UNIVERSIDADE FEDERAL DE SÃO CARLOS
CENTRO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM ECOLOGIA E RECURSOS NATURAIS

Demografia e variação genética de *Puma concolor* (Linnaeus, 1771) na região nordeste do estado de São Paulo

Renata Alonso Miotto

São Carlos, SP
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Tese apresentada ao Programa de Pós-Graduação em Ecologia e Recursos Naturais, Centro de Ciências Biológicas e da Saúde, Universidade Federal de São Carlos, como parte dos requisitos para a obtenção do título de doutor em Ciências.

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SUMÁRIO

Resumo

A região nordeste do estado de São Paulo era originalmente coberta por savanas e floresta semi-decídua, mas durante dois séculos passou por diversos ciclos de exploração de seus solos férteis. Apesar da grande perda de hábitats naturais e da fragmentação da vegetação remanescente em conseqüência de atividades agrícolas, algumas espécies de grandes carnívoros, como a onça-parda (*Puma concolor*), ainda persistem na região. Nesse trabalho, utilizando como ferramentas o DNA fecal e marcadores moleculares, procuramos agregar informações demográficas e genéticas da população de onças-pardas presente na região nordeste do estado de São Paulo a fim de contribuir para a persistência em longo prazo dessa espécie na região. Durante os anos de 2004 e 2008, identificamos 17 animais (13 fêmeas e quatro machos) ocupando dois dos últimos refúgios naturais da região; dentre estes, reconhecemos cinco fêmeas residentes adultas sugerindo que esses refúgios atuam como uma área fonte de indivíduos na paisagem. Com exceção de três indivíduos, todos os animais amostrados eram em algum grau aparentados e observamos ainda, evidências de filopatria, comportamento já descrito em populações da América do Norte. Por meio da aplicação de modelos de marcação e recaptura, estimamos uma densidade de 9.36 ± 2.54 animais/100 km^2 no interior dessas áreas e entorno próximo (~260 km^2), a mais alta densidade já descrita na literatura para a espécie. O grande número de animais concentrados nessas áreas provavelmente está relacionado à ausência de competidores diretos, bem como à grande disponibilidade de presas e à ausência de hábitats de qualidade similar na região; especialmente fêmeas tendem a tolerar maior sobreposição de territórios quando há grande disponibilidade de recursos. Considerando que onças devem migrar ao longo da matriz a partir dessas áreas, quantificamos aspectos que poderão vir a ter grande influência na viabilidade da espécie na região nordeste do estado e que exigem ações mitigatórias, como atropelamentos em rodovias (11; 10 machos e uma fêmea) e conflitos entre esses animais e atividades humanas (seis eventos). Onças-pardas, principalmente os machos, são animais que dispersam longas distâncias a partir de sua área natal para ocupar novos territórios, evitando o endocruzamento e

mantendo altos níveis de fluxo gênico. Desse modo, a fim de qualificar o sucesso de movimentos de dispersão das onças entre diferentes manchas de hábitat, em uma escala geográfica maior, amostramos 37 animais e testamos a existência de estruturação genética na região. Observamos que as onças-pardas que ocupam a região nordeste do estado constituem uma única população, possuem altos níveis de diversidade genética (H_E=0.79; média de 10 alelos por loco), são em sua grande maioria relacionadas entre si, e detectamos ainda, evidências de gargalo populacional. Apesar da intensa transformação da paisagem em decorrência das atividades humanas, concluímos que ainda é mantido um fluxo gênico entre as Unidades de Conservação com mais de 2,000 ha da região e mais de 70 km distantes umas das outras, e que ações conservacionistas devem ser baseadas na manutenção desse fluxo. Considerando os resultados obtidos, sugerimos que o manejo da espécie deve ser conduzido em uma escala de paisagem visando o aumento da conectividade entre hábitats a partir (1) da criação de novas áreas protegidas; (2) do manejo alternado de plantações de eucalipto da região; (3) da manutenção de fragmentos de vegetação em propriedades particulares (Reserva Legal); (4) da construção de estruturas de passagem de fauna em rodovias; e (5) de ações educacionais que mudem a percepção da comunidade em relação à presença de grandes carnívoros.

Abstract

The northeast area of São Paulo state was originally covered by cerrado and semideciduous forest, but for the last two centuries, it has experienced distinct cycles of human exploitation of its fertile soils. Despite of intensive human activities, large habitat loss and fragmentation of the native vegetation cover, pumas (*Puma concolor*) still inhabit remnant habitat fragments in the northeastern area of the state. In this study, by using fecal DNA and molecular markers, we investigated demographic and genetic issues on pumas to aggregate basic information for conservation efforts to maintain long term viable subpopulations of this top-predator in the region. Between 2004-2008, we identified 17 animals (13 females and four males) inhabiting two of the last natural refuges in the area; five females represented resident adults indicating that both refuge areas may act as a source of individuals in the landscape. Only three animals did not exhibit any degree of relatedness, and we found evidence of philopatry, behaviour already described in North American populations. By using mark-recapture-DNA-based methods, we estimated a density of 9.36 ± 2.54 animals/100 km^2 inside and surrounding these areas (~260 km^2), the largest density estimate for this species reported in the literature. The high abundance may reflect an absence of direct competitors, as well as large prey availability and absence of similar high quality patches in the matrix; mainly females tend to tolerate higher home-range overlap when many resources are available. Considering that pumas may disperse throughout the matrix, we quantified aspects that will probably have influence on the species viability in the area, such as roadkills (11; 10 males and one female) and puma-human conflicts (six events). Especially male pumas tend to disperse long distances from their natal area to occupy new home-ranges, avoiding inbreeding and maintaining high levels of gene flow. Thus, to qualify the dispersers' movement success, in a higher geographic scale, we sampled 37 pumas and tested the hypothesis of lack of genetic structure among the natural areas in the region. We observed that pumas in the northeast area of São Paulo state constitute a single population, have high levels of genetic diversity (H_E=0.79; mean of 10 alleles per locus) and are related to each other. We also found evidence of a recent bottleneck event in this

population. In spite of the huge landscape transformation in consequence of human activities, we concluded that pumas still maintain some levels of gene flow among the protected areas of the study area larger than 2,000 ha and more than 70 km distant one from another, so conservation efforts should be concentrated on the maintenance of this flow. We recommend that puma management should be conducted at the landscape level by increasing habitat connectivity, such as, (1) creating new protected areas; (2) applying an alternate cutting approach in eucalyptus plantations; (3) maintaining habitat patches in private properties; (4) creating structures to allow highway crossing of pumas; (5) designing educational actions to change community perception of large carnivores.

INTRODUÇÃO

Um dos objetivos da biologia da conservação é o de possibilitar a sobrevivência, em longo prazo, das espécies e dos ecossistemas dos quais elas dependem (Wayne e Morin 2004). A introdução de espécies exóticas, a poluição, a fragmentação de hábitats e o extermínio da fauna e flora por ações humanas são os principais fatores responsáveis pela perda da diversidade biológica mundial (Pimm e Gilpin 1989; Frankham *et al.* 2002).

Dentro das comunidades biológicas, certas espécies são importantes para determinar a persistência de muitas outras (Primack e Rodrigues 2001). Predadores de topo de cadeia afetam a organização de comunidades com base apenas na quantidade de indivíduos ou biomassa (Janzen 1986) e são importantes no controle de populações de herbívoros (Redford 1992; Sergio *et al.* 2008). Podem ainda, ser indicadores de alta biodiversidade uma vez que dependem e afetam direta ou indiretamente uma complexa rede de fatores bióticos e abióticos dentro de uma comunidade (Sergio *et al.* 2008). Desse modo, a proteção de predadores de topo de cadeia, como os grandes carnívoros, deve ser prioridade para os esforços de conservação, uma vez que a perda de suas áreas de vida pode influenciar e levar à perda de muitas outras espécies que se encontram em níveis tróficos inferiores (Terborgh 1992; Simberloff 1998; Primack e Rodrigues 2001).

O declínio das populações de grandes carnívoros hoje é um problema global (Weber e Rabinowitz 1996, Karanth *et al.* 2006). Na Ásia, na África e nas Américas, a maior parte dessas populações sofre múltiplas pressões, como a degradação dos seus hábitats, a caça, doenças transmitidas por animais domésticos e o comércio ilegal de partes de seus corpos (Weber e Rabinowitz 1996). Assim como em todo o planeta, populações brasileiras de grandes carnívoros também sofrem as conseqüências do crescente desenvolvimento de centros urbanos e da expansão das fronteiras agrícolas no país, já que essas atividades implicam na remoção e substituição da vegetação natural, ou seja, de seus hábitats naturais (Palmeira *et al*. 2008). O estado de São Paulo em particular é o estado mais rico e populoso do Brasil, mas em contrapartida ao grande desenvolvimento urbano, industrial e agrícola teve a sua superfície

transformada nos últimos séculos (Biota/Fapesp 2008). Os remanescentes naturais do estado são poucos, a maioria é pequena (têm menos de 50 ha) e não está conectada entre si (Biota/Fapesp 2008), o que em linhas gerais reduz a disponibilidade de recursos, a possibilidade de dispersões e migrações, e consequentemente, a viabilidade de populações de grandes carnívoros em longo prazo. Áreas de intensa atividade antrópica, como a região nordeste do estado, já testemunharam a extinção de ao menos uma espécie de grande carnívoro, a onça-pintada (*Panthera onca*). Espécies de hábitos mais generalistas com a onça-parda (*Puma concolor*) e o lobo-guará (*Chrysocyon brachyurus*) ainda persistem, mas a maneira com que têm ocupado fragmentos remanescentes e sobrevivido na região ainda é pouco compreendida e investigada.

O estudo de grandes carnívoros é dificultado pela baixa densidade de indivíduos, além dos hábitos elusivos e de difícil observação que apresentam (Kohn e Wayne 1997). Uma nova possibilidade para monitorar a demografia dessas espécies utiliza materiais orgânicos que os animais deixam para trás (Wayne e Morin 2004). Novas técnicas moleculares de extração de DNA aplicadas a materiais como fezes, pêlos e ossos permitem uma análise não-invasiva dos indivíduos (Morin e Woodruff 1996) e oferecem uma alternativa para a contagem e a identificação individual em populações, além da determinação do sexo e dos seus deslocamentos (Kohn e Wayne 1997). Estudos não-invasivos também são métodos muito promissores de monitorar populações ameaçadas, pois evitam os danos causados pela captura (Wayne e Morin 2004).

Em longo prazo, o essencial é que essas espécies sejam preservadas como entidades dinâmicas capazes de se adaptarem às mudanças ambientais (Frankham *et al.* 2002). Planos de conservação baseados em informações obtidas de maneira não-invasiva podem então, integrar informações genéticas, ecológicas e fenotípicas para maximizar as chances de populações persistirem a futuras mudanças, assim como preservar seu legado histórico (Moritz 2002).

1.1 O histórico de ocupação e degradação da cobertura vegetal do estado de São Paulo

Há aproximadamente 500 anos, na época do descobrimento do Brasil, 83% da superfície do estado de São Paulo era recoberta por diferentes fitofisionomias de Mata Atlântica e 14% por fitofisionomias de Cerrado (Kronka *et al.* 1998). Desde essa época, o estado passou por diversos ciclos de exploração de ouro, açúcar e café (Dean 1996), mas foi a partir de 1810, com a expansão da cafeicultura, que o interior do estado passou a ser efetivamente ocupado (Kronka *et al*. 2005). A partir da década de 1970, com a criação pelo governo militar do programa Pró-Álcool, o interior paulista passou por uma expansão avassaladora de plantações de cana-de-açúcar, substituindo principalmente as áreas de pastagens e plantações de café (Martinelli e Filoso 2008), seguida pela expansão da citricultura na década seguinte (Joly *et al.* 2008). De sua cobertura vegetal original, em 1962 o estado contava com 29,26%, passando para 17,72% em 1971/73, e 13,43% em 1993 (Nalon *et al.* 2008).

Em consequência das atividades humanas, da Mata Atlântica restam hoje aproximadamente 16% da sua cobertura original (Ribeiro *et al.* 2009) e somente na Serra do Mar e no Vale do Ribeira há remanescentes significativos da vegetação original (Joly *et al.* 2008). Da cobertura de Cerrado, 90% de sua área foi destruída restando hoje somente 230 mil hectares subdivididos em 8.300 fragmentos, mais de 4.000 deles com menos de 10 hectares, e somente 47 com uma área superior a 400 hectares (Kronka *et al*. 1998; Kronka *et al.* 2005). Em geral, a cobertura vegetal original, que chegou a ocupar mais de 80% do território do estado, está hoje reduzida a 13,94% (Joly *et al*. 2008; Biota/Fapesp 2008), sendo que apenas 25% dessa cobertura está protegida sob a forma de Unidades de Conservação controladas pelo poder público (Biota/Fapesp 2008). O restante encontra-se em propriedades privadas, grande parte pertencente ao setor agrícola (Biota/FAPESP 2008).

Não só no estado de São Paulo, como em todo o país, a contínua degradação dos biomas do Cerrado e da Mata Atlântica os levou a serem classificados como dois dos 25 “pontos quentes” terrestres para a conservação no planeta (Myers *et al.* 2005), devido ao número de espécies endêmicas que possuem e à

grande ameaça de integridade as quais os seus ecossistemas estão expostos. Devido à sua posição geográfica, na transição entre as regiões tropical e subtropical, e ao seu relevo, a biodiversidade do estado de São Paulo está entre as mais elevadas do país (Joly *et al.* 2008), mas toda a diversidade remanescente está ameaçada principalmente pelo desmatamento e pela caça (Cullen Jr *et al*. 2000; Kierulff *et al.* 2008). Apesar do aprimoramento da legislação ambiental e de seus mecanismos de fiscalização, atualmente a taxa de desmatamento ainda é muito elevada (Joly *et al.* 2008).

1.2 A região nordeste do estado de São Paulo

Em meados do século XIX, a vegetação natural da região nordeste do estado de São Paulo passou a ser substituída pelo plantio de café (Shida 2005). Especialmente na região de Ribeirão Preto, o complexo cafeeiro levou ao desenvolvimento de uma rede urbana e de uma malha ferroviária para escoamento da produção (Shida 2005). O início da atividade ferroviária foi também responsável pelo aumento da taxa de desmatamento na região, uma vez que aumentou a demanda por madeira (Dean 1996; Shida 2005). De acordo com Dean (1996), nessa época apenas a região de Ribeirão Preto contava com mais de 100 indústrias extrativistas que forneciam lenha para o escoamento da produção.

Apesar do início da decadência do café em 1896, as áreas cultivadas continuaram a crescer, concomitante ao início da atividade pecuarista (Shida 2005). Com a quebra da Bolsa de Nova York em 1929 e o incentivo à pecuária leiteira, monoculturas da região passaram a policulturas, cujos principais produtos eram amendoim, feijão, soja, arroz, milho, laranja, cana-de-açúcar e algodão (Shida 2005). No final da década de 1940, deu-se início à expansão da agroindústria açucareira que atingiu o seu ápice na década de 1970, com subsídios gerados pelo governo militar (Dean 1996). Desde então a atividade açucareira continua a crescer em todo o estado.

Nos últimos 50 anos, a area plantada com cana-de-açúcar no Brasil aumentou de aproximadamente 1.4 milhões para sete milhões de hectares (Martinelli e Filoso 2008). Apenas o estado de São Paulo possui

mais de 50% de toda a área plantada do país, com plantações crescendo a uma taxa de aproximadamente 85,000 hectares por ano (Martinelli e Filoso 2008). Em adição ao cultivo de eucalipto, ao estabelecimento de pastagens e malhas rodoviárias, e ao progressivo desenvolvimento de centros urbanos, essa atividade transformou severamente a cobertura vegetal original da região nordeste do estado resultando numa paisagem altamente fragmentada.

Apesar da intensa perda de hábitats e da fragmentação dos remanescentes florestais, algumas espécies de grandes carnívoros, como a onça-parda, ainda persistem na região (Lyra-Jorge *et al*. 2008; Miotto *et al.* 2007). O modo com essa espécie tem se adaptado e resistido a essas transformações ainda é pouco compreendido e investigado. Provavelmente seus hábitos generalistas e a ausência de competidores diretos, como por exemplo, a onça-pintada, espécie já extinta na região, contribuem para a manutenção da espécie na área.

Embora ainda estejam presentes na região, as onças-pardas, assim como outros carnívoros, podem estar sofrendo ou podem vir a sofrer conseqüências relativas à intensa atividade humana. As conseqüências diretas dessas atividades são a redução de hábitats e a fragmentação, a primeira levando à redução dos tamanhos populacionais, diminuição de recursos disponíveis, e consequentemente extinções locais; a fragmentação levando à inviabilização de migrações, e assim ao isolamento das populações (Kierulff *et al.* 2008). Uma vez reduzidas e isoladas, populações tornam-se mais susceptíveis a fatores estocásticos ambientais, demográficos e genéticos comprometendo sua viabilidade em longo prazo (Frankham *et al*. 2002).

Por possuírem hábitos generalistas e conseguirem se locomover mesmo na descontinuidade de hábitats, como por exemplo, através de plantações de eucalipto ou canaviais (Miotto *et al.* 2007; Lyra-Jorge *et al*. 2008), aparentemente onças-pardas são mais susceptíveis à perda de hábitats do que à fragmentação. Mas cabe ressaltar que na região, com o isolamento dos fragmentos remanescentes, ao dispersarem pela matriz as onças estão sujeitas a atropelamentos e a encontros diretos com seres

humanos como criadores de rebanhos domésticos, o que quase invariavelmente resulta na morte do animal (Verdade e Campos 2004).

A região nordeste do estado possui quatro Unidades de Conservação com mais de 2,000 ha em que essa espécie está presente: a Estação Ecológica de Jataí, e o Parque Estadual do Vassununga, a Estação Ecológica de Itirapina e a Floresta Estadual Edmundo Navarro de Andrade (FEENA) (Figura 1). A distância entre a Estação Ecológica de Jataí o Parque Estadual do Vassununga é de apenas três quilômetros. Juntas, essas áreas estão aproximadamente 70 km distantes da Estação Ecológica de Itirapina, e 90 km distantes da FEENA. Os trabalhos aqui apresentados foram realizados com base em animais amostrados no interior, no entorno próximo ou na matriz entre essas Unidades de Conservação e segue-se então, uma breve caracterização de cada uma dessas áreas.

