplo, obter as primeiras amostras de onça-pintada de um dos últimos fragmentos populacionais desta espécie na Mata Atlântica Costeira. Uma maior amostragem nesta área permitirá avaliar a viabilidade das onças-pintadas neste remanescente florestal, assim como acessar a diversidade genética e o grau de endocruzamento desta população isolada e criticamente ameaçada.

No manuscrito do capítulo IV amostras fecais identificadas como pertencentes à onça-pintada, empregando-se a metodologia proposta no capítulo III, foram utilizadas para testar a possibilidade de identificar-se a coloração de indivíduos selvagens e de cativeiro desta espécie através do DNA fecal. A ocorrência de uma relativamente alta frequência de onças-pintadas melânicas em certas regiões da sua distribuição, como por exemplo, no Parque Estadual do Morro do Diabo (SP), parece indicar que esta característica tornou-se comum seja devido a uma vantagem adaptativa dos indivíduos escuros, ou a um efeito estocástico gerado pela redução no seu tamanho populacional. Em ambos os casos, a elucidação deste fenômeno tem relevância para a elaboração de uma estratégia eficiente para a conservação da onça-pintada, e deverá ser o foco de investigações moleculares mais aprofundadas. Até o momento nenhum estudo científico envolvendo esta característica foi realizado em qualquer parte da distribuição da espécie, impossibilitando determinar-se a

importância de fatores genéticos, históricos e/ou ecológicos envolvidos na coloração desta espécie.

Considerando a dificuldade de até mesmo observar indivíduos melânicos em vida livre, o uso de amostragem não-invasiva parece ser uma das poucas (senão a única) alternativas para realizar pesquisas com populações naturais que envolvam esta característica. Os resultados obtidos no manuscrito do capítulo IV são de grande relevância, demonstrando que é possível identificar de forma confiável a coloração de onças-pintadas utilizando-se DNA fecal. Estes resultados abrem novas perspectivas para a investigação aprofundada deste polimorfismo, com ênfase na ocorrência e dinâmica desta característica em populações naturais. Um local bastante propício para que se inicie este tipo de abordagem é o Parque Estadual Morro do Diabo. Nesta área, dados de armadilhas fotográficas e captura de animais utilizando cães treinados têm indicado uma alta frequência de indivíduos melânicos em relação a outras áreas de sua ocorrência (Cullen 2007). Amostras de sangue de onças-pintadas melânicas e com padrão de coloração selvagem obtidas desta área (utilizadas no capítulo V) foram recentemente genotipadas para o alelo mutante *MC1R-Δ15* que causa o melanismo na espécie. Com a utilização de DNA fecal será viável ampliar a amostragem de indivíduos melânicos nesta região para que estudos mais aprofundados possam ser iniciados.

Uma abordagem bastante promissora em Genética da Conservação é a utilização, em conjunto com marcadores neutros, de genes que estejam diretamente envolvidos na variação fenotípica e adaptação a diferentes ambientes. Desta maneira, será possível estimar a distância adaptativa entre populações naturais embasando a definição de unidades intra-específicas (Smith & Wayne 1996; Eizirik *et al*. 2006). No entanto, para que isto se torne possível, é necessário primeiramente investigar genes envolvidos em

polimorfismos fenotípicos com potencial adaptativo em uma dada espécie. A investigação do gene *MC1R*, relacionado à coloração das onças-pintadas, é um exemplo de enfoque a ser explorado.

No manuscrito do capítulo V é apresentado um estudo de genética de populações naturais de onça-pintada na ecorregião do Alto Rio Paraná, uma das últimas regiões de Mata Atlântica de Interior onde ainda se encontram populações desta espécie. Primeiramente, foi padronizado um conjunto de 13 locos de microssatélites para a espécie (artigo reproduzido no Anexo 1), o qual exibiu grande eficiência de amplificação e conteúdo informativo, com alto poder para a genotipagem individual, permitindo a sua aplicação inclusive em trabalhos baseados em amostragem não-invasiva. Os resultados do capítulo V sugeriram que, apesar da alta capacidade de dispersão da espécie e da proximidade geográfica dos fragmentos populacionais estudados, já existe perda de diversidade genética, assim como diferenciação considerável entre os remanescentes florestais. Provavelmente isto se deve à ação intensa da deriva genética, que, por sua vez, é induzida pelo pequeno tamanho efetivo em cada uma das áreas e pelo crescente isolamento entre as mesmas devido a ações antrópicas. Por outro lado, foram identificados indivíduos migrantes entre algumas localidades, bem como mistura ancestral entre elas, indicando que este processo natural de conectividade deve ser mantido para que estas populações, recentemente isoladas, possam ser viáveis a longo prazo. Estes resultados serão integrados ao plano de manejo que vem sendo desenvolvido para este felídeo nesta região, subsidiando a elaboração e efetivação de esforços urgentes para a sua conservação na Mata Atlântica de Interior.

Todos os aspectos investigados nesta tese representam apenas o início de estudos moleculares que visam, em conjunto com estudos ecológicos, obter informações

importantes de populações naturais de *Panthera onca* visando a sua conservação a longo prazo. Será de grande importância estender as análises moleculares para populações desta espécie da Mata Atlântica Costeira, Caatinga e Pantanal, com a concomitante avaliação de níveis de diversidade genética intra e interpopulacionais. Em particular, será possível avaliar a situação atual de populações mais ameaçadas (por exemplo, Mata Atlântica) em relação a áreas contínuas e com um número significativamente maior de indivíduos, como o Pantanal, o qual poderá ser utilizado para estimar os níveis "normais" de variabilidade intrapopulacional para esta espécie.

No artigo reproduzido no Anexo 1 foram fornecidos dados preliminares de um estudo genético com populações de onça-pintada do Pantanal. A diversidade genética estimada foi de moderada a alta e comparável àquelas estimadas nos maiores fragmentos populacionais da ecorregião do Alto Rio Paraná, porém mostrou-se maior que àquelas estimadas nos menores fragmentos desta ecorregião (manuscrito do capítulo V). No entanto, a análise restringiu-se a somente 23 amostras de duas localidades. São necessários estudos em outras populações deste bioma para que se possa avaliar adequadamente sua diversidade genética.

Com a continuidade dos estudos, integrando dados genéticos e ecológicos, será possível alcançar resultados robustos e propor recomendações de manejo mais pragmáticas para esta espécie. Somente por meio de uma abordagem multidisciplinar será possível compreender a dinâmica populacional deste felídeo e desenvolver estratégias de conservação e manejo bem sucedidas.

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ANEXO 1

JAGUAR CONSERVATION GENETICS

Eduardo Eizirik, Taiana Haag, Anelisie S. Santos, Francisco M. Salzano, Leandro Silveira, Fernando C. C. Azevedo & Mariana M. Furtado

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Jaguar Conservation Genetics

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Information on genetic aspects of jaguar populations is still scarce. Initial studies have surveyed genetic diversity parameters and assessed the geographic differentiation among individuals on a continental or sub-continental scale, but so far little has been accomplished with respect to investigating regional or local jaguar populations. Moreover, different studies have employed different sets of molecular markers, posing potential problems for the future development of comparative analyses across study sites and ecosystems. Here we review the current status of jaguar genetic studies, present a new set of microsatellite markers that may be useful for jaguar population genetic studies, and survey the molecular diversity of two adjacent wild jaguar populations, sampled in the Brazilian Pantanal region. Our results suggest that this set of markers is highly efficient for jaguar genetic studies, and that moderate to high levels of variability are present in wild jaguar populations, at least in the surveyed areas of the Pantanal. This contribution may be useful as a review of jaguar genetics, as well as a baseline empirical work that might support future in-depth investigations of these and other free-ranging populations of this felid.