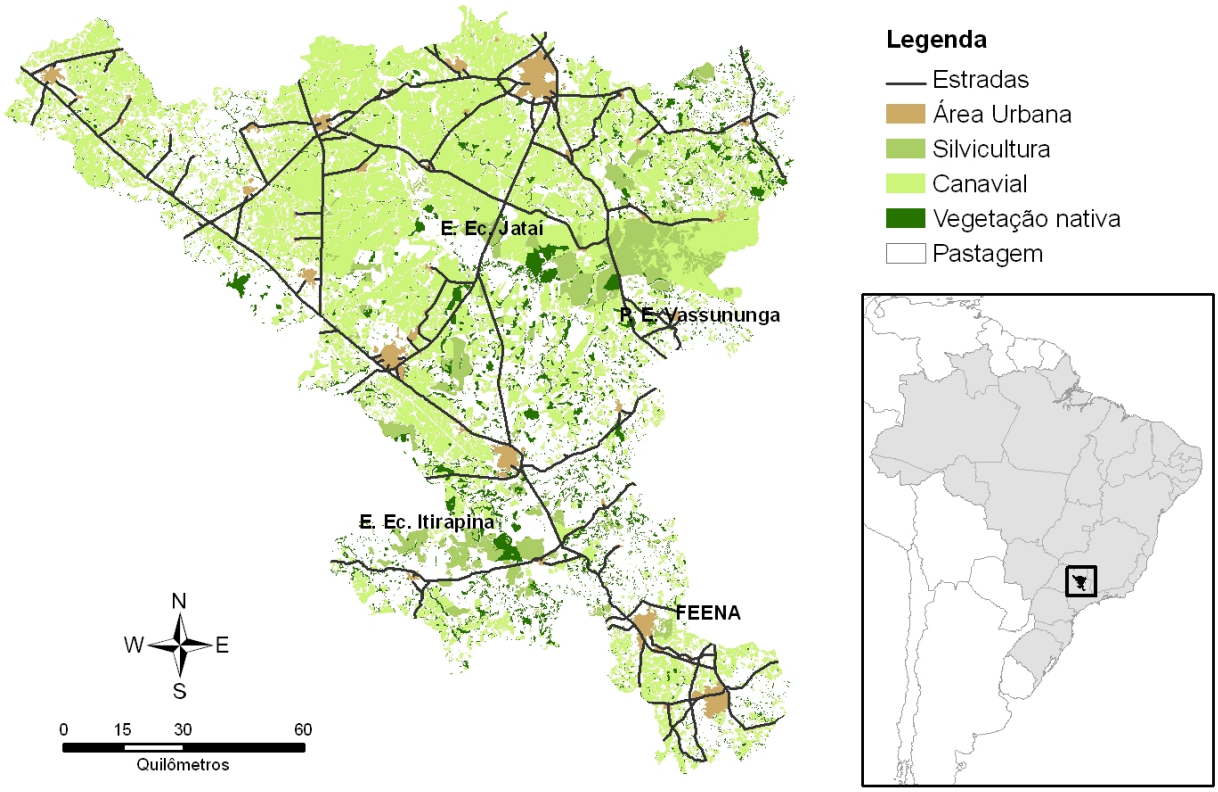

Figura 1. Região nordeste do estado de São Paulo, suas classes de uso do solo e Unidades de Conservação com áreas maiores do que 2,000 ha.

1.2.1 Estação Ecológica de Jataí (EEJ)

A EEJ está localizada no município de Luís Antônio (21º35'S–47º48'O). Originalmente com 4,532.18 ha de área, em 18/09/2002 por meio do Decreto Lei 47.096/SP, passou a possuir 9,010.7 ha com a incorporação de parte da Estação Experimental de Luís Antônio, tornando-se então a maior unidade de conservação do estado de São Paulo com área contínua de cerrado *lato sensu*. Conhecida como Fazenda Jataí até o final da década de 50, a área pertencia à extinta Companhia Mogiana de Estradas de Ferro (Santos *et al.* 2000). Em 1959 a área foi adquirida pelo Instituto Florestal do estado de São Paulo para a criação de uma unidade de conservação devido a "necessidade da preservação do remanescente de vegetação ciliar do rio Mogi-Guaçu, do conjunto lagunar e do ecossistema de cerrado de grande valor cultural e científico" (decreto Lei 18.997, SP-15/06/1982).

A EEJ apresenta uma grande diversidade de hábitats, distribuídos desde os ambientes verdadeiramente aquáticos como rios, córregos e lagoas, passando por banhados e formações periodicamente alagáveis, até as florestas e morros permanentemente livres de inundações (Santos *et al*. 1995). Os ecossistemas terrestres são representados, principalmente, por fisionomias de cerrado (cerradão e cerrado *sensu stricto*), além de trechos de floresta estacional semidecidual (Toppa *et al*. 2002). A EEJ é cercada por intensa atividade de silvicultura e por plantio de cana-de-açúcar. Enfrenta problemas com a caça recreacional, a pesca predatória, a contaminação por agroquímicos e o risco de incêndios devido às práticas agrícolas no entorno (Pires e Santos 1995).

1.2.2 Parque Estadual do Vassununga (PEV)

O PEV está localizado no município de Santa Rita do Passa Quatro (21º41'S-47º34'O). A área total do Parque é de 2,069.42 ha e este é subdividido em seis glebas distintas: Capão da Várzea (12.10 ha), Capetinga Oeste (327.83 ha), Praxedes (152.75 ha), Maravilha (127.08 ha), Capetinga Leste (236.56 ha) e Pé-de-Gigante (1,212.92 ha) (Korman 2003). O PEV foi criado em 26/10/1970 pelo decreto lei 52.546/70 e

abriga diversas formações vegetais (Korman 2003). A gleba Pé-de-Gigante é composta por fisionomias de cerrado, desde o campo sujo até o cerradão, além de mata ciliar, floresta estacional semidecídua e campo de várzea (Batalha 1997). As demais glebas são todas compostas por floresta estacional semidecidual (Korman 2003). O uso e ocupação das terras da região podem ser subdivididos em seis classes: atividades agropecuárias, atividades agroflorestais, vegetação florestal de interflúvio, campo sujo, cerrado e vegetação ripária (Shida e Pivello 2002).

Apesar das áreas do PEV e da EEJ estarem cercadas por intensas atividades de silvicultura e plantio de cana-de-açúcar ainda mantém alta diversidade de fauna (Martuscelli e Olmos 1993; Talamoni *et al.* 2000; Jorge *et al*. 2001; Munari *et al*. 2001) e flora (Batalha 2000; Toppa *et al*. 2002). Martuscelli e Olmos (1993) realizaram um levantamento faunístico preliminar da região e encontraram 172 espécies de aves e 19 de mamíferos, muitas delas ameaçadas como, por exemplo, o urubu-rei (*Sarcoramphus papa*), o gavião de cabeça-cinza (*Leptodon caynnensis*), o curió (*Oryzoborus angolensis*), o macaco sauá (*Callicebus personatus*), e o lobo-guará (*Chrysocyon brachyurus*). Mesmo as divisas das unidades de conservação com os canaviais e reflorestamentos apresentam alta riqueza de espécies como foi demonstrado por Jorge *et al*. (2001) e Munari *et al*. (2001) que registraram a presença de mamíferos de pequeno, médio e grande porte como, por exemplo, tamanduá-mirim (*Myrmecophaga tridactyla*), tamanduá-bandeira (*Tamanduá tetradactyla*), cachorro-do-mato (*Cerdocyon thous*), quati (*Nasua nasua*), jaritataca (*Conepatus chinga*), irara (*Eira barbara*), veado-campeiro (*Ozotoceros bezoarcticus*), entre outras.

1.2.3. Estação Ecológica de Itirapina (EEI)

A EEI está localizada nos municípios de Itirapina e Brotas (22º15'S-47º49'O) e foi criada em 1984 pelo decreto estadual 22.335. Possui 2,300 ha em que predominam fisionomias de cerrado como campos sujos, campos cerrados e campos limpos com porções alagáveis na estação chuvosa, além de áreas de cerrado *sensu-stricto*, mata galeria e brejos. A EEI apresenta ainda, um dos últimos campos limpos naturais

remanescentes do estado de São Paulo. As altitudes na área variam entre 700 e 827 metros e o inverno é seco. O entorno da EEI possui monoculturas de *Eucalyptus spp.* e *Pinus spp.*, cítricos, canaviais e pastos. Faz limite também com a Represa do Lobo (Broa) e com um remanescente de campo cerrado de 300 ha na divisa leste-nordeste.

Motta-Junior *et al.* (2008) registraram a ocorrência de 231 espécies de aves na EEI, cerca de 27% das espécies de aves listadas para o domínio do Cerrado, e 30% das espécies registradas para o estado de São Paulo. A EEI possui ainda uma grande diversidade de mamíferos como, por exemplo, o lobo-guará (*C. brachyurus*), o tatu-de-rabo-mole (*Cabassous unicinctus*), o tatu-peludo (*Euphractus sexcinctus*), o cachorro-do-mato (*C. thous*) e a capivara (*Hydrochaeris hydrochaeris*) (Tozetti 2002).

1.2.4 Floresta Estadual Edmundo Navarro de Andrade (FEENA)

A FEENA está localizada no município de Rio Claro (22º25'S-47º33'O). Possui 2,314 ha e foi criada em 2002 pelo decreto estadual 46.819 quando deixou de ser classificada como um horto florestal e passou à categoria Florestal. Apresenta remanescentes de floresta estacional semidecídua e vegetação ripária, além do plantio em talhões de *Eucalyptus spp.* e *Pinus spp.* com sub-bosques em diferentes níveis de regeneração (Begotti 2008). O entorno é caracterizado por áreas urbanas e plantações de cana-de-açúcar.

Em um levantamento faunístico da área, Begotti (2008) registrou a ocorrência de 22 espécies de médios e grandes mamíferos em seu interior e entorno próximo (500 m), como por exemplo, jaguatirica (*Leopardus pardalis*), gato-mourisco (*Puma yagouaroundi*), cachorro-do-mato (*C. thous*), paca (*Cuniculus paca*), irara (*E. barbara*) e o veado-catingueiro (*Mazama guazoubira*).

1.3 A espécie *Puma concolor* (Linnaeus 1771)

A onça-parda (*Puma concolor*) (Figura 2) é uma das oito espécies representantes da família Felidae que ocorre em território brasileiro (Oliveira e Cassaro 1999). A espécie tem uma distribuição muito ampla

(Figura 3), desde o oeste do Canadá até o extremo sul do continente sul-americano, passando por todo o Brasil (Oliveira e Cassaro 1999). Popularmente é conhecida como onça-vermelha, leão-baio, suçuarana, puma ou leão-da-montanha (Oliveira e Cassaro 1999). O hábitat é variado, incluindo florestas tropicais e subtropicais, Caatinga, Cerrado e Pantanal, bem como sua dieta, que inclui, geralmente, mamíferos, desde roedores até filhotes de pecuária doméstica (Oliveira e Cassaro 1999).

Figura 2. A espécie *Puma concolor*. Fotografias obtidas por meio de armadilhas fotográficas na Estação Ecológica de Jataí em 2009, e gentilmente cedidas por Giordano Ciocheti.

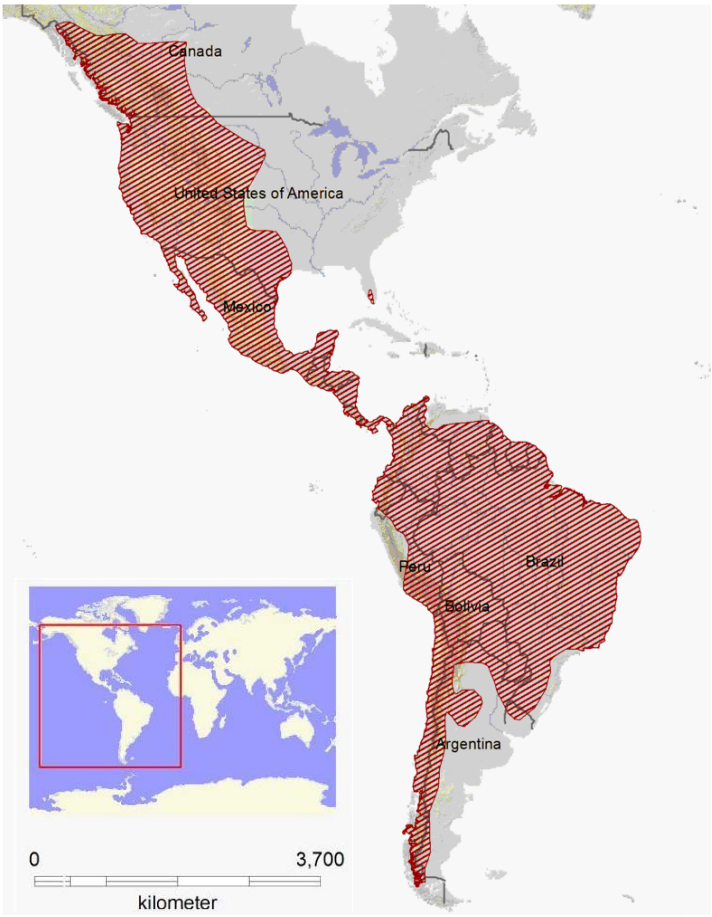

Figura 3. Distribuição atual de *Puma concolor* pelas Américas (modificado de IUCN 2010).

A onça-parda é um predador generalista e, provavelmente, sua grande adaptabilidade permitiu que a espécie resistisse às extinções do Pleistoceno que eliminaram os outros grandes felinos da América do Norte (IUCN 2005). As principais presas dos pumas são diferentes de acordo com a variação latitudinal da sua área de vida. Particularmente nos trópicos, presas de tamanho pequeno a médio parecem ser mais importantes enquanto na América do Norte, a predação é preferencial sobre grandes ungulados. Iriarte *et al.* (1990) sugeriu que o menor tamanho corporal das onças-pardas nos trópicos, e a baixa taxa de predação de presas grandes, estão ligados à competição interespecífica da espécie com a onça-pintada (*Panthera onca*), o maior felino americano. Farrel *et al*. (2000), estudando a dieta de carnívoros simpátricos

a partir da análise do conteúdo de presas nas fezes, também encontraram mínima sobreposição de presas entre as duas espécies.

Pumas são animais territorialistas que têm o hábito de marcar o seu território depositando pequenos volumes de fezes em locais proeminentes como trilhas, rochas ou ninhos já desocupados (Chame 2003). Essa prática se deve ao fato de que, quando defeca, a maioria dos carnívoros produz secreções odoríferas na glândula anal que aderem às fezes (Chame 2003). Uma vez que cada espécie produz um odor característico, a marcação territorial por meio das fezes permite que sejam reconhecidas informações interespecíficas de um território individual, sexo, e estado reprodutivo desempenhando importante papel na comunicação social desses animais (Gorman e Trowbridge 1989).

As onças-pardas necessitam de grandes áreas de hábitat, geralmente maiores do que 100 km^2 (Sweanor *et al.* 2000), e quando em forrageamento, podem viajar, em média, 9 km por noite (Beier 1993). São animais que dispersam por longas distâncias, até mesmo na presença de grande descontinuidade em seu hábitat (Ruth *et al*. 1998). Já foram documentadas, para machos subadultos, dispersões maiores do que 450 km (Anderson *et al*. 2004).

Pumas são carnívoros solitários que exibem a poliginia como estratégia reprodutiva onde machos tipicamente dominantes acasalam com fêmeas que residem na sua área de vida (Murphy 1998 *apud* Anderson *et al.* 2004; Logan e Sweanor 2001). Aparentemente, não há uma estação reprodutiva bem definida e acasalamentos podem ocorrer durante todo o ano (Ross e Jalkotzy 1992; Logan e Sweanor 2001). Estima-se que a sobrevivência desses animais seja de 12 a 13 anos (Currier 1983; Logan e Sweanor 2001).

Os machos agressivamente defendem o seu território contra outros machos intrusos enquanto as fêmeas permitem maior sobreposição com coespecíficos (Ross e Jalkotzy 1992; Logan e Sweanor 2001). A área de vida das fêmeas tende a ser grande o suficiente para prover presas a elas e a seus filhotes, mas o território dos machos tende a ser maior ainda, se sobrepondo ao de várias fêmeas, aparentemente para

maximizar o seu sucesso reprodutivo (Murphy 1998 *apud* Anderson *et al.* 2004). Para evitar o endocruzamento, os machos dispersam mais frequentemente e a maiores distâncias do que as fêmeas, que muitas vezes exibem filopatria (Sweanor *et al.* 2000; Logan e Sweanor 2001). A filopatria em machos foi documentada somente na Flórida, onde restrições severas ao seu hábitat aparentemente forçaram os machos a retornarem às vizinhanças da área de nascimento após tentativas frustradas de dispersão (Maehr 1997 *apud* Sweanor *et al*. 2000).

Em ambientes natural ou artificialmente fragmentados, geralmente as populações de onças-pardas exibem estrutura de metapopulação (Beier 1995; Sweanor *et al.* 2000) e os jovens entre 10 e 33 meses de idade dispersam a partir da área de vida da mãe à procura de um novo local apropriado para se estabelecerem (Sweanor *et al.* 2000). Apesar de estudos de telemetria e de estimativas de dispersão e migração confirmarem a tendência de estruturação em metapopulações (Beier 1995; Sweanor *et al.* 2000), Anderson *et al*. (2004) encontraram dados consistentes com uma grande população panmítica de pumas nos EUA exibindo alto fluxo gênico entre subpopulações e baixa estruturação. Esses autores sugerem que a dispersão dos machos em corredores naturais de vegetação nas regiões do Colorado, sul de Dakota e Wyoming (EUA) está possibilitando a conectividade entre subpopulações.

1.3.1 Conservação de *Puma concolor*

Embora já tenha sido considerada pela IUCN como uma espécie 'próxima à ameaçada' em 2002 (IUCN 2002), atualmente a onça-parda se enquadra em uma categoria de menor grau de ameaça denominada '*least concern*' (IUCN 2010). De acordo com a lista brasileira de espécies ameaçadas, assim como as demais espécies de felinos que ocorrem no país, a onça-parda se encontra na categoria 'ameaçada' (IBAMA 2003). As principais ameaças a essa espécie são a perda e fragmentação de hábitat, a caça, e a diminuição das suas principais presas alimentares (Mazzolli *et al*. 2002).