The use of molecular tools to investigate genetic, ecological and behavioral aspects of wildlife populations has gained immense popularity in recent years, allowing unprecedented probing into multiple components of organismal biology which were previously inaccessible. In addition to its scientific relevance, knowledge of such aspects is often a critical component for the design of adequate conservation strategies on behalf of species and ecosystems. Genetic data are required to understand long-term demographic history and dynamics, and to characterize social structure and patterns of dispersal and territoriality. They are also useful for assessing evolutionary potential and inferring census and effective population sizes, which are important components of Population Viability Analyses. The field of Conservation Genetics encompasses a diverse array of methodological approaches involving the use of genetic information to tackle these and other issues of conservation concern.

Jaguars (Fig. 1) are an elusive species whose population biology has been historically difficult to study, and only recently has been the focus of in-depth investigation made possible by technological and analytical innovations. If ecological investigations of jaguars are now the focus of multiple studies at various field sites, genetic analyses of this species are still in their infancy, having been severely limited by the practical difficulty in sampling biological materials representative of natural populations. A range-wide assessment of genetic diversity and evolutionary history has been performed, and studies addressing regional or local-level issues are starting to become feasible, as improved methods for biological sampling become incorporated in this scientific discipline. Here we (i) review the history of jaguar conservation genetics and the current state of the field, (ii) discuss the advantages and prospects of developing a set of molecular markers that can become standardized for jaguar population genetics, and (iii) present novel preliminary data describing the levels of microsatellite diversity in a natural jaguar population, that of the southern Brazilian Pantanal.

Although the jaguar had been included in previous genetic studies addressing phylogenetic questions with the use of molecular markers (e.g. Johnson & O'Brien 1997), its intraspecific levels of diversity had not been investigated until 2001. In that year, a study employing mitochondrial DNA (mtDNA) sequences encompassing a segment of the control region (CR) and 29 nuclear microsatellite loci addressed the genetic diversity and demographic history of jaguars, based on 44 individuals sampled from Mexico to southern Brazil (Eizirik *et al*. 2001). That study revealed that this species exhibits a shallow mtDNA structure, compared to other felids, with low differentiation among geographic regions. The shallow structure, with low inter-regional differentiation, was inferred to have been caused by a rather recent population expansion, *ca.* 300,000 years ago, followed by a history of demographic connectivity over a continental scale. No support was observed for the classically recognized jaguar subspecies, a finding that had also been reported on the basis of morphological data (Larson 1997). The major pattern that emerged from that data set was a phylogeographic partition between the northern and southern portions of the range, likely a function of reduced historical gene flow across the Amazon River. The levels of diversity detected in the hypervariable microsatellite loci were quite high and also indicative of large scale gene flow across the range of the species. No ma-

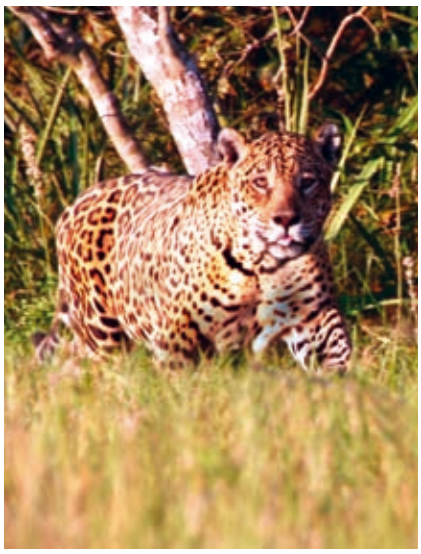

Fig. 1. Female wild jaguar in its natural habitat in the Pantanal (Photo L. Leuzinger, Fazenda Barranco Alto).

jor partitions were detected with those markers, but four moderately differentiated regional groups could be discerned. The partition likely induced by the Amazon River could still be detected, but its intensity was lower than that observed with the female-transmitted mtDNA marker, suggesting that male-mediated gene flow across the river could play a role in the historical geographic homogenization in this species. This hypothesis has so far not been thoroughly tested (but see Ruiz-Garcia *et al.* 2006), and requires more detailed sampling of local populations, particularly throughout the Amazon region. Likewise, the precise magnitude of genetic differentiation among any regional populations could not be fully tested in that study, due to the sparse sampling available for each locale, and the range-wide scope of the analyses.

Subsequent to that study, to our knowledge only three scientific papers have addressed genetic aspects of jaguar populations (Moreno *et al*. 2006, Ruiz-Garcia *et al*. 2006, Soares *et al.* 2006). All three studies have employed microsatellite loci as molecular markers, allowing an assessment of the performance of these hypervariable nuclear segments to investigate this species. These loci are currently the markers of choice for population level studies of most wildlife species, as their high mutation rates and Mendelian inheritance allow the detailed probing into demographic, behavioral and ecological questions. We will briefly review the scope and findings of these three papers, and focus on the comparison of the microsatellite loci employed, aiming to evaluate the current status of marker standardization among studies.

Moreno *et al*. (2006) analyzed 39 jaguar individuals sampled in Brazilian zoos, using four microsatellite loci, three of which had been used by Eizirik *et al*. (2001). These three loci presented high levels of allelic diversity in this captive population (no analysis of natural populations was included), with 9-12 alleles identified in each of them. Ruiz-Garcia *et al*. (2006) addressed the population genetics of Colombian jaguars, including a total of 62 individuals from that country and 22 additional samples. Twelve microsatellite loci were employed, four of which had been previously used by Eizirik *et al*. (2001), and three overlapping with those of Moreno *et al*. (2006) (one of which did not overlap with Eizirik *et al*. [2001]). They also found high levels of diversity and some evidence of genetic continuity (*i.e.* no differentiation) between areas located to the north and to the south of the Amazon River. This finding might disagree with the initial inference by Eizirik *et al.* (2001), but the sampling schemes and geographic scopes were different between the two studies, and so were most of the molecular markers employed. Further analyses with designed sampling and standardized markers are still required to test this hypothesis. Finally, Soares *et al*. (2006) employed seven microsatellite loci (all of which had previously been used by Eizirik *et al.* [2001]) to perform a paternity analysis in a jaguar population in the Brazilian Cerrado biome. Only four individuals were analyzed, and three of them were related to each other, so little inference can be made on the levels of genetic diversity in that population using these data.

An overall conclusion of this brief assessment is that still very few studies have been performed on jaguar genetics, highlighting the need for further work on this topic. Moreover, many of the employed markers were not shared among studies, precluding direct comparisons of the levels of genetic diversity identified in different areas. It would be thus important to develop a set of markers that is standardized for jaguar genetics, presenting high amplification success and allelic diversity in this species, and allowing for cross-study comparisons of variability measures. Although such rough comparisons of diversity could be made across studies as long as the loci were the same, a more refined goal would be to have data sets that could be integrated in meta-analyses.

One challenge to such integration is the lack of reproducibility of the precise allele sizes across different laboratories and genotyping devices, especially in the case of dinucleotide microsatellite markers (whose repeat unit is 2 nucleotides long). This type of locus is more difficult to score reliably, and more prone to inter-lab variation in allele assignment (E.E., personal observation). However, they are very abundant in the genome, and more frequently identified in screens for variable markers than other types of repeats. Most of the microsatellite markers originally described for the domestic cat (*Felis catus*) were dinucleotides (*e.g.* Menotti-Raymond *et al.* 1999), and this set of loci served as the basis for most population genetic studies performed with wild felids so far. As a consequence, most loci applied in the studies reviewed above were dinucleotide repeats: 27 out of 29 loci in Eizirik *et al*. (2001), four out of four loci in Moreno *et al*. (2006), 11 out of 12 loci in Ruiz-Garcia *et al*. (2006), and six out of seven loci in Soares *et al*. (2006). In spite of the variability reported for these markers in these studies, it may be better to base a standardized microsatellite set for jaguars on other types of loci, such as tetranucleotides (composed of 4-bp repeat units), whose allele scoring is more reliable and reproducible. Given that several trinucleotide and tetranucleotide loci have been reported for the domestic cat (*e.g.* Menotti-Raymond *et al*. 1999, 2005), we aimed to assess their performance in jaguars, and to test whether they may serve as a basis for a standardized panel of population-level markers for this species.