Weaver *et al*. (1996) estimaram que 75% da mortalidade de pumas adultos é resultado de conflitos com humanos. A predação de animais domésticos por felinos como a onça-parda e a onça-pintada é pouco documentada no Brasil (Conforti e Azevedo 2003), mas ocorre em locais em que os criadouros e as suas áreas de vida estão muito próximos (Schaller e Crawshaw 1980; Mazolli *et al*. 2002). Muitos são mortos por fazendeiros que vêm nesses animais uma ameaça às suas criações (Conforti e Azevedo 2003; Verdade e Campos 2004).

Animais domésticos tornam-se presas em potencial em conseqüência ao desflorestamento. O declínio dos refúgios e do número de presas (Mazolli *et al*. 2002) pode afetar negativamente o comportamento de forrageamento de predadores de topo de cadeia (Novack *et al*. 2005) e esse desequilíbrio aumenta o conflito entre felinos e humanos (Mazolli *et al*. 2002). Conforti e Azevedo (2003) relataram um aumento na predação de animais domésticos nas redondezas do Parque Nacional do Iguaçu coincidente ao aparente desaparecimento de uma das principais presas dos felinos, o queixada (*Tayassu pecari*).

No Brasil, foram realizados poucos estudos intensivos a respeito da ecologia de onças-pardas em seu ambiente natural ou em áreas de grande atividade humana. A maioria deles concentra informações relativas aos seus hábitos alimentares e ao uso de hábitats (Mantovani 2001; Ciocheti 2007; Emmons 1987; Lyra-Jorge et al. 2008), ou relacionadas a conflitos com a atividade pecuarista (Azevedo 2008; Conforti e Azevedo 2003; Mazzolli *et al.* 2002; Michalski *et al.* 2005).

Ao contrário do Brasil, pumas vêm sendo intensamente estudadas no continente Norte Americano há mais de duas décadas, uma vez que a expansão urbana está promovendo o desaparecimento dos animais e gerando conflitos entre estes e a população humana. Até o início da colonização européia, pumas distribuíam-se amplamente em praticamente toda a América do Norte (Anderson 1983 *apud* McRae *et al*. 2005; Pimm *et al*. 2006a). Hoje em dia muitos estados americanos abrigam somente pequenas subpopulações remanescentes (McRae *et al*. 2005) e os efeitos das grandes reduções populacionais e do alto grau de endocruzamentos dentro dessas populações já se tornaram mensuráveis. Roelke *et al*. (1993)

relataram, com base em diversos marcadores de DNA, que as populações da Flórida têm, nos dias de hoje, a mais baixa variação genética já encontrada para qualquer população de felinos estudada, menor até mesmo do que a dos guepardos africanos (O'Brien *et al*. 1983). Além da baixa variação genética, os animais aparentemente enfrentam ainda dificuldades reprodutivas e de sobrevivência em consequência ao endocruzamento (Roelke *et al*. 1993). Dentre os principais problemas estão a baixa qualidade do sêmen produzido pelos machos, o criptorquidismo (a não descida dos testículos a partir do tórax em direção ao escroto), deficiência cardíaca e maior susceptibilidade a doenças infecciosas (Roelke *et al*. 1993). Para tentar reverter esse quadro, na década de 1990 agências americanas de conservação e manejo da vida selvagem introduziram na Flórida 8 animais de populações 'mais saudáveis' provenientes do Texas, apesar dessa medida de ter gerado grande controvérsia e de os resultados serem difíceis de serem investigados (Maehr e Caddick 1995; Pimm *et al*. 2006 a,b; Mills 2006; Creel 2006; Maehr *et al*. 2006). Na ocasião, foi considerado imprescindível incrementar a diversidade genética da população, bem como aumentar o seu tamanho já que o estado possuía apenas 30 indivíduos remanescentes (Pimm *et al*. 2006a). Por outro lado, surgiram questões relativas à falta de áreas naturais que pudessem abrigar esses novos animais, assim como a possibilidade de ocorrência de 'depressão exogâmica' (Maehr *et al*. 2006).

1.4 Amostragem não-invasiva de grandes carnívoros

A fim de minimizar o impacto de estudos em populações e, ao mesmo tempo, permitir a detecção de grandes carnívoros de hábitos elusivos e que naturalmente ocorrem em baixas densidades, desde a década passada vêm sendo desenvolvidos métodos não-invasivos para o estudo populacional por meio de marcadores moleculares (Morin e Woodruff 1996; Kohn e Wayne 1997). Métodos não-invasivos baseados no estudo do DNA foram propostos recentemente para estimar o tamanho populacional com grandes vantagens já que as amostras podem ser coletadas sem a necessidade de visualizar ou perturbar o animal (Taberlet *et al*. 1997).

Uma alternativa não-invasiva para a identificação do indivíduo em estudos de campo é a utilização de fezes para a obtenção de DNA (Ernest *et al*. 2000). Fezes são abundantes, sua coleta não é invasiva e poucos gramas contêm DNA proveniente de milhares de células da mucosa intestinal (Albaugh *et al*. 1992). Uma vez que cada indivíduo pode ser caracterizado por um genótipo *multilocus* único, a análise do DNA fecal torna possível a determinação do número de animais diferentes que foram amostrados e permite que seja realizada uma estimativa do tamanho populacional (Bellemain *et al*. 2005). A grande variação de algumas porções do DNA mitocondrial entre espécies distintas também permite que as amostras possam ser individualizadas dentre diversos *taxa* (Farrel *et al*. 2000).

A análise de DNA das fezes de qualquer carnívoro apresenta a dificuldade de se discernir entre o DNA da espécie de interesse e o material genético de suas presas (Ernest *et al*. 2000). Outra condição à técnica molecular é que esta seja aplicável ao DNA degradado assim como ele é encontrado nas fezes (Palomares *et al*. 2002). Esses problemas podem ser resolvidos por meio de amplificações espécie-específicas de seqüências de DNA relativamente curtas (Palomares *et al*. 2002), já que a chance de amplificação de um fragmento íntegro de DNA aumenta à medida que o tamanho do fragmento diminui (Frantzen *et al*. 1998; Broquet *et al.* 2007).

Estudos sobre a distribuição dos genótipos obtidos a partir de análises não-invasivas têm demonstrado resultados similares àqueles encontrados em estudos com rádio-telemetria que determinaram o modo de deslocamento e as áreas de vida de espécies de grandes carnívoros (Wayne e Morin 2004). Estudando ursos negros da Noruega, Bellemain *et al*. (2005) compararam as estimativas de tamanho populacional fornecidas por dados genéticos com dados coletados em campo em uma mesma escala temporal e encontraram grande correspondência entre os dois métodos de amostragem.

Na última década, um grande número de estudos populacionais sobre as mais diversas espécies se baseou em análises genéticas não-invasivas como, por exemplo: com linces ibéricos (*Lynx pardinus*) (Pires e Fernandes 2003; Palomares *et al*. 2002); com chimpanzés (*Pan troglodytes verus*) (Bradley *et al*. 2000,

2001; Morin *et al*. 2001); gorilas (*Gorilla gorilla gorilla*) (Bradley *et al*. 2000, 2001); ursos-negros americanos (Wasser *et al*. 1997); aves (*Otis tarda*) (Idaghdour *et al*. 2003); leões marinhos (*Eumetopias jubatus*) (Deagle *et al*. 2005); mustelídeos (Riddle *et al*. 2003; Gómez-Moliner *et al*. 2004; Hedmark *et al*. 2004); raposas (*Vulpes vulpes*) (Dalén *et al*. 2004); elefantes africanos (*Loxodonta cyclotis*) (Eggert *et al*. 2003); leopardos asiáticos (*Panthera pardus*) (Perez *et al*. 2006); leopardos-das-neves (*Panthera uncia*) (Janečka *et al*. 2008).

Apesar do número crescente de publicações, dados obtidos a partir de análises não-invasivas podem estar associados a erros de genotipagem gerados pela baixa quantidade e/ou qualidade do DNA utilizado (Taberlet *et al*. 1996; Broquet e Petit 2004). Tais erros são revelados por diferenças entre os genótipos obtidos de forma independente, isto é, em reações distintas, a partir de uma mesma amostra e devem ser incorporados aos dados. Particularmente para microssatélites, problemas como a falha na amplificação de um dos dois alelos de um indivíduo, fenômeno conhecido como *alellic dropout,* ou ainda a amplificação de alelos falsos (Taberlet *et al*. 1996; Prugh *et al*. 2005) podem acarretar uma individualização errônea das amostras e, consequentemente, distorções no tamanho populacional estimado. Nos últimos anos têm sido propostas algumas medidas para acessar e quantificar esses vieses das análises não-invasivas por meio de probabilidades e múltiplas amplificações (Miller *et al*. 2002; Creel *et al*. 2003; Piggott *et al*. 2004; Broquet e Petit 2004; Prugh *et al*. 2005; Roon *et al*. 2005). Falta de amplificação e a não detecção alélica foram observadas em praticamente todos os estudos não-invasivos com taxas que variaram entre 0,03 e 0,11 para não amplificação e 0,00 a 0,39 para não detecção alélica (Taberlet *et al*. 1996, 1999; Kohn e Wayne 1997; Gagneux *et al.* 1997; Ernest *et al*. 2000; Morin *et al.* 2001).

A estimativa da probabilidade de identidade (P_{ID}) vem sendo utilizada para acessar a confiança estatística da identificação individual (Kohn *et al*. 1999, Mills *et al*. 2000) e pode ser particularmente útil para o planejamento de estudos que necessitam de individualização, já que pode ser estimada para diferentes números de *loci* sem a necessidade de ter a comprovação forense dos genótipos em mãos

(Waits *et al*. 2001). A P_{ID} demonstra se o número de *loci* analisados foi eficiente para a identificação de indivíduos, e valores compreendidos entre 0,01 – 0,0001 são considerados satisfatórios (Paetkau *et al.* 1998, Waits *et al.* 2001).

1.5 Marcadores Moleculares

A existência de variabilidade genética permite a comparação entre indivíduos, populações ou espécies diferentes (Solé-Cava 2001). Muitos biólogos populacionais estão aderindo às técnicas baseadas em DNA com o intuito de obter marcadores genéticos altamente informativos (Parker *et al*. 1998), isto é, que permitam acessar a variação genética, e dessa maneira estudar indivíduos e/ou seus genes sob as condições do campo. O trabalho com marcadores de DNA pode fornecer uma "impressão digital" de cada indivíduo (Parker *et al.* 1998), informações sobre a estrutura populacional e diferenciação geográfica (Avise 2004), além da determinação das áreas de vida, tamanho territorial e tamanho populacional (Kohn e Wayne 1997). Ferramentas moleculares podem ser úteis quando o objetivo é elaborar um plano para a manutenção da diversidade genética e para elucidar aspectos demográficos e ecológicos de espécies ameaçadas (Haig 1998).

Nos últimos anos, com o desenvolvimento da técnica da Reação em Cadeia da Polimerase (PCR – *Polymerase Chain Reaction*) (Mullis e Faloona 1987; Saiki *et al*. 1988), um grande número de novas ferramentas moleculares tornou-se disponível a estudos genéticos ao nível populacional (Parker *et al*. 1998; Sunnucks 2000) como, por exemplo, RFLP (Restriction Fragment Lengh Polimorphisms), RAPD (Randon Amplified Polymorphic DNA), minissatélites (VNTR – Variable Number of Tandem Repeats) e microssatélites (SSR – Simple Sequence Repeats) (Ferreira e Gratapaglia 1995). A tecnologia de PCR envolve a síntese enzimática *in vitro* de milhares de cópias de uma seqüência específica de DNA e isso permitiu a obtenção de material suficiente para a análise genética, mesmo de fontes que forneçam uma quantidade mínima de DNA (Perez-Sweeney *et al*. 2003).

Cada marcador genético apresenta características próprias que o torna mais adequado à resolução de uma questão específica (Perez-Sweeney *et al*. 2003). Marcadores diferentes podem ter taxas de substituição/evolução diferentes, de modo que, com a escolha do marcador adequado, torna-se possível solucionar desde problemas de identificação de indivíduos à identificação de espécies crípticas ou formulação de hipóteses filogenéticas em grupos supraespecíficos (Solé-Cava 2001). Marcadores que evoluem rapidamente são úteis para o estudo de indivíduos, famílias e populações, enquanto que evoluem mais lentamente são melhor utilizados no estudo de espécies ou taxa supraespecíficos (Solé-Cava 2001). O método a ser usado na abordagem de um problema depende então da adequação do grau de variabilidade do marcador molecular escolhido ao nível de divergência que se deseja estudar (Solé-Cava 2001). Dentre os marcadores moleculares mais utilizados atualmente destacam-se as seqüências de DNA mitocondrial e os microssatélites.

1.5.1 Microssatélites

Microssatélites (SSR – Simple Sequence Repeats) consistem em pequenas seqüências de 1 a 6 nucleotídeos de comprimento repetidas lado a lado (Ferreira e Grattapaglia 1995). São abundantes no genoma nuclear de eucariotos (Weber e Wong 1993) e têm herança codominante, isto é, cada um dos alelos de um *locus* de um indivíduo pode ser identificado separadamente (Sunnucks 2000). Microssatélites são detectados por meio da amplificação via PCR utilizando-se *primers* específicos que se ligam a regiões conservadas delimitando então, a seqüência de DNA que contém a região repetitiva (Perez-Sweeney *et al*. 2003). Os fragmentos resultantes da amplificação são então separados por eletroforese e, uma vez marcados com fluorescência, podem ser visualizados em seqüenciador automático (Perez-Sweeney *et al*. 2003).

Microssatélites são marcadores largamente utilizados em estudos populacionais, pois poucos *loci* podem fornecer muitos alelos a serem examinados (Parker *et al*. 1998). Devido a uma grande variação no

número de unidades repetitivas para qualquer *locus* entre membros de uma espécie, os *loci* de microssatélites exibem alto polimorfismo (Culver *et al*. 2001). Acredita-se que este alto nível de polimorfismo em *loci* de microssatélites seja resultado de erros de pareamento durante a replicação do DNA, causando o ganho ou a perda de uma ou mais unidades repetitivas (Culver *et al*. 2001). Esse mecanismo de mutação produz diferentes tamanhos de fragmentos repetitivos que por sua vez são diferentes entre indivíduos (Culver *et al*. 2001). A variação em microssatélites tornou-se, então, uma classe útil de marcadores genéticos em populações, já que permite a identificação individual e o estabelecimento de relações de parentesco (Goldstein e Pollock 1997), além de serem indicadores de mudanças na diversidade genética em curtos períodos de tempo (Haig 1998).

1.5.2 DNA mitocondrial

As células da maioria dos eucariotos contêm um DNA nuclear de herança biparental, e também um DNA organelar (em mitocôndrias e cloroplastos) que, usualmente, tem herança uniparental (materna) (Sunnucks 2000; Parker *et al*. 1998). Essa diferença na transmissão e ainda outras em relação às propriedades evolutivas faz com que o DNA nuclear e o DNA organelar reflitam aspectos diferentes da biologia e da história de uma população. Devido à sua alta taxa de evolução, à ausência de recombinação e ao alto polimorfismo de algumas de suas porções entre espécies distintas, o DNA mitocondrial é o marcador adequado para se investigar a diferenciação entre espécies intimamente relacionadas (Hedrick e Miller 1992; Perez-Sweeney *et al*. 2003; Avise 2004). Menor do que o DNA nuclear, haplóide e de morfologia circular, o DNA mitocondrial (15-17 kb) propicia o diagnóstico de um determinado *taxa* mais rapidamente (Sunnucks 2000) além de fornecer informações sobre efeitos fundadores, hibridizações e introgressões (Parker *et al*. 1998).

A avaliação direta da variação genética existente entre organismos, espécies ou populações pode ser realizada por meio da determinação da seqüência de bases de algumas porções do DNA mitocondrial. A

técnica de seqüenciamento envolve o isolamento do DNA, a amplificação da porção de interesse via PCR e a resolução da reação de sequenciamento em um seqüenciador automático (Perez-Sweeney *et al*. 2003).

1.6 Objetivos

A paisagem atual da região nordeste do estado de São Paulo é caracterizada por intensa atividade humana, com poucos e isolados fragmentos de vegetação remanescente imersos numa matriz de plantações de cana-de-açúcar, eucaliptos e pastagens. Apesar da intensa perda de hábitats naturais e da fragmentação na região, algumas espécies de grandes carnívoros, como a onça-parda (*P.concolor*), ainda ocupam esses fragmentos remanescentes. Nesse cenário, por meio de um método não-invasivo de amostragem, a análise do DNA fecal, procuramos compreender um pouco mais como animais dessa espécie ainda persistem nessa paisagem antropizada, além de elucidar aspectos relativos à sua biologia e ao seu comportamento. Os objetivos desse trabalho foram:

1. Considerando que em áreas fragmentadas populações da espécie *P.concolor* podem exibir uma estrutura de metapopulações (Beier 1993; Sweanor *et al.* 2000; Logan e Sweanor 2001), testar a hipótese de a Estação Ecológica de Jataí e o Parque Estadual do Vassununga, o maior conjunto de fragmentos remanescentes da região, atuarem juntos como uma área fonte indivíduos que dispersariam ao longo da matriz para colonizar fragmentos adjacentes;
2. Estimar a proporção sexual de *P.concolor* nessas áreas;
3. Investigar as relações de parentesco entre os indivíduos de *P. concolor* que ocupam essas áreas;
4. Estimar a abundância e a densidade de *P. concolor* nessas duas Unidades de Conservação e entorno próximo;
5. Quantificar a ocorrência de atropelamentos de *P. concolor* e de eventuais conflitos entre essa espécie e atividades humanas na região nordeste do estado de São Paulo;
6. Em conseqüência aos hábitos generalistas da espécie e à sua grande capacidade de dispersão mesmo em descontinuidade de hábitats (Ruth *et al.* 1998; Miotto *et al.* 2007; Lyra-Jorge *et al.* 2008),

testar a hipótese da ausência de estruturação genética entre as subpopulações de *P. concolor* na região nordeste do estado;

7. Propor medidas de manejo de diferentes elementos da paisagem a fim de contribuir para a persistência e viabilidade em longo prazo da espécie *P. concolor* na região nordeste do estado.