Materials and Methods

Assessment of tetranucleotide microsatellite loci for jaguar population genetics

We tested 20 trinucleotide/tetranucleotide microsatellite loci developed for

the domestic cat (Menotti-Raymond *et al.* 1999, 2005). Two of them (FCA441, FCA453) had been previously used by Eizirik *et al*. (2001), and another (FCA391) was employed by Ruiz-Garcia *et al.* (2006). Five loci (FCA749, FCA751, FCA748, FCA732 e FCA559) did not present efficient amplification in jaguars in pilot runs, and were excluded from further testing. Another locus (FCA424) was monomorphic (*i.e.* bearing no variation) in the pilot sample, and locus FCA738 presented only two alleles; both of them were also excluded from further analyses. We thus focused on a panel of 13 loci (FCA742, FCA741, FCA740, FCA723, FCA453, FCA441, FCA391, F146, F124, F98, F85, F53 and F42) that presented good results for jaguars sampled across their range (not shown), and initiated an assessment of their performance in population-level studies. We are currently employing these markers in jaguar population genetic studies focusing on multiple sites located in the Brazilian Atlantic Forest, Pantanal and Amazon biomes. We describe below preliminary results from a screen for genetic variation in these markers in the southern Pantanal, based on samples collected at two nearby locations.

Genetic diversity of natural jaguar populations: the Brazilian Pantanal

Blood samples from 23 wild-caught jaguar individuals were obtained in two nearby areas within a seasonally flooded habitat in the southern region of Pantanal, Mato Grosso do Sul state, Brazil. The field sites were the Caiman Ecological Refuge (19.80° S / 56.27° W; n = 12) and San Francisco ranch (20.08º S / 56,60º W; n = 11) where field projects addressing jaguar ecology and conservation are currently being carried out.

Blood samples were preserved with EDTA and in some cases with a salt saturated solution (100mM Tris, 100mM EDTA, 2% SDS), and stored at 4°C or –20°C for most of the time prior to DNA extraction. Total DNA was extracted from blood samples following a standard phenol-chloroform protocol (Sambrook *et al*. 1989), and its quality and yield were assessed by analysis on an agarose gel. DNA extracts were amplified by PCR for the 13 microsatellite loci listed above. Every forward primer was 5'-tailed with an M13 sequence (Boutin-Ganache *et al*. 2001), and used in combination with an M13 primer that had the same sequence but was dye-labeled on its 5' end. PCR reactions were carried out for each locus separately, and products from 1 to 3 loci were diluted and pooled together based on yield, size range and fluorescent dye. Microsatellite genotyping was performed using a MegaBACE 1000 automated sequencer and the ET-ROX 550 size standard (GE Healthcare), and then analyzed utilizing the accompanying software Genetic Profiler 2.2.

We calculated the number of alleles, polymorphic information content (*PIC*), observed (H_O) and expected (H_E) heterozygosity for each locus, and tested for any evidence of departures from expectations of Hardy-Weinberg Equilibrium (HWE) and linkage equilibrium using CERVUS 2.0 (Marshall *et al*. 1998) and ARLEQUIN 3.1 (Excoffier *et al*. 2006). To quantify the power of individual identification with the set microsatellite markers applied here, we estimated the probability of identity (P_{ID}) index, *i.e.* the probability of any two individuals in the population randomly sharing identical genotypes for all the analyzed loci (Paetkau *et al*. 1998).

Results and Discussion

Of 13 primer pairs used, ten presented allele intervals compatible with a tetranucleotide repeat (FCA741, FCA740, FCA723, FCA453, FCA441, FCA391, F124, F85, F53, F42), two were trinucleotide repeats (F146 and F98) and one was a dinucleotide repeat (FCA742). One additional tetranucleotide locus (FCA741) was found to be monomorphic in this jaguar sample and was removed from the study.