Apresentamos os resultados desse trabalho nos três capítulos subseqüentes, sendo que cada um deles já está em formato de publicação.

CAPÍTULO 1

Monitoring a Puma (*Puma concolor*) Population in a Fragmented Landscape in the Brazilian Southeast

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ABSTRACT

The northeast area of São Paulo state was intensively deforested, resulting in a highly fragmented landscape composed of a few large patches and several small patches of natural vegetation surrounded by sugarcane, eucalyptus and citrus plantations. In this scenario, we investigated the puma's (*Puma concolor*) population size, sex ratio and relatedness in two of the last and largest natural refuges in the area using a noninvasive method during 2004-2008. By collecting and individualizing feces samples by microsatellites, we identified 17 distinct pumas in these areas; there were 13 females (76.4%) and four males (23.6%). Five females were sampled in distinct years and over an extended time and probably represented resident adults. By investigating the relatedness among individual pumas inhabiting the area, we found that only three animals were not related to each other. We also found evidence that young females may establish an adjacent/overlapping territory to their mothers (philopatry). During the sampling period, we registered 11 road-killed pumas in the region, 10 males and one female, and six events of human-puma conflict. The study area may acts as source of individuals that disperse across the matrix to occupy new home ranges, maintaining some degree of gene flow in a source-sink metapopulation structure. Finally, we recommend that puma management should be conducted at the landscape level to provide effective puma conservation in the northeast area of São Paulo state.

Key words: fecal DNA; metapopulation; microsatellites; noninvasive sampling; relatedness; road-kills; sex ratio.

THE NORTHEAST AREA OF SÃO PAULO STATE WAS ORIGINALLY COVERED BY CERRADO (SOUTH America savanna) and semideciduous forest (one of Atlantic Forest phytophysiognomy), but for the last three centuries, it has experienced distinct cycles of human exploitation of its fertile soils. During this period, coffee plantations, sugarcane crops, and the increasing urban population have led to an intensive deforestation of the original vegetation cover (Dean 1996). This occupation resulted in a disturbed landscape composed of few large native vegetation patches (>1000 ha) and diverse small patches (<50 ha), the minority of which are protected by the government (Ribeiro *et al.* 2009).

In this scenario, natural habitats were reduced or eliminated, forcing large carnivores to adapt to a new fragmented landscape. How these carnivores have adapted in the northeast area of São Paulo state is poorly investigated. Sensitive species like the jaguar (*Panthera onca*) went completely extinct, but more plastic species such as the puma (*Puma concolor*) and the maned wolf (*Chrysocyon brachyurus*) still inhabit small patches of natural vegetation in the area (Talamoni *et al.* 2000, Mantovani 2001, Miotto *et al.* 2007, Lyra-Jorge *et al.* 2008). In this context, populational, ecological and behavioral information is crucial for an adequate species management and to measure the success of these actions. With this kind of information, populational trends could be predicted to determine whether protected or remnant areas are maintaining viable populations of these species (Caughley 1994) and to guide efforts towards this goal.

Puma genetics, ecology, social organization and population dynamics have been extensively studied in North America (see as example, Ross and Jalkotzy 1992, Beier 1993, Roelke *et al.* 1993, Lindzey *et al.* 1994, Beier *et al.* 1995, Ruth *et al*. 1998, Ernest *et al.* 2000, Sweanor *et al.* 2000, Logan and Sweanor 2001, Anderson *et al.* 2004, MacRae *et al.* 2005), but there is a lack of studies in South America. In Brazil, the studies focus on food habits, habitat use (Emmons 1987, Ciocheti 2007, Lyra-Jorge *et al.* 2008), and puma-human conflicts (Mazzolli *et al.* 2002, Conforti and Azevedo 2003, Azevedo 2008).

Counting and identifying individual pumas is extremely difficult because they are elusive animals and live in low densities. Usually, estimates of puma population sizes, sex ratios or population dynamics are based on capture-recapture methods that track individuals through telemetry data (Beier *et al.* 1995, Sweanor *et al.* 2000) or on camera-trapping methods (Kelly *et al.* 2008, Paviolo *et al.* 2009). As an alternative, organic materials that individuals leave behind can be used to demographically monitor elusive species (Wayne and Morin 2004). New molecular techniques of DNA extraction applied to materials such as hair and feces allow us to analyze organisms in a noninvasive way (Morin and Woodruff 1996) and offer an alternative to identify and count individuals, in addition to giving information on sex ratio and dispersers (Kohn and Wayne 1997).

In a two-year pilot study, we detected the puma presence and minimum population size in two protected areas and their surroundings in the northeast area of São Paulo state (Miotto *et al*. 2007), but many questions remained unanswered, especially on the distribution of pumas in this fragmented area. In natural or artificial fragmented areas, pumas may exhibit a metapopulation structure (Beier 1993, Sweanor *et al.* 2000), simply defined as a network of subpopulations connected and dynamically affected by migration (Hanski and Simberloff 1997). Thus, we hypothesized that large remnant patches in the São Paulo human-disturbed landscape could act as a source of individuals that maintain a regional metapopulation structure. Because females may exhibit some degree of philopatry (Ross and Jalkotzy 1992, Logan and Sweanor 2001), we hypothesized that related females could be residents (*i.e.*, using the area during an extended and continuous period) in large-patch remnants, while some pumas, especially subadult or adult males, could disperse throughout the matrix to occupy distinct patches and maintain some degree of gene flow among these areas.

To test this hypothesis, we monitored a puma population inhabiting the largest cerrado vegetation patch of São Paulo state and some smaller patches surrounding it using a noninvasive method. By collecting and individualizing puma feces samples using microsatellites between 2004 and 2008, we aimed to recognize transient and resident individuals inhabiting the area. We also investigated the

relationship among the identified pumas to elucidate patterns of social organization, including philopatry and sex distribution in fragmented landscapes such as the northeast area of the state. Finally, considering that pumas may disperse throughout the matrix, we recorded the number of puma-human conflicts and road-killing events in the region during this period, aspects that may influence puma population maintenance and suggested some conservation actions for the species persistence in this landscape-structure scenario.

METHODS

STUDY AREA.—The study area is located in the Luís Antônio and Santa Rita do Passa Quatro municipalities, both in the northeast region of São Paulo state (21°30'–21°45' S, 47°20'–47°55' W) (Fig. 1). The area is located in a transition zone between cerrado and semideciduous vegetation in an approximately 20,000 ha area. It consists of two protected areas 3 km apart, the Jataí Ecological Station (JES) and the Vassununga State Park (VSP), in addition to some habitat patches on private properties. The JES (21°35'S, 47°48'W) encompasses 9010 ha and is the largest protected area of the state with continuous cerrado vegetation. The VSP total area (21°41'S, 47°34'W) is 2069 ha, subdivided in six distinct patches composed of cerrado physiognomies and semideciduous forest (Korman 2003). These areas are surrounded by sugarcane crops, cattle ranches, eucalyptus plantations, dirt roads and highways.

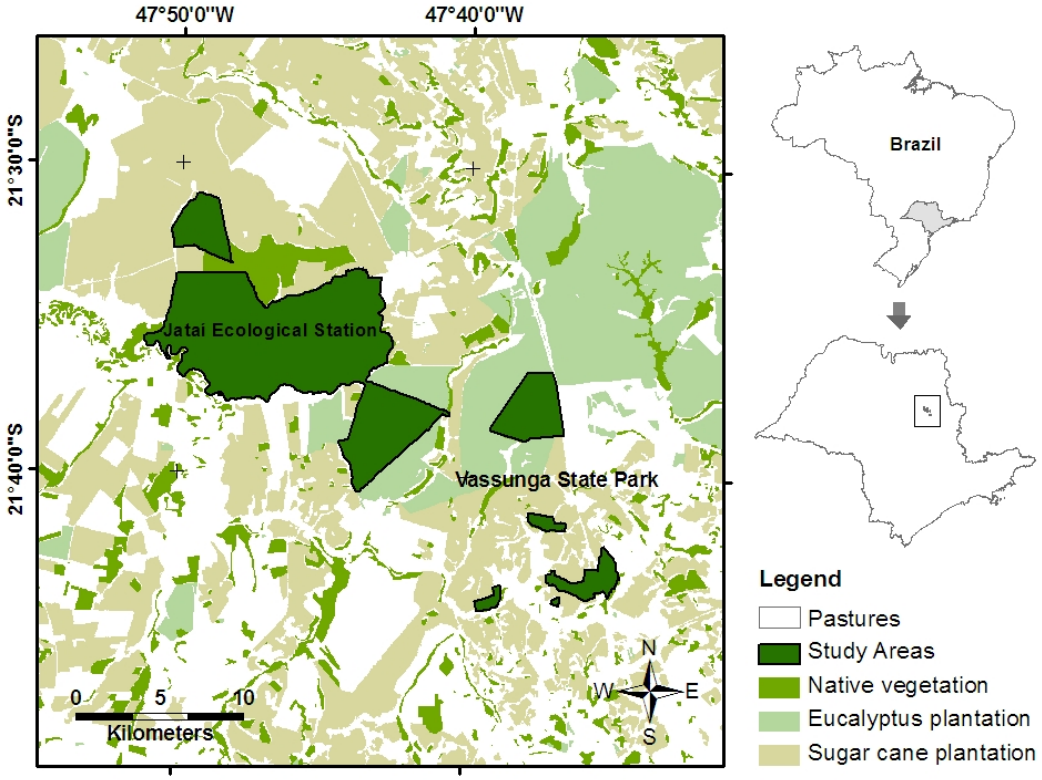

FIGURE 1. Northeast area of the São Paulo state region and its land-use classes.

SAMPLE COLLECTION.—Between October 2004 and December 2007, we conducted two-day field expeditions in the dry season of each year, when it is easier to find fresh samples (R. Miotto, pers. obs.). In 2008, we conducted field expeditions every month. Fragments from the study area are surrounded by dirt roads, while the JES in particular is intersected by dirt roads throughout, so during each expedition, we looked for feces on all of the dirt roads inside and surrounding the protected areas, including eucalyptus plantations and sugarcane crops, covering approximately 500 km of roads. All field work was conducted by two researchers. Based on tracks and the feces diameter/morphology (Chame 2003), we collected 75 potential puma fecal samples and recorded the coordinates (UTM-Datun SAD69) of all collection points in a Global Positioning System (GPS). Samples were stored in sterile preservative-free plastic tubes without any conservative solution and were kept at -22ºC in the laboratory until the DNA extraction was performed.

DNA EXTRACTION.—We extracted fecal DNA using the QIAmp DNA Stool Mini Kit (Qiagen) or PSP Spin Stool DNA Kit (Invitek) according to the manufacturer's recommendations. For the blood or tissue DNA extractions used as reference sequences in the genetic analysis, we followed the phenol/chloroform/isoamylic alcohol protocol proposed by Sambrook *et al.* (1989).

SPECIES IDENTIFICATION.—To confirm the species origin of the collected feces samples, we amplified a 146 bp portion from cytochrome b gene of the mitochondrial DNA (mtDNA) using primers described by Farrel *et al.* (2000) and methods described in Miotto *et al.* (2007).

SAMPLE FECES INDIVIDUALIZATION.—To individualize each fecal sample, we amplified a set of 12 species-specific microsatellite loci with primers developed by Kurushima *et al.* (2006): Pco C209, Pco D217, Pco D103, Pco C217, Pco C112, and Pco C108, all tetranucleotide-repeat microsatellite loci, and Pco B010, Pco B210, Pco A339, Pco A208, Pco A216, and Pco B003, dinucleotide-repeat microsatellite loci. Primers were marked with universal fluorescent M13 tails following Schuelke (2000). Each PCR reaction (15 μL) contained 7.5 μL of *GoTaq Master Mix* (Promega), containing 1x buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1u *Taq polymerase*, 8 pmol of reverse primer, 2 pmol of forward primer, 8 pmol of M13 sequence marked with the 6-FAM fluorophore, and 150 μg/mL of BSA. The remaining volume of the 15 μL reaction comprised DNA. We performed amplifications in a PTC-100 Thermocycler (MJ Research, Inc.) according to the following program (for all primer pairs): an initial denaturation cycle at 94°C for 5 min, 40 cycles at 92°C for 1 min, 48°C for 1 min, 72°C for 1 min, and a final 30 min extension at 72°C. Negative controls were included in all reactions to monitor possible contaminants. The resulting genotypes were analyzed in a MegaBACE ET-550R size standard automatic sequencer (GE Healthcare) with the Genetic Profile software. We conducted individual identifications of genotypes using the Gimlet software (Valière 2002).

To prevent misidentification due to allelic dropout, in which one of the two alleles of the individual is not detected (Taberlet *et al.* 1996), we genotyped a homozygote sample in three to five independent PCR reactions and genotyped heterozygotes twice randomly. For feces individualization, only samples that were successfully genotyped at least five loci were included in the analysis. To quantify the power of discrimination of the individuals through the microsatellite loci used, we determined the identity probability ($P_{(ID)}$), *i.e.*, the probability of two individuals in a population randomly sharing identical genotypes for all the analyzed loci (Paetkau *et al.* 1998, Waits *et al.* 2001). The $P_{(ID)}$ values were calculated for each locus in the Gimlet software (Valière 2002) and then multiplied by the total number of loci to obtain a total $P_{(ID)}$ (Paetkau *et al.* 1998). Once we expected to sample related animals in the study area, we estimated both the $P_{(ID)unbiased}$ and $P_{(ID)sib}$ equations, taking in account the population size and the presence of related individuals, respectively (Waits *et al.* 2001). We determined the total and per-locus genotyping error rates (allelic dropout) by dividing the number of detected errors by the number of cases in which an error might have been detected (*i.e.*, the total number of genotyping reactions).

SAMPLE FECES SEX IDENTIFICATION.—To identify the sexes of samples, we amplified a portion of the amelogenin gene present in both sex chromosomes with primers described by Pilgrim *et al.* (2005). In this gene fragment, males have a 20 bp deletion in the Y-chromosome copy, and consequently produce PCR products of different sizes, while females amplify fragments of the same size. As suggested by Pilgrim *et al.* (2005) and Lynch and Brown (2006), to prevent false positive for females, we only conducted sex identification reactions in samples with consistent microsatellite amplification and also repeated each reaction three times to avoid allelic dropout.

RELATEDNESS.—We estimated the genetic relatedness and relationships among individual pumas using the ML-Relate software (Kalinowski *et al.* 2006). This program calculates maximum likelihood estimates

of relatedness (*r*) (Blouin 2003) and discriminates four common pedigree relationships: unrelated (U), half siblings (HS), full siblings (FS) and parent-offspring (PO). We estimated a putative relationship between individual pairs; when a relationship was different from the unrelated category, we used this program to assess the uncertainty (p-values) surrounding estimates by testing two a priori hypotheses: a putative relationship with a higher likelihood (HS, FS or PO) against an alternative hypothesis that the pumas were unrelated. When pairwise comparisons had a low p-value ($p<0.05$), we excluded the alternative hypothesis and accepted the relationship with the maximum likelihood (HS, FS or PO). Considering that we did not have a group of animals with known relationships to calibrate the program, rather than define exact relationships categories, we instead aimed to verify the existence of related animals in our data set.

PUMA ROAD-KILLINGS AND HUMAN CONFLICTS.—Considering that pumas from the studied area may disperse throughout the matrix, during the feces sample collection period, we also recorded all human conflict and road-kill events in the region by maintaining contact with the Forest Police, highways administration services and veterinary hospitals that might receive injured or dead animals. We recorded the date, sex and coordinates of each dead or captured animal and also collected blood or tissue samples for further genetic analysis.

RESULTS

Between October 2004 and December 2008, we collected 75 feces samples in the study area and successfully extracted DNA from 62 samples (82.6%). In 47 samples (75.8%), the mtDNA fragment was amplified, allowing us to identify the sample species origin. By comparing cytochrome b fragments obtained from fecal DNA with puma and other carnivore reference sequences (Miotto *et al.* 2007), we identified 6 samples belonging to ocelots (*Leopardus pardalis*), a sympatric species, and 41 belonging to pumas (Fig. 2). There was low similarity between the sequences obtained from feces, and the

sequences of other carnivores present in the study area (data not shown). We only amplified microsatellites for the 41 feces whose species were identified as puma.

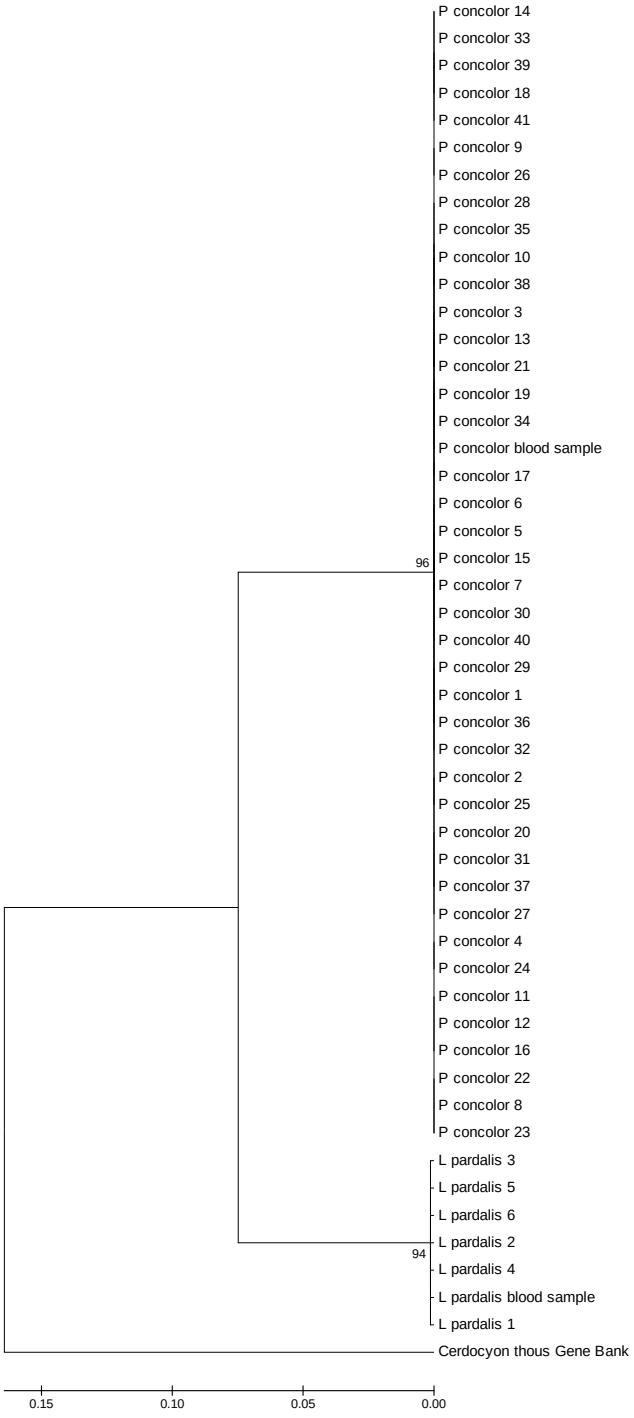

FIGURE 2. Neighbor-joining tree of reference blood samples and fecal cytochrome *b* sequences with *Cerdocyon thous* as an outgroup. The bootstrap values, based on 1,000 replicates, are shown over the branches.

To individualize feces samples, we initially tested a set of 12 microsatellite primers, but five primer pairs (Pco C209, Pco D217, Pco D103, Pco C217, and Pco C112, all tetranucleotide-repeats) had low amplification success and were removed from the analysis. For the seven remaining loci, the genotyping success was higher than 80% per locus. We quantified a general rate of 10.03% for the allelic dropout rate and estimated a total $P_{(ID)unbiased}$ of 6.994×10^{-9} and a total $P_{(ID)sib}$ of 1.853×10^{-3}. The range size, number of alleles per locus, allelic dropout rates and $P_{(ID)}$ values per locus are described in Table 1.

TABLE 1. *The range size, number of alleles, probability of identity, and total and per locus allelic dropout rates for the seven microsatellite loci analyzed.*

Locus	Range size (bp)	Alleles number	$P_{(ID)unbiased}$ values	$P_{(ID)sib}$ values	Allelic dropout (%)
C108	124-160	4	1.442×10^{-1}	4.626×10^{-1}	10.81
B010	203-229	8	5.010×10^{-2}	3.998×10^{-1}	5.40
B210	165-177	7	6.356×10^{-2}	4.040×10^{-1}	15.9
A216	237-251	6	4.686×10^{-2}	3.738×10^{-1}	6.06
A208	187-201	6	8.092×10^{-2}	4.117×10^{-1}	17.7
B003	279-303	6	1.231×10^{-1}	4.497×10^{-1}	5.1
A339	264-280	7	3.265×10^{-2}	3.583×10^{-1}	9.3
Mean/Total	-	-	6.994×10^{-9}	1.853×10^{-3}	10.03

For the 41 analyzed feces, two samples were amplified at fewer than five loci and were removed from the final individualization analysis. Among the remaining 39 analyzed feces, 17 distinct pumas were identified in the study area (Fig. 3). Only three individuals were sampled once; the other 14 were sampled at least twice. The individualized samples, their sex, and the sampling replication dates are described in Table 2.

More females (13; 76.4%) were sampled than males (4; 23.6%). Considering that male and female subadult pumas may disperse from their natal home-range with 16 - 22 months of age (Logan and Sweanor 2001), we identified five females that are probably residents in the study area: Pumas 1, 2, 4, 6 and 15. These females were sampled in distinct years and over an extended time period (Table 2),

longer than the maternal care period (14-19 months; Logan and Sweanor 2001); thus, they were probably not cubs anymore. On the other hand, male replicates did not exceed 11 months between sampling dates (the maximum sampling period for Puma 3). We did not find evidence of resident males, but this may be a consequence of the sampling effort.

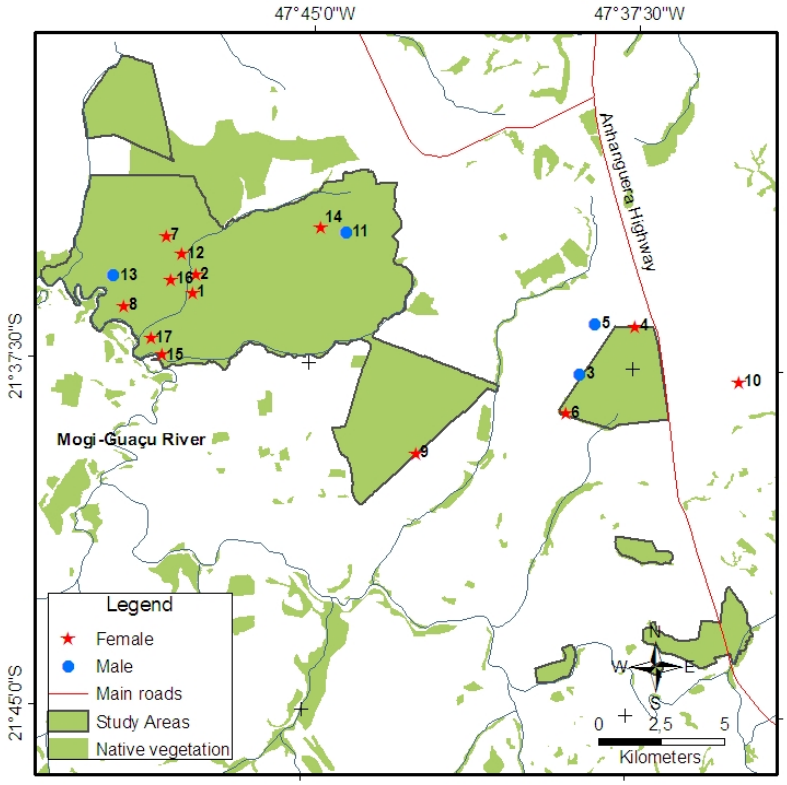

FIGURE 3. Individual pumas (n=17) identified through fecal DNA analyses in the Jataí Ecological Station, Vassununga State Park and surrounding areas. Each number corresponds to one animal.

By investigating the genetic relationship patterns among all possible pair combinations, we found the following pedigree categories for pumas inhabiting the study area: 2.9% PO, 5.1% FS, 3.6% HS and 88.4% U. Because we did not calibrate the program with previously known relationships, the PO relationship may be the most accurate. By excluding mutation, the PO pairs must share at least one allele at every locus (Blouin *et al.* 1996). In this sense, we found evidence that a young female may establish an adjacent/overlapping territory to her mother (philopatry): Pumas 1 and 2 are mother and

offspring, and both are residents in the area (Table 2). Only three animals did not exhibit any degree of relatedness with the other sampled puma: Pumas 5, 9 and 10; all of the remaining animals were related to each other.

TABLE 2. *Puma fecal samples (n=39) attributed to 17 distinct individuals inhabiting the Jataí Ecological Station, Vassununga State Park and surrounding areas and the sex and date of each collected sample/replicate.*

Individual	Sex	1st Collection	1st Replicate	2nd Replicate	3rd Replicate	4th Replicate
Puma 1	Female	Oct 2004	Jun 2005	Jul 2006	Aug 2008	Oct 2008
Puma 2	Female	Oct 2004	Nov 2005	May 2008	-	-
Puma 3	Male	May 2005	Apr 2006	-	-	-
Puma 4	Female	May 2005	Apr 2007	Mar 2008	-	-
Puma 5	Male	Jun 2008	Jul 2008	Aug 2008	-	-
Puma 6	Female	Jul 2006	Jul 2008	-	-	-
Puma 7	Female	Apr 2007	May 2007	May 2008	-	-
Puma 8	Female	May 2007	Jun 2007	-	-	-
Puma 9	Female	Mar 2008	Aug 2008	-	-	-
Puma 10	Female	Mar 2008	Dec 2008	-	-	-
Puma 11	Male	Jun 2008	Sep 2008	-	-	-
Puma 12	Female	May 2008	Jul 2008	-	-	-
Puma 13	Male	May 2008	-	-	-	-
Puma 14	Female	May 2008	-	-	-	-
Puma 15	Female	May 2005	Apr 2007	Jul 2008	-	-
Puma 16	Female	Sep 2008	-	-	-	-
Puma 17	Female	Oct 2008	Dec 2008	-	-	-

During the fecal sampling period, we registered 11 road-killed pumas in the region, 10 males and one female (Fig. 4). We also recorded six events of human-puma conflict. One male was captured near a residential area in the Araraquara municipality; another male was captured close to the urban area of Sales Oliveira; in addition, two females were captured by farmers after livestock predation in the Rincão and Analândia municipalities. These four animals were released into the Jataí Ecological Station or into the surrounding areas. Two male cubs were captured in a sugarcane plantation during the land burning

in Jaboticabal. A third male cub was captured in a sugarcane plantation in Serrana after his mother was run over by a harvest machine. These cubs were transferred to zoos.

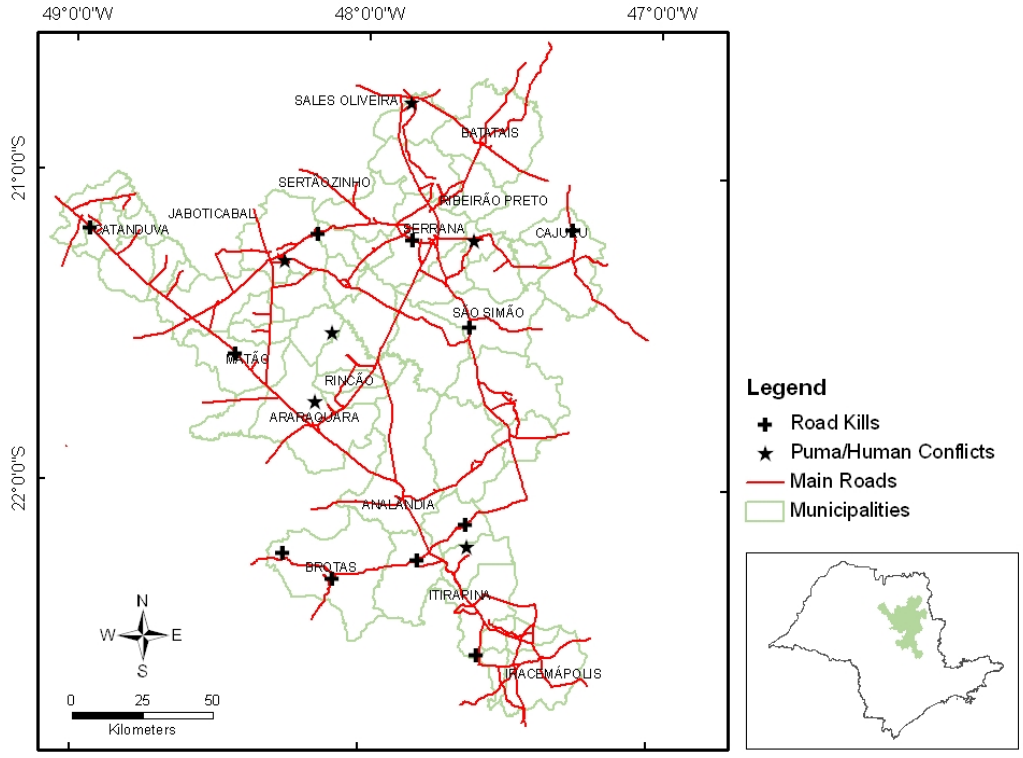

FIGURE 4. Localization of road-kill and puma-human conflict events in the northeast area of São Paulo state during the scat collection period (2004-2008).

DISCUSSION

GENETIC ANALYSIS.—In noninvasive analyses, special attention must be paid to errors that commonly occur in the microsatellite analysis of materials, such as hair and feces, because they may affect the correct identification of individual samples (Taberlet *et al.* 1996, 1999; Waits *et al.* 2001, Creel *et al.* 2003, Broquet and Petit 2004, Prugh *et al*. 2005). To avoid errors in our analysis, we established some conditions to obtain consistent genotypes: (1) we randomly genotyped the majority of heterozygotes twice and confirmed homozygotes genotypes with three to five independent PCR reactions; (2) we included a locus in our analysis only if more than 80% of samples were successfully genotyped; and (3)

in the final analysis, we only included those samples that were genotyped for at least 80% of all the analyzed loci.

Allelic dropout has been reported in almost all noninvasive studies with rates from 0 to 39% (Gagneux *et al.* 1997, Kohn and Wayne 1997, Taberlet *et al.* 1996, 1999; Ernest *et al.* 2000, Morin *et al.* 2001, Miotto *et al.* 2007). In this study, we observed allelic dropout at all of the analyzed loci, from low to moderate rates (5.1%-17%). To identify and quantify these events, several genotyping replicates are recommended in the literature (Taberlet *et al.* 1999, Waits *et al.* 2001, Creel *et al.* 2003, Broquet and Petit 2004, Piggot *et al.* 2004, Prugh *et al*. 2005, Roon *et al.* 2005). Although we repeated each amplification up to five times, in most cases we obtained consistent genotypes from three repetitions, the same number of repetitions reported by Janečka *et al.* (2008) as a reasonable and cost-effective number.

The estimated values for the probability of identity were a $P_{(ID)unbiased}$ of 6.994×10^{-9} and a $P_{(ID)sib}$ of 1.853×10^{-3} for the seven analyzed loci. These values indicate that the analyzed loci were able to successfully distinguish each individual because both values are less than or similar to the values that are considered satisfactory for individualization in noninvasive genotyping (0.001; Waits *et al.* 2001). Considering that some sampled individuals in the study area were related to each other, the $P_{(ID)sib}$ estimate may be more appropriate because it is a less biased estimate for populations composed of closely related individuals (Waits *et al*. 2001).

PUMAS SEX RATIO.—Pumas have a polyginous and promiscuous mating system. Sex ratio for pumas litters are typically 1:1 (Logan and Sweanor 2001). In the study area, we observed a higher number of females (76.4%) than males (23.6%). Unfortunately, we could not verify the sex ratio according to the age structure, but a higher number of adult females in North American puma populations has been described in the literature (Ross and Jalkotzy 1992, Lindzey *et al.* 1994, Logan and Sweanor 2001).

Subadult sex ratios tend to favor females probably due to higher mortality and emigration rates among males (Logan and Sweanor 2001).

In a large continuous desert mountain range, Logan and Sweanor (2001) estimated the chance of a male subadult surviving to the age of adulthood at 56%. In contrast to large continuous areas in which dispersing subadults primarily must avoid encounters with territorial adult males to survive (Logan and Sweanor 2001), subadult males dispersing in fragmented and human-dominated landscapes also must cross inhospitable habitats to establish their new home range. As we observed in the northeast area of São Paulo state, dispersers are adversely affected by conflicts with humans or human activities such as vehicle collisions on roads. The majority of road-killed pumas we documented were subadult males, and this may have contributed to the female-biased sex ratio in the area.

From the 17 identified pumas in the study area, only three animals did not exhibit some degree of relatedness (Pumas 5, 9, 10). By analyzing the sampling periods and relationship categories, we clearly distinguished a phylopatric case in which a female resident parent-offspring pair established an adjacent or overlapping home range (Pumas 1 and 2). Female philopatry has been documented in previous studies (Ross and Jalkotzy 1992, Logan and Sweanor 2001). In contrast, males tend to disperse to distant areas to avoid competition and inbreeding (Logan and Sweanor 2001). Logan and Sweanor (2001) documented several cases of mothers and phylopatric adult daughters occupying adjacent or overlapping home ranges and forming matrilines, while male offspring dispersed on average eight times the distance of females from their natal home ranges to adult home ranges. These authors found that matrilineal females had higher reproductive success and suspect that philopatry was an adaptative behaviour because cubs of matrilineal mothers had higher survival rates (67%) than cubs of non-matrilineal mothers (56%). By examining the puma spatial distribution in the JES (Fig. 3), we noticed that several females used an area closer to the Mogi-Guaçú River and some small oxbow lakes surrounding the southern portion of the protected area. Due to the proximity of scat from different females, it may be possible that pumas also form matrilines in the JES as well, but unfortunately,

defining age structure is not possible using molecular markers, and matrilines could not be identified. Mark/recapture methods would be more appropriate for obtaining this kind of information.

Some authors suggest that pumas may exhibit a metapopulation structure (Beier 1993, Sweanor *et al.* 2000). According to this theory, a space could be divided into patches of habitat surrounded by an unsuitable matrix that individuals could transverse; thus, dispersing and migrating individuals connect demographically distinct subpopulations into a network (Levins 1969, Elmhagen and Angerbjörn 2001). Even though metapopulations are difficult to detect in large mammals populations (see Elmhagen and Angerbjörn 2001), in some situations, a metapopulation-dynamics approach could be useful for conservation biology.

In the northeast area of São Paulo state, geographic patches differ in their quality, and the application of source/sink metapopulation dynamics model may be reasonable for conservation planning. In this modern theorical model, some patches consist of source habitats where local populations have positive growth rates, while other patches consist of sink habitats with negative growth rates (Elmhagen and Angerbjörn 2001). By identifying resident pumas and a large number of animals concentrated in a relatively small but high-quality area, and considering that the JES and VSP areas are the largest patches in the region, we suggest that these protected areas together may act as a source of individuals in a regional source/sink metapopulation structure. Pumas are probably dispersing throughout the matrix and colonizing/recolonizing surrounding smaller patches.

CONSERVATION IMPLICATIONS.—Since its colonization three centuries ago, the northeast area of São Paulo state has been covered by coffee plantations. In the 1970's, the Brazilian government created the *Pró-álcool* program to substitute the use of fossil fuels with ethanol in vehicles. With government incentives, a large number of sugarcane plantations were established in the northeast area of São Paulo. Today, this number is still increasing, especially to supply the production of biofuels, which are thought to be a less harmful fuel alternative for environment pollution. For almost 40 years, remnant

natural vegetation patches and coffee plantations have been replaced by sugarcane plantations that now dominate the land-use in the area, followed by eucalyptus and citrus plantations.

The JES and VSP are two of the last and largest natural refuges for many species of ungulates such as peccaries and deer, important prey items in the puma diet, in the northeast area of São Paulo state (Mantovani 2001, Novack *et al*. 2005, Ciocheti 2007). With high prey abundance, these refuges represent high-quality habitat patches in the matrix, which probably allows pumas to be concentrated in the area and to tolerate overlapping home ranges. Females (the majority of sampled pumas) tend to require smaller areas and tolerate higher home-range overlap when many resources are available (Logan and Sweanor 2001).