All loci were in linkage equilibrium in both sampling locales after Bonferroni adjustments (Rice 1989 [α = 0.05]). Deviations from HWE expectations were tested for each of the two locations separately, and then combined. One locus (FCA441) was found to be out of HWE in the Caiman ranch population and another one (FCA742) in the San Francisco ranch population. In both cases, the deviation from HWE was no longer significant after application of the sequential Bonferroni correction. When both populations were combined in a joint analysis, a third locus (FCA740) appeared to depart from HWE expectations, but again the statistical significance of this result was lost after applying the sequential Bonferroni correction. These results indicate that the deviations observed prior to the correction may not bear any biological relevance, and for the present time we can infer that these markers meet HWE expectations for these populations.

The overall analysis of the 12 selected loci, employing the total sample of 23 individuals captured in both locales, revealed moderate to high levels of genetic diversity, with an average expected heterozygosity (H_e) of 0.7171, mean number of alleles per locus of 5.83, and mean Polymorphic Information Content (PIC) of 0.6592 (Table 1). Both populations exhibited considerable diversity (Table 2), a finding which will be refined with additional sampling in the

Table 1. Measures of diversity at 12 microsatellite loci characterized in this study for *Panthera onca* in the southern region of the Pantanal biome, Brazil.

Locus	*N*	No. of alleles	Allele size range	Ho[1]	He[2]	PIC[3]
FCA742	19	11	142-178	0.947	0.876	0.838
FCA740	23	5	300-316	0.652	0.739	0.681
FCA723	23	6	200-244	0.783	0.653	0.580
FCA453	22	6	192-216	0.818	0.715	0.656
FCA441	22	4	165-177	0.500	0.589	0.520
FCA391	23	6	215-243	0.870	0.776	0.727
F146	23	3	173-182	0.304	0.382	0.318
F124	23	7	203-231	0.870	0.769	0.715
F98	23	3	189-195	0.565	0.641	0.552
F85	22	7	139-183	0.773	0.834	0.790
F53	21	5	164-196	0.762	0.803	0.748
F42	22	7	251-275	0.864	0.830	0.785

[1]Observed heterozygosity; [2]Expected heterozygosity;

[3]Mean polymorphic information content.

Table 2. Measures of diversity at 12 microsatellite loci in two local populations of *Panthera onca* from the Brazilian Pantanal.

Population	*n*	Average expected heterozygosity	Average No. of alleles per locus	PIC*	No. of private alleles
Caiman E. R	12	0.6962	5.33	0.6226	17
San Francisco ranch	11	0.7088	4.42	0.6248	6

* Mean polymorphic information content

future. Since this is the first assessment of jaguar genetic diversity performed for local wild populations, and most of our molecular markers are different from those employed previously, the observed levels of variability cannot yet be directly compared to other studies. However, this scenario should change in the near future as other populations are currently being analyzed with these same markers. Given that jaguars are believed to be more abundant in the southern Pantanal region than in many other parts of their distribution, these preliminary data from this biome may serve as a baseline which may be helpful when assessing current levels of diversity in small, fragmented jaguar populations.

The estimated probability of identity (P_{ID}) using these markers in the joint Pantanal sample was 2×10^{-13}, indicating that it is extremely unlikely that any two individuals may bear the same composite genotypes at these loci (*i.e.* this estimate would imply that one would need to sample > 1 trillion jaguars to find two individuals with identical composite genotypes). This is very important in the context of allowing the individual identification of jaguars using molecular markers, such as in the case of non-invasive samples (e.g. scats, hairs) and forensic specimens, which are of direct interest to studies addressing ecological, behavioral and conservation-related issues (*e.g.* density estimates, kinship and social structure, patterns of dispersal and population connectivity). Given the power observed in this panel of 12 microsatellites, it is likely that a subset of these markers will still have very high precision in the discrimination of jaguar individuals in any local population, allowing the investigation of ecological and behavioral questions using non-invasive sampling (which often requires that one selects a smaller number of loci to minimize error rates and to facilitate thorough genotype checking via redundancy). We conclude that this set of markers holds good promise for building a standardized panel for jaguar population genetic studies, either by itself or in combination with some loci selected from previous studies.

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