A previous study in the area demonstrated the use of eucalyptus plantations by pumas in their movements (Lyra-Jorge *et al*. 2008). These plantations probably do not act as habitats but may contribute to puma dispersion throughout the landscape because they may be one of the most permeable elements in human-dominated landscapes (Lyra-Jorge *et al.* 2008). However, some management practices are necessary to maintain animal movements. In Brazil, eucalyptus plantations are usually composed of several square stands, and they are managed approximately every seven years. Acting as corridors for puma dispersion, we suggest an alternate management of these stands because, when a large number of them are cut down, all animals are frightened away from the area (R. Miotto, pers. obs.). An alternating cutting approach may not result in large open areas that discourage animal movements and may instead increase the connectivity, as suggested by Lindenmayer *et al.* (2000) and Fischer *et al.* (2006).

Unfortunately, this type of information is not available for sugarcane plantations. Although Dotta and Verdade (2007) did not find any puma tracks in sugarcane crops in an area close to our study area, we collected some fecal samples and registered two events of very young puma cubs rescued on plantations, indicating that pumas are dispersing across these areas and even may be using them to raise young. Sugarcane plantation management occurs every seven months, typically in the form of

burning. There are no available data on animal deaths during burning periods, but we believe that pumas and other species may be strongly affected by this practice.

Pumas tend to avoid paved roads (Dickinson *et al.* 2005, Markovhick-Nicholls *et al.* 2008), but roads do not prevent their movement (Dickinson *et al*. 2005). In fact, we recorded only one puma road-kill on a major road in the study area, the Anhanguera Highway, with approximately 8000 cars/day (DER 2008). Also, Puma 10 was sampled on the other side of this road (Fig. 3), and we found no evidence of this animal crossing it in the JES direction. However, the absence of suitable habitat in the area is probably forcing pumas to cross roads more frequently. The majority of vehicle collision events happened on smaller paved roads connecting municipalities (Fig. 4).

Logan and Sweanor (2001) observed that, in a large, naturally protected area, intraspecies conflict (*i.e.*, pumas killing other pumas to defend their cubs or territory or direct competition for food) is the major cause of male and female puma mortality, followed by disease, accidents and old age. As these authors suggest, intraspecies conflict may occur in exploited and non-exploited populations, but apparently, in human-disturbed areas, vehicle strikes may be an important factor contributing to puma mortality. Even not knowing the influence of different factors on puma mortality in the area, such as hunting, prey abundance, strife or disease, we assume that those 11 registered cases indicate that vehicle strikes have a strong influence on puma survival, especially for subadult males. Thus, the implementation of highway crossing structures, such as underpasses, may be crucial for puma persistence in the northeast area of the state. Because many more roads in the state are being built, the construction of crossing structures in preferred habitats should be a prerequisite for these agencies when they request their governmental environmental license for road construction.

Today, Brazilian laws state that owners must conserve riparian vegetation, hilltops and at least 20% of all property area with natural vegetation, but government control is scarce and rules are not always respected. High-quality patches are 70 km distant from JES and VSP areas; thus, the conservation of small habitat patches on every property could contribute to puma dispersion because it should reduce

the risks of crossing anthropogenic habitats, increasing gene flow and maintaining the puma evolutionary potential. Large remnants like the JES and VSP are extremely important because they can act as source areas, but is necessary to connect them with different core areas to maintain viable populations.

During a four-year sampling period in an approximately 20,000 ha area, we identified 17 distinct pumas. This result suggests that the JES and VSP are high-quality patches and also that pumas may be confined in these areas once there are no surrounding patches of similar quality. Fragments larger than 2,000 ha are at least 70 km distant from the JES and VSP areas, and there are urban centers among them. Smaller patches could enhance the connectivity among these areas and surrounding ones, increasing the landscape complexity by acting as stepping stones or creating networks of functionally connected areas, not just for pumas but for a variety of species. In a source/sink metapopulation structure, the persistence of demographically isolated subpopulations depends on immigration. As the quality of the matrix increases, barriers to migration decrease, and thus, the migration rate increases (Vandermeer and Carvajal 2001). Being good disperses and generalist animals, pumas are capable of crossing a diversity of land-use classes, but some issues may mitigate the high levels of vehicle collisions and human conflicts observed in the study area. Future studies on the identification and mapping of elements that could enhance connectivity (Vogt *et al.* 2009) and possible dispersal routes (Wikramanayake *et al.* 2004, Epps *et al.* 2007) and proposals of mitigation actions on preferred habitats could provide support for conservation planning. We strongly recommend that effective puma conservation in the northeast area of São Paulo state should be based primarily on a landscape-scenario management.

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CAPÍTULO 2

Running head: Puma density in southeastern Brazil

ESTIMATING PUMA *(PUMA CONCOLOR)* DENSITY IN A HUMAN-DISTURBED LANDSCAPE IN SOUTHEASTERN BRAZIL BASED ON DNA-MARK-RECAPTURE DATA

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ABSTRACT

Genetic and ecological aspects of pumas are well documented in North America, but there is a lack of studies in South America, including Brazil. By means of a noninvasive method, fecal DNA analysis, we estimated puma abundance and density in two protected areas embedded in a human-disturbed landscape in the northeast area of São Paulo state, Brazil. In eight months of mark-recapture feces sampling, fifteen animals were individualized for seven microsatellite loci ($P_{(ID)unbiased}$= 6 × 10^{-9}; $P_{(ID)sib}$ = 0.001). The estimated abundance of pumas with the Jolly-Seber open population model was 24.34 (± 6.62 *SE*). The estimated density of pumas per 100 km^2 was 9.36 ± 2.54 *SE*. This is the first density estimate of pumas in a human-dominated landscape in Brazil and the highest estimate among previous South American studies. The high number of pumas concentrated in a relatively small area (260 km^2) is probably a consequence of the absence of suitable habitats in the northeast area of the state and the absence of direct competitors. The high abundance may also reflect large prey availability in the two protected areas that formed the study area because the landscape was dominated by extensive sugarcane field crops and eucalyptus plantations. The density estimated in this study is one of the principles that may contribute to the monitoring of puma populations over the years, and combined with other ecological, behavioral and genetic data, it may guide conservation actions that could maintain viable populations in the future in the northeast region of São Paulo state.

Key-words: fecal DNA, Jolly-Seber model, MARK, microsatellites, mitochondrial DNA, noninvasive analysis

In the last century, the northeast area of São Paulo state has changed drastically (Dean 1996; Martinelli and Filoso 2008). The development of urban centers, the emerging sugarcane and eucalyptus plantations, and the extensive enlargement of the road network has resulted in considerable habitat loss and fragmentation of natural vegetation cover.

Despite this intensive human landscape transformation, some large carnivore species, such as the puma (*Puma concolor*), still inhabit the area (Lyra-Jorge et al. 2008; Miotto et al. 2007). How these animals still occupy the remnant fragments and their population sizes are poorly investigated. The population size and density estimates of puma populations are important for planning conservation and management actions, especially in human-dominated landscapes. Also, monitoring population size over the years may allow managers to predict population tendencies and the success of conservation actions (Caughley 1994; Karanth et al. 2006; Kelly et al. 2008).

Usually, mark-recapture methods have been used to estimate population size and abundance of large carnivores based on camera-traps (Karanth and Nichols 1998; Kelly et al. 2008; Paviolo et al. 2009; Silveira et al. 2009; Silver et al. 2004; Trolle et al. 2003, 2007) or radio-telemetry tracking data (Franklin et al. 1999; Soislaio and Cavalcanti 2006; Sweanor et al. 2000).

As an alternative, noninvasive genetic sampling has became a powerful tool for studying and monitoring elusive and low-density species (Schwartz et al. 2007; Waits and Paetkau 2005). DNA from sources such as hair or feces can be used as molecular tags in mark-recapture population censuses (Bellemain et al. 2005; Boulanger et al. 2008; De Barba et al. 2010; Prugh et al. 2005). Collecting noninvasive samples is equivalent to capturing the animal that deposited the genetic sample (Prugh et al. 2005); therefore the probability of encountering feces can be referred to as the capture probability. Because each animal has a unique multilocus genotype, each individual can be identified; then, closed or open-population models can be applied according to the sampling period, births, deaths, and emigration and immigration occurrences to estimate population parameters such as abundance, survival or recruitment.

The puma is the most widely terrestrial mammal distributed in the Americas (Iriarte et al. 1990). Their populations have been extensively studied in North America (see Anderson et al. 2004; Beier 1993; Beier et al. 1995; Ernest et al. 2000; Lindzey et al. 1994; Logan and Sweanor 2001; MacRae et al. 2005; Roelke et al. 1993; Ross and Jalkotzy 1992; Ruth et al. 1998), but such studies have been lacking in South America. Puma population sizes and densities were estimated by a few studies, including Franklin et al. (1999) in Patagonia, Chile, Kelly et al. (2008) in Bolívia, Argentina and Belize, and Paviolo et al. (2009) in the Green Corridor of Atlantic Forest in Misiones Province in Argentina, but there have been no studies of population size in Brazilian territory, especially in human-disturbed areas. In Brazil, studies about pumas have concentrated exclusively on their food habits or habitats use (Ciocheti 2007; Emmons 1987; Lyra-Jorge et al. 2008), movements (Mantovani 2001; Schaller and Crawshaw 1980) and puma-human conflicts (Azevedo 2008; Conforti and Azevedo 2003; Mazzolli et al. 2002).

Here, we present population size and density estimates of pumas inhabiting a human-disturbed landscape in the northeastern region of São Paulo state, Brazil, based on a DNA mark-recapture method. Through microsatellites, we individualized marked and recaptured puma feces samples and estimated population abundance by applying an open-population model framework.

MATERIAL AND METHODS

Study area.— The study area encompasses approximately 260 km^2 and is located in the Luís Antônio and Santa Rita do Passa Quatro municipalities, both in the northeastern region of São Paulo state (21°30'–21°45'S and 47°20'–47°55'W) (Fig. 1). The area is characterized as transitional between cerrado and semideciduous forest vegetation and possesses two protected areas 3 km apart, the Jataí Ecological Station (JES) and the Vassununga State Park (VSP), in addition to some habitat patches on private properties. The JES (21°35'S–47°48'W) encompasses 9,000 ha and is the largest protected area of the state with continuous cerrado vegetation. The total area of the VSP (21°41'S-4734'W) is

2,070 ha, subdivided in six distinct patches composed of cerrado physiognomies and semideciduous forest (Korman 2003). In this protected area, we focused the collection effort only in the higher patch, the 'Pé-de-Gigante' patch, which has an area of 1,210 ha. These areas are surrounded by sugarcane crops, cattle ranches, eucalyptus plantations, dirt roads and highways but still retain high faunal diversity (Talamoni et al. 2000).

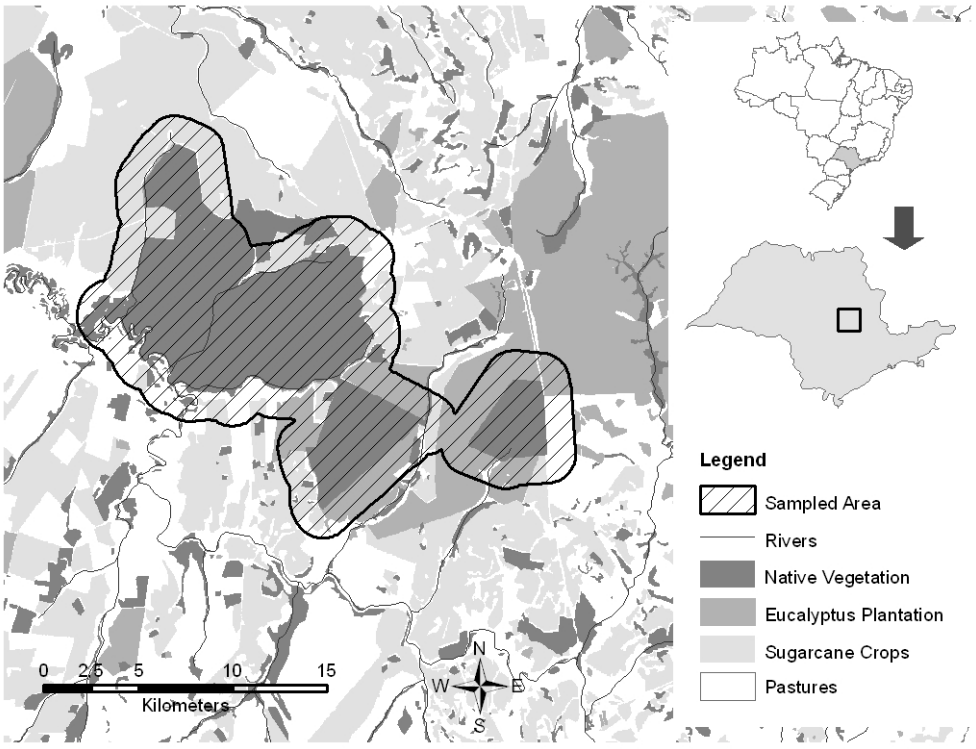

Fig. 1—Northeastern region of São Paulo state and its land use classes.

Sample collection.— We conducted monthly two-day field expeditions from March to October 2008. Fragments from the study area are surrounded by dirt roads, while the JES in particular is intersected by dirt roads throughout. Pumas tend to use these roads to move from place to place (Dickinson et al. 2005) and usually defecate on them, probably to mark their territory. Thus, we looked for feces on all of the dirt roads inside and surrounding the protected areas, including eucalyptus plantations and sugarcane crops, covering approximately 500 km of roads. Based on tracks and the feces diameter/morphology, we collected 37 potential puma fecal samples and recorded the coordinates of all

collection sites in a Global Positioning System (GPS). Samples were stored in sterile, preservative-free plastic tubes without any conservation solution and kept at -22°C in the laboratory until the DNA extraction was performed.

DNA extraction.— The fecal DNA was extracted using the QIAmp DNA Stool Mini Kit (Qiagen) or PSP Spin Stool DNA Kit (Invitek), following manufacturer's recommendations. For the blood and tissue DNA extractions used as reference sequences in the genetic analysis, we followed the phenol/chloroform/isoamylic alcohol protocol proposed by Sambrook et al. (1989).

Species identification.— To confirm the species origin of the collected feces samples, we amplified a 146 bp portion from the cytochrome b gene of the mitochondrial DNA (mtDNA) using primers described by Farrel et al. (2000). The amplifications were performed in a PTC-100 Thermocycler (MJ Research, Inc.), and negative controls were included in all reactions to monitor possible contaminations. The PCR products were sequenced in a MegaBACE ET-550R Size Standard automatic sequencer (GE Healthcare). Obtained sequences were aligned using the Clustal X 1.81 software (Thompson et al. 1997) and visually verified and edited in BioEdit 5.9 software (Hall 1999). The sequences were then compared with puma reference sequences available in GenBank (AF266475, DQ469952) and with sequences from other sympatric carnivores as described in Miotto et al. (2007).

Feces sample individualization.— To individualize each fecal sample, we amplified a set of seven species-specific microsatellite loci with primers developed by Kurushima et al. (2006): Pco C108, a tetranucleotide-repeat microsatellite locus, and Pco B010, Pco B210, Pco A339, Pco A208, Pco A216, and Pco B003, which were dinucleotide-repeat microsatellite loci. Primers were marked with universal fluorescent M13 tails following Schuelke (2000). Each PCR reaction (15 μL) contained 7.5 μL of *GoTaq Master Mix* (Promega), containing 1x buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 u *Taq polymerase*; 8

pmol of reverse primer, 2 pmol of forward primer, 8 pmol of M13 sequence marked with the 6-FAM fluorophore, and 150 μg/mL of BSA. The remaining volume of the 15 μL reaction was comprised of DNA. Amplifications were performed in a PTC-100 Thermocycler (MJ Research, Inc.) according to the following protocol (for all primer pairs): an initial denaturation cycle at 94°C for 5 min, 40 cycles at 92°C for 1 min, 48°C for 1 min, and 72°C for 1 min, and a final 30 min extension at 72°C. Negative controls were included in all reactions to monitor possible contamination. The resulting genotypes were analyzed in a MegaBACE ET-550R size standard automatic sequencer (GE Healthcare) with the Genetic Profile software. We conducted individual identifications of genotypes using the Gimlet software (Valière 2002).

To prevent misidentification due to allelic dropout, in which one of the two alleles of the individual is not detected (Taberlet et al., 1996), we genotyped a homozygote sample in three to five independent PCR reactions and genotyped heterozygotes twice randomly. For feces individualization, only samples that were successfully genotyped for at least five loci were included in the analysis. To quantify the power of discrimination of the individuals through the microsatellite loci used, we determined the identity probability ($P_{(ID)}$), i.e., the probability of two individuals in a population randomly sharing identical genotypes for all the analyzed loci (Paetkau et al., 1998; Waits et al., 2001). The $P_{(ID)}$ values were calculated for each locus in the Gimlet software (Valière, 2002) and then multiplied by the total number of loci to obtain a total $P_{(ID)}$ (Paetkau et al., 1998). Because we expected to sample related animals in the study area, we estimated both the $P_{(ID)unbiased}$ and $P_{(ID)sib}$ equations, taking into account the population size and the presence of related individuals, respectively (Waits et al., 2001). We determined the total and per-locus genotyping error rates (allelic dropout) by dividing the number of detected errors by the number of cases in which an error might have been detected (i.e., the total number of genotyping reactions).

Abundance and density of pumas.— After individualizing puma feces samples, a capture-recapture history of each animal was established for the eight month sampling period. To estimate the abundance,

we opted for a non-closed population model because we assumed that animals could leave or immigrate into the study area during the sampling period. Pumas are vagile and have wide home ranges, and although we may have sampled resident animals in the study area, we had previous information that suggested that some animals may frequently disperse throughout the matrix (Miotto et al. in prep.). We estimated abundance with the Jolly-Seber probability model (JS) (Jolly 1965; Seber 1965) using the POPAN formulation (Schwarz and Arnason 1996), implemented in the MARK software (White and Burnham 1999). This open population model assumes the existence of a 'super-population' (N) in which (i) unmarked animals have the same probability of capture as marked animals in the population (assumption of equal catchability); (ii) survival rates are homogeneous for marked and unmarked animals; (iii) the study area is constant; and (iv) there is no loss of mark (genetic tag in our case) during the sampling period. Parameters of this model are (i) p_i , the probability of capture of marked and unmarked individuals on occasion i; (ii) ϕ_i,, the probability of survival of marked and unmarked individuals from occasion i to i+1; and (iii) b_i, the probability of a new animal entering the population between occasions i and i+1. We selected between eight models, all nested inside the global model, allowing for temporal variation in all three parameters [p(t)ϕ(t)b(t)N(*)]. The tested models are described in Table 1. Models were ranked using the Akaike Information Criterion (AICc) adjusted for small population sizes. The model with the lowest AICc value was considered the model that best balanced bias and precision (White and Burnham 1999) and, therefore, the most appropriate model for an accurate abundance estimation, which was estimated as a derived parameter. To estimate puma density, we divided estimated abundance by the effective sampled area (~260 km^2), including the JES and VSP, plus the eucalyptus plantations and sugarcane crops surveyed.

TABLE 1. Model selection results for the Jolly-Seber model analysis of pumas capture data in the northeast area of the São Paulo state.

Model	AICc	Delta AICc	AICc Weight	Model likelihood	Parameters	Deviance
p(*)ϕ(*)b(t) N(.)	85.5812	0.0000	0.9999	1.0000	4	8.0974
p(t) ϕ(*)b(*)N(.)	108.0453	22.4641	0.0001	0.0000	9	9.8096
p(t)ϕ(*)b(t) N(.)	117.3066	31.7254	0.0000	0.0000	11	5.9280
p(*)ϕ(t)b(t) N(.)	117.6137	32.0325	0.0000	0.0000	11	6.2351
p(t)ϕ(t)b(t) N(.)	245.3125	128.0099	0.0000	0.0000	18	5.1379
p(*)ϕ(t)b(*)N(.)	4221.5951	4136.0139	0.0000	0.0000	8	4128.6165
p(*)ϕ(*)b(*)N(.)	4222.9016	4137.3204	0.0000	0.0000	3	4148.3231
p(t)ϕ(t)b(*)N(.)	4283.9946	4198.4134	0.0000	0.0000	15	4126.6160

RESULTS

Genetic analysis.— We collected 37 feces samples in the study area and successfully extracted DNA from 32 samples (86.48%). In 27 samples, the *mtDNA* was amplified (84.37%). By comparing *mtDNA* sequenced fragments, we identified 24 puma feces. Three samples belonging to ocelots (*Leopardus pardalis*), a sympatric species present in the study area (DQ469953 for an ocelot reference sample), were recognized and discarded from further analysis. Genotyping success for the set of seven microsatellite loci was higher than 80%. We quantified a general rate of 10.03% for allelic dropout and estimated a total $P_{(ID)unbiased}$ of 6×10^{-9} and a total $P_{(ID)sib}$ of 0.001. The range size, number of alleles per locus, and P_{ID} values per locus are described in Table 2. Finally, in 24 completely analyzed feces samples, we identified 15 different pumas inhabiting the study area. Table 3 presents the mark-recapture data (encounter history) for these 15 animals.

Abundance and density of pumas.— Model selection in the MARK software resulted in p(*)ϕ(*)b(t)N(*) (constant capture probability, constant survival rate, and temporal variation in the probability of new animals entering the population between occasions) being the most appropriate model (ACIc=85.58) (Table 1). The high Delta AICc values for all the remaining models allowed distinguishing the first model as the only one that sufficiently supported the data and, therefore, as the only appropriate model for

obtaining further estimates. The estimated abundance (N) using this model was 24.34 ± 6.62 *SE*, with a 95% confidence interval. The density of pumas per 100 km^2 was 9.36 ± 2.54 *SE*.

TABLE 2.—The range size, number of alleles, probability of identity, and total and per locus allelic dropout rate for the seven microsatellite loci analyzed.

Locus	Range size (base pairs)	Allele number	$P_{(ID)unbiased}$	$P_{(ID)sib}$
Pco C108	124 - 160	4	1.612×10^{-1}	4.792×10^{-1}
Pco B010	203 - 229	8	5.023×10^{-2}	4.119×10^{-1}
Pco B210	165 - 177	7	5.831×10^{-2}	4.014×10^{-1}
Pco A216	237 - 251	6	4.745×10^{-2}	3.816×10^{-1}
Pco A208	187 - 201	6	6.920×10^{-2}	4.042×10^{-1}
Pco B003	279 - 303	6	1.099×10^{-1}	4.430×10^{-1}
Pco A339	264 - 280	7	3.436×10^{-2}	3.654×10^{-1}
Mean/Total	-	6.28	6×10^{-9}	0.001

TABLE 3.—Encounter history of 15 individualized pumas at the studied area after 8 fecal collection field expeditions. Number 1 indicates marked individuals and 0 indicates nonmarked individuals.

	Occasions							
Individual	1	2	3	4	5	6	7	8
Puma 1	0	0	0	0	1	0	1	0
Puma 2	0	1	0	0	0	0	0	0
Puma 3	1	0	1	0	0	0	0	0
Puma 4	0	0	1	1	1	0	0	0
Puma 5	0	0	0	1	0	0	0	0
Puma 6	0	1	0	0	0	0	0	0
Puma 7	1	0	0	0	1	0	0	0
Puma 8	1	0	0	0	0	0	0	1
Puma 9	0	0	1	0	0	1	0	0
Puma 10	0	1	0	1	0	0	0	0
Puma 11	0	1	0	0	0	0	0	0
Puma 12	0	1	0	0	0	0	0	0
Puma 13	0	0	0	1	0	0	0	0
Puma 14	0	0	0	0	0	1	0	0
Puma 15	0	0	0	0	0	1	0	1

DISCUSSION

Noninvasive genetic analysis.— Counting pumas is difficult because they are elusive animals and extremely wary of people (Franklin et al. 1999; Logan and Sweanor 2001). Estimates of their population size usually require extensive time in the field to quantify them by capturing and marking individuals (Logan and Sweanor 2001), but in the last years, molecular tools and noninvasive sampling have became an alternative for monitoring elusive and low density mammals (Bellemain et al. 2005; Janečka et al. 2008; Prugh et al., 2005; Schwartz et al. 2007; Waits and Paetkau 2005). Despite high costs and intensive and time consuming laboratory work, noninvasive mark-recapture methods have become powerful tools for monitoring population trends over time. Also, noninvasive analysis has the major advantage that genetic samples are easily collected without seeing or disturbing the animal (Taberlet et al. 1999).

The estimated values of probability of identity were low for the seven analyzed loci ($P_{(ID)unbiased}$ of 6×10^{-9} and $P_{(ID)sib}$ of 0.001). Both values are considered satisfactory for individualization in noninvasive genotyping (Waits et al. 2001) and indicate that the analyzed loci successfully distinguished each individual, even in the presence of closely related animals in the study area (Miotto et al. in prep.).

Mainly due to low DNA quality and quantity, microsatellite analyses of noninvasive samples, such as feces, are commonly affected by genotyping errors, such as allelic dropout or false allele amplification, and consequently, the sample individualization and population size estimates could be biased (Broquet and Petit 2004; Creel et al. 2003; Prugh et al. 2005; Taberlet et al. 1996, 1999; Waits et al. 2001). To avoid errors in our analysis, we established some conditions to obtain consistent genotypes: (i) we randomly genotyped the majority of heterozygotes twice and confirmed homozygotes' genotypes with three to five independent PCR reactions; (ii) we included a locus in our analysis only if more than 80% of samples were successfully genotyped; and (iii) in the final analysis, we only included those samples that were genotyped for at least 80% of all the analyzed loci. Following these rules, we successfully distinguished 15 animals in the study area during the eight months sampling period.

Unlike other felid species, pumas have no pelage patterns, such as the rosettes that are features of jaguars and ocelots (Silveira et al. 2009; Silver et al. 2004; Trolle et al. 2003, 2007) or tiger stripes (Karanth and Nichols 1998; Karanth et al. 2006) that could allow reliable individualization in camera-trap studies. Kelly et al. (2008) proposed a protocol to identify different pumas based on body patterns analyzed by distinct researchers. This protocol was later used by Paviolo et al. (2009), but some subjectivity in individualization remained once each researcher identified a distinct number of animals (Kelly et al. 2008). Thus, despite its high monetary and time costs, as long as a rigid amplification protocol against genotype misidentification is applied (i.e., reducing allelic dropout or false allele occurrence), noninvasive genetic analysis may guarantee more accuracy in animal individualizations, especially for species without evident pelage patterns, and avoid a possible under- or overestimation of population size.

Population size estimator.— Several statistical frameworks are available to estimate wildlife population sizes. The most common methods used in noninvasive genetic analysis are the rarefaction method described by Kohn et al. (1999), which considers several sample sessions as a single one and calculates the population size as the asymptote of the relationship between the cumulative number of unique genotypes and the number of samples typed, and capture-mark-recapture methods (CRM—Seber 1982). Because some studies indicated that the rarefaction method may overestimate population size (Bellemain et al. 2005; Petit and Valiere 2006), we opted to use a CRM estimator.

The best model in our selection analysis supports temporal variation in recruitment (i.e., variation in the pattern by which new animals enter into the population, either by births or immigration), while it implies constancy in survival, as well as in capture probability. Apparently, pumas do not have a specific reproductive season, with mating occurring throughout the year (Logan and Sweanor 2001). Male pumas especially tend to disperse from natal areas, while females may exhibit philopatry or disperse over short distances (Logan and Sweanor 2001); therefore, the detected variation could be a direct

response to reproduction, with the entry of males into the area for mating and the consequential entry of newborns, or a response of subadults leaving the area after the maternal care period, which may occur at 16 - 22 months of age (Logan and Sweanor 2001). Unfortunately, by using molecular markers we were not able to establish age structure and, consequently, could not recognize cubs, juveniles or adults in our data set. In a previous study (Miotto et al. in prep.), however, we detected few males and many resident females, suggesting that males may be more responsible for the flow of individuals into and out of the area.

Puma abundance and density.— North American puma population densities vary from ~0.3 animals/100 km^2 in Utah (Hemker et al. 1984; Lindzey et al. 1994) to 5.03 animals/100 km^2 in the northeastern region of Washington state (Robinson et al. 2008). Although well documented on this continent, important environmental and socioecological differences exist between North and South American countries (Paviolo et al. 2009), rendering comparison between these two regions difficult and not useful for conservation strategies design.

There are few studies estimating puma density in South America, and all of these were conducted in larger areas than our study area, with the exception of Franklin et al. (1999). These authors collared and tracked 13 animals in a 200 km^2 area of Patagonia, Chile and estimated a density of 6 animals/100 km^2, although they argued that this number could have been higher (~30 pumas/100 km^2) few years before their study. Kelly et al. (2008) estimated densities in three study sites in Bolívia (5.13 ± 8.01 pumas/100 km^2), Argentina (0.5 ± 0.81 pumas/100 km^2), and Belize (2.35 ± 4.91 pumas/100 km^2). Finally, Paviolo et al. (2009) estimated densities ranging from 0.33 to 2.89 pumas/100 km^2 along the Green Corridor of the Upper Parana Atlantic Forest.

Our population size estimates were high (24.34 ± 6.62 *SE*). This value is higher than the number of animals sampled in a previous monitoring study in the area (17 animals; Miotto et al. in prep.). Even with few studies carried out in human occupied areas, all of which were in North America, our density

estimate (9.36 ± 2.54 animals/100 km^2) is one of the highest puma density estimates reported in the literature, and we believe that this value is strongly influenced by the scenario in which the puma population sampled is embedded.

In the last 50 years, the area planted with sugarcane in Brazil increased from ~1.4 million to 7 million ha (Martinelli and Filoso 2008). The São Paulo state possesses more than 50% of the country's sugarcane land cover, with plantations increasing at a rate of ~85,000 ha/yr (Martinelli and Filoso 2008). In addition to eucalyptus plantations, pastures and the development of urban centers, this intensive human activity has severely transformed the original vegetation cover of the northeast region of the state. Today, the area is characterized by a high number of small patches and a few large patches surrounded by human-disturbed areas. Despite these huge landscape transformations, pumas are still present in the area, probably as a consequence of their generalist habits.

In general, carnivore densities are positively correlated with prey biomass (Carbone and Gittleman 2002; Franklin et al. 1999; Logan and Sweanor 2001; Karanth et al. 2006). Our study area includes two protected areas that represent two of the last largest natural refuges of many species of ungulates and peccaries in the northeast area of São Paulo state, which are abundant prey items in the puma diet (Ciocheti 2007; Novack et al. 2005). With high prey abundance, they represent high-quality habitat patches in the matrix what probably allows pumas to concentrate in the area and tolerate overlapping territories (Miotto et al. in prep.). Intraspecific strikes may contribute to puma survival/mortality, but when prey availability is high, pumas, mainly females, tend to have smaller home-ranges (Logan and Sweanor 2001). Even embedded in a relatively permeable matrix, with eucalyptus plantations and sugarcane crops, pumas may be confined to these areas once there are no surrounding patches with similar quality. Fragments surrounding the protected areas are more susceptible to hunt pressure that leads to decreased prey biomass and abundance (Cullen Jr et al. 2000; 2001). Also, the absence of direct competitors, such as the jaguar (*Panthera onca*), may contribute to the maintenance of a high density of pumas in the study area because the coexistence between different felid species may regulate their

population sizes (Donadio and Buskirk 2006). Even though they avoid contact with humans and have nocturnal/crepuscular habits, several pumas were frequently sighted in these areas; thus, the high abundance was expected. Such high density may also directly contribute to the high number of road-killing and puma- human conflict events taken place in the last five years surrounding the study area (Miotto et al. in prep.).

The closest protected area larger than 1,000 ha is almost 70 km from JES and VSP. Thus, in maintaining the puma population, the priority in the northeast region of São Paulo state should be improving puma functional connectivity by establishing new protected areas and increasing pre-existing ones, like JES and VSP. Landscape management should also require the improvement of habitat quality on private properties, with recovery and conservation of remnant forest fragments, mainly in areas occupied by the sugarcane and wood pulp industries.

Habitat loss and degradation are the major threats to species persistence in South America (Paviolo et al. 2009). In areas with intensive human activities such as the northeast of São Paulo state, density estimates are one of the concepts that may contribute to monitoring puma populations over the years and, combined with other ecological, behavioral and genetic data, may guide conservation actions that could maintain viable populations in the future.

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CAPÍTULO 3

Running head: Genetic structure of pumas in southeastern Brazil

Genetic diversity and population structure of pumas in southeastern Brazil: implications for conservation in a human-dominated landscape

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Abstract

São Paulo is the most populous, developed and industrialized state of Brazil. Despite of intensive human activities, large habitat loss and fragmentation of the native vegetation cover, pumas (*Puma concolor*) still inhabit remnant habitat fragments in the northeastern area of the state. We investigated the occurrence of genetic structuring and levels of genetic variability on pumas to aggregate basic information for conservation efforts to maintain long term viable populations of this top-predator in the region. By analyzing microsatellite loci variation, we corroborated the hypothesis of absence of genetic structuring, and estimated high levels of genetic diversity (H_E=0.79; mean of 10 alleles per locus). In spite of the increasing number of roadkilling and puma-human conflicts in the area, apparently pumas still maintain some level of gene flow between protected areas of the region. The observed excess of heterozygotes suggests a recent bottleneck event in this population, probably a consequence of the profound landscape transformation of the studied area during the last century; another possibility is this may be due to the observed deviation in the population sex ratio, which may be influencing the pumas' mating system. We propose that: (1) landscape management in the study area should be focused on increasing habitat connectivity, creating protected areas and structures to allow highway crossing of pumas; (2) educational actions should be undertaken to change community perception of large carnivores, and possibly the implementation of compensatory actions to ranchers.

Key-words: fecal DNA, genetic variability, landscape management, microsatellite, noninvasive analysis, protected areas.

Introduction

The importance of large carnivores on the functioning and structure of the ecosystems in which they occur is unquestionable. Top-predators influence biodiversity by initiating trophic cascades through the community (top-down effects) (Terborgh et al., 2002; Sergio et al., 2008), and may also be predictors of major ecosystem dysfunctions, such as chemical pollution, habitat fragmentation and other anthropogenic disturbances, as they depend on complex biotic and abiotic conditions to thrive (Sergio et al., 2008). Thus, protection of top-predators should be a priority for conservation efforts, since their extinction may influence the persistence of many species in lower trophic levels (Terborgh, 1992; Simberloff, 1998).

The present decline of large carnivore populations is a global issue, mainly as a consequence of habitat degradation, hunting, persecution and conflicts with human population (Weber and Rabinowitz, 1996; Treves and Karanth, 2003). In this context, conservation strategies require basic knowledge of the genetics, ecology, behavior and landscape use of threatened species. Since 2004 we have been gathering such information on pumas (*Puma concolor*) that still inhabit a human-dominated landscape in the northeastern region of the São Paulo state, southeastern Brazil. The expansion of urban centers and agricultural limits during the last century resulted in extensive habitat loss and fragmentation in the area (Dean, 1996; Ribeiro et al., 2009). Today, the resulting landscape possesses few, small and poorly connected natural vegetation patches (Biota/Fapesp, 2008; Ribeiro et al., 2009), reducing prey availability and successful dispersion movements of wide-ranging large carnivores. In past studies in this region we observed an increasing number of roadkilled animals and puma-human conflicts (Miotto et al., in prep.), which will probably greatly influence puma persistence in the area. Larger carnivore species, such as the jaguars (*Panthera onca*), are already extinct in the area.

The northeastern region of the São Paulo state possesses few protected areas larger than 2,000 ha (20 km^2) and these are not structurally connected. By investigating the existence of genetic structuring among pumas inhabiting these protected areas and surrounding habitat fragments, we aimed to obtain

information on the efficiency of the dispersing movements of these animals in the region, and consequently subsidize conservation actions.

Considering the generalist habits and the great ability of pumas to disperse even in discontinuous habitats (Ruth et al., 1998; Miotto et al., 2007; Lyra-Jorge et al., 2008), despite the increasing number of roadkilling and puma-human conflicts in the region (Miotto et al., in prep.), we tested the hypothesis that these animals still maintain some gene flow among the protected areas, constituting a single population. We also estimated levels of genetic variability among pumas in the region, investigated recent bottleneck events and suggested actions that may mitigate deaths and contribute to the species persistence in this human-dominated landscape.

Material and methods

Study area

The study area was composed of 15 municipalities in approximately 1,700 km^2 area from the northeastern region of São Paulo state, Brazil (Fig. 1). Together, the urban areas of the region have approximately 1,600,000 habitants (IBGE, 2009) and there is an extensive road network connecting these municipalities. Four protected areas larger than 2,000 ha (20 km^2) are present: the Jataí Ecological Station (JES; 21°35'S-47°48'W; Luís Antônio municipality), with 9,010 ha (90.1 km^2) representing the largest protected area of the state with continuous cerrado vegetation; the Vassununga State Park (VSP; 21°41'S-47°34'W; Santa Rita do Passa Quatro municipality), only 3 km apart from JES, with 2,069 ha (20.7 km^2) subdivided in six distinct patches of cerrado and semideciduous Atlantic forest; the Itirapina Ecological Station (IES; 22°11'S-47°51'W; Itirapina and Brotas municipalities), with 2,300 ha (23 km^2) of cerrado vegetation; and the Edmundo Navarro de Andrade State Forest (FEENA; 22°25'S-47°33'W; Rio Claro municipality) with 2,314 ha (23.1 km^2) and composed by a mixture of several *Eucalyptus* and *Pinus* species, semideciduous Atlantic forest and riparian vegetation. Due to their proximity, we considered two of these areas as a continuum core area (JES/VSP) (Miotto et al., in

prep.). In a straight line, IES is approximately 70 km distant from the JES and VSP areas, and 40 km distant from FEENA. The JES/VSP areas are 90 km distant from FEENA. The remaining habitat fragments throughout the study area are smaller than 2,000 ha. According to Biota Fapesp Program database (Biota/Fapesp, 2008), the study area possesses 3,100 fragments larger than 5 ha (0.05 km^2), and these have an average area of 22.26 ha (0.22 km^2). The matrix is composed of sugarcane crops, eucalyptus plantations, cattle ranches and citriculture.

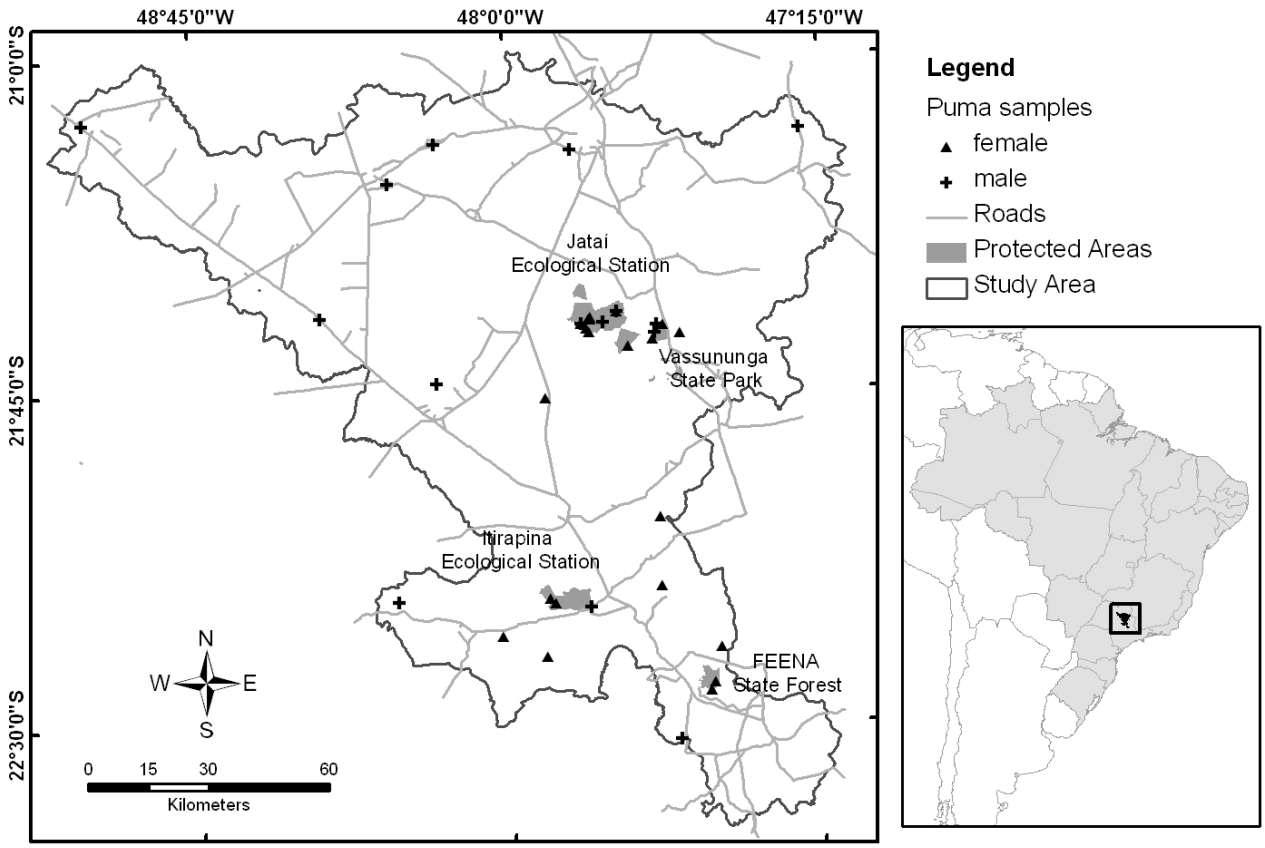

Figure 1. Geographic distribution of protected areas larger than 2,000 ha and studied pumas (n=37), northeastern São Paulo state, Brazil.

Sample collection

We collected a total of 111 samples (100 feces, 1 hair and 10 tissue samples). From October 2004 to December 2008, we collected 75 fecal samples on roads and trails inside and surrounding the

JES/VSP protected areas, as part of a puma ecological monitoring program (Miotto et al., in prep.). During the same period, we collected another 11 samples (1 hair sample, 2 blood samples, 8 muscle samples) from roadkilled pumas in the study area by maintaining contact with the local Forest Police and veterinary hospitals that could receive injured or dead animals. From 2006 to 2007 we collected 15 fecal samples in dirt roads from FEENA; from 2008 to 2009 we collected 6 fecal samples in dirt roads from IES and surrounding areas, and 5 fecal samples in small habitat patches in the Analândia and São Carlos municipalities. Blood or muscle samples were stored in 100% ethanol; hair and feces samples were stored in sterile preservative-free plastic tubes without any conservative solution. All samples were kept at -22^{o}C until DNA extraction.

DNA extraction

We extracted fecal DNA using the QIAmp DNA Stool Mini Kit (Qiagen) or PSP Spin Stool DNA Kit (Invitek), following manufacturers' recommendations. For tissue and hair DNA extractions, we followed the phenol/chloroform/isoamylic ethanol protocol proposed by Sambrook et al. (1989).

Species identification

To confirm the source species of the collected fecal samples, we amplified a 146 bp portion from cytochrome b gene of mitochondrial DNA using primers described by Farrel et al. (2000). We performed amplifications in a PTC-100 Thermocycler (MJ Research, Inc.); negative controls were included in all reactions to monitor possible contaminations. PCR products were sequenced in a MegaBACE ET-550R Size Standard automatic sequencer (GE Healthcare). Obtained sequences were aligned using the Clustal X 1.81 (Thompson et al. 1997), and visually verified and edited with the BioEdit 5.9 (Hall 1999). The sequences were then compared with reference puma sequences available in GenBank (AF266475, DQ469952), as well as sequences from other sympatric carnivores as described in Miotto *et al.* (2007).

Fecal samples individualization and genetic variability analysis

To individualize each fecal sample and estimate genetic variability, we amplified a set of 12 species-specific microsatellite loci with primers developed by Kurushima et al. (2006): Pco C209, Pco D217, Pco D103, Pco C217, Pco C112, Pco C108, all tetranucleotide-repeat microsatellite loci; and Pco B010, Pco B210, Pco A339, Pco A208, Pco A216, Pco B003, dinucleotide-repeat microsatellite loci. Primers were marked with universal fluorescent M13 tails according to Schuelke (2000). Each PCR reaction (15 μL) contained: 7.5 μL of *GoTaq Master Mix* (Promega), containing 1x buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1u *Taq polymerase*; 8 pmol of reverse primer, 2 pmol of forward primer, 8 pmol of M13 sequence marked with the 6-FAM fluorophore, and 150 μg/mL of BSA. The remaining volume of the 15 μL reaction was completed with sample DNA. We performed amplifications in a PTC-100 Thermocycler (MJ Research, Inc.), according to the following program (for all primer pairs): an initial denaturation cycle at 94°C for 5 min, 40 cycles at 92°C for 1 min, 48°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 30 min. Negative controls were included in all reactions to monitor possible contaminations. Produced genotypes were analyzed in a MegaBACE ET-550R size standard automatic sequencer (GE Healthcare) with the Genetic Profile. We conducted individual identification of genotypes with the Gimlet (Valière, 2002).

Sex determination

For the sex determination from fecal samples, we amplified a portion of the amelogenin gene present in both sex chromosomes with primers described by Pilgrim et al. (2005). In this gene fragment, males have a 20 bp deletion in the Y-chromosome, and consequently, produce two PCR products with different sizes, while females amplify fragments with the same size. To prevent false positive for females, we only conducted sex determination reactions in samples with consistent microsatellite amplification, and also repeated each reaction three times. Molecular sex determination was not

necessary for tissue and hair samples as these samples were obtained from visualized roadkilled animals.

Relatedness

We estimated genetic relationships between individualized pumas using the ML-Relate (Kalinowski et al., 2006). The maximum likelihood estimate of relatedness (*r*) (Blouin, 2003) was used to discriminate four common pedigree relationships: unrelated (U), half siblings (HS), full siblings (FS) and parent-offspring (PO).

When estimated putative relationships among individual pairs were different from unrelated, we used the same software to access the uncertainty (p-values) of these estimates by testing two *a priori* hypothesis: a putative relationship with higher likelihood (HS, FS or PO) against an alternative hypothesis. Since we did not have a group of animals with known relationships to calibrate the program, rather than defining accurate relationships categories our goal was to verify the existence of related animals in our dataset, which is why we considered only the 'unrelated' relationship as an alternative hypothesis. When pair comparisons had a low p-value ($p<0.05$), we excluded the alternative hypothesis and accepted the relationship with maximum likelihood (HS, FS or PO).

Data analysis

Several genotyping errors have been frequently associated to noninvasive genetic analyses due to low quantity and quality of DNA in these types of samples (Taberlet et al., 1996). Since this study was based on fecal DNA, to avoid errors such as the allelic dropout, where there may be failure to detect one of the two alleles of an individual (Taberlet et al., 1996), we established some *a priori* conditions to obtain consistent genotypes: (a) homozygote samples were genotyped for three to five independent PCR reactions; (b) heterozygote samples were randomly genotyped twice; (c) only samples that were

successfully genotyped for at least five loci were included in the fecal individualization analysis; (d) only loci genotyped for at least 70% of samples were included in the analyses.

To quantify the discrimination power of the microsatellite dataset, we determined the probability of identity ($P_{(ID)}$), *i.e.*, the probability of two individuals in the population to randomly share identical genotypes for all the analyzed loci (Paetkau et al., 1998; Waits et al., 2001). The $P_{(ID)}$ values were calculated for each locus with the Gimlet (Valière 2002) and then multiplied among the loci to obtain a total $P_{(ID)}$ (Paetkau et al., 1998). As we expected to sample related animals in the study area, we used the $P_{(ID)sib}$ index, a more conservative estimate (Waits et al., 2001).

We investigated null alleles, allelic dropout and stutter occurrence with the Micro-Checker 2.2.3 (Van Oosterhout et al., 2004). We used the Genepop 3.4 (Raymond and Rousset, 1995) to estimate expected (H_E) and observed (H_O) heterozygosity, to test each locus for linkage disequilibrium and deviations from Hardy–Weinberg Equilibrium (HWE) proportions (Markov chain exact test; Guo and Thompson, 1992), all of them with 10,000 dememorization, 1,000 batches and 10,000 interactions *per* batch. With the FStat 2.9.3 (Goudet, 1995), we estimated the allelic richness and the F_{IS} coefficient (Weir and Cockerham, 1984) as measure of the inbreeding level in the population.

To verify the existence of genetically structured populations, as well as the number of genetic diversity based populations (different clusters sampled), we used the Structure 2.2 (Pritchard et al., 2000) assuming the 'Admixture Model' and 'correlated allelic frequencies'. By assuming HWE and linkage equilibrium within subpopulations, the Bayesian clustering method implemented in this software allowed us to investigate spatial structure of genetic diversity without defining geographic groups *a priori*. We ran the software without any previous geographic information and estimated the probability of our data to fit the hypothesis of 1-3 clusters (K) by performing 30 independent runs for each K with a burn-in period of 50,000 steps and 200,000 Markov Chain Monte Carlo interactions.

The Bottleneck 1.2.02 was used to test for heterozygosity excess as a consequence of a recent bottleneck using a 'Wilcoxon test', a test with high statistical power that can be used with as few as four

polymorphic loci and any number of individuals (Cornuet and Luikart, 1996). We assumed a Two-Phase Mutation Model (TPM) incorporating 10, 20 and 30% of Infinite Allele Mutation Model (IAM), with 10,000 iterations. The test was also repeated assuming IAM and Stepwise Mutation Model (SMM).

Results

We successfully extracted DNA, identified the source species as *P. concolor,* and genotyped a total of 52 samples out of the original 100 fecal samples. From 12 microsatellite loci initially tested, only seven had an amplification success higher than 70%, so we excluded loci Pco C209, Pco D217, Pco D103, Pco C217 and Pco C112 from the analyses. Among the 52 fecal samples, we identified 25 distinct pumas and 68% of these individuals were sampled at least twice. The probability of identity estimate for the seven analyzed loci indicated that our microsatellite panel was efficient to discriminate individuals ($P_{(ID)sib}$=9.873×10^{-4}), as values lower than 0.001 may be considered satisfactory (Waits et al., 2001). One hair and 10 tissue samples were successfully genotyped and represented different animals from those individualized in fecal samples. Thus, we conducted genetic variability analysis in a total 37 animals, 15 males and 22 females (Table 1). Observed allelic frequencies for the seven analyzed loci are presented in Table 2.

In the analysis of relatedness, only three individuals did not present any level of relationship with other studied animals. For all possible pair combinations of individuals, significant relationships were represented by 89.4% U, 7.3% FS, 2.8% PO and 0.5% HS (P<0.05). Because we did not calibrate the program with genotypes from animals known to be related, higher confidence may be expected in PO relationships, as in the absence of mutations PO pairs must share at least one allele at every locus (Blouin et al., 1996).

Our data presented no evidence of null alleles, allelic dropout or stutters. Genetic variability analysis indicated a mean of 0.82 and 0.79 for H_O and H_E, respectively. Number of alleles per locus ranged from 7 (Pco A208 and Pco A216) to 12 (Pco B010), with a mean of 10 alleles per locus. Range size, number

of alleles, allele richness, and observed and expected heterozygosities for the analyzed loci are presented in Table 3.

Table 1. Details on puma samples collected in the northeastern region of São Paulo state, Brazil, 2004-2009. Numbers in parenthesis for feces indicate the number of replicates collected for each individual (consensus genotypes). (M) Male (F) Female

Individual	Sample	Sex	Geographic coordinate (Datum SAD69)	Municipality
Pco ram-02	Feces (5)	F	21°37' 30"S - 47°48' 04"W	Luís Antônio
Pco ram-05	Feces (3)	F	21°35' 44"S - 47°47'56"W	Luís Antônio
Pco ram-09	Feces (2)	M	21°37' 39"S - 47°38' 44"W	Sta. Rita Passa Quatro
Pco ram-10	Feces (4)	F	21°36' 35"S - 47°37'29"W	Sta. Rita Passa Quatro
Pco ram-11	Feces (3)	M	21°36' 34"S - 47°38' 24"W	Sta. Rita Passa Quatro
Pco dot-09	Feces (2)	F	21°38' 30"S - 47°39' 01"W	Luís Antônio
Pco dot-12	Feces (3)	F	21°35' 29"S - 47°47' 58"W	Luís Antônio
Pco dot-23	Muscle	M	22°32' 25"S - 47°35' 37"W	Iracemápolis
Pco dot-24	Hair	M	21°36' 13"S - 47°46' 09"W	Luís Antônio
Pco dot-25	Blood	F	22°11' 45"S - 47°38' 11"W	Analândia
Pco dot-27	Feces (2)	F	21°36' 22"S - 47°49' 17"W	Luís Antônio
Pco dot-29	Muscle	M	22°14' 26"S - 47°48' 24"W	Itirapina
Pco dot-31	Feces	F	22°25' 51"S - 47°31' 15"W	Rio Claro
Pco dot-41	Feces	F	22°20' 06"S - 47°29' 38"W	Rio Claro
Pco dot-42	Muscle	M	21°35' 09"S - 48°26' 49"W	Matão
Pco dot-43	Muscle	M	21°08' 32"S - 49°00' 30"W	Catanduva
Pco dot-44	Muscle	M	21°10' 12"S - 47°17' 39"W	Cajuru
Pco dot-46	Muscle	M	21°12' 49"S - 47°50' 34"W	Ribeirão Preto
Pco dot-51	Muscle	M	21°11' 55"S - 48°10' 01"W	Sertãozinho
Pco dot-52	Feces	F	22°13' 57"S - 47°53' 37"W	Itirapina
Pco dot-53	Feces	M	22°13' 30"S - 48°16' 14"W	Brotas
Pco dot-55	Feces (2)	F	21°39' 25"S - 47°42' 28"W	Luís Antônio
Pco dot-58	Feces	F	22°02' 29"S - 47°38' 12"W	Analândia
Pco dot-60	Feces (2)	F	21°37' 44"S - 47°35' 02"W	Sta. Rita Passa Quatro
Pco dot-64	Feces (2)	M	21°34' 42"S - 47°44' 12"W	Luís Antônio
Pco dot-66	Feces (2)	F	21°35' 29"S - 47°47' 57"W	Luís Antônio
Pco dot-68	Feces	M	21°36' 22"S - 47°49' 17"W	Luís Antônio
Pco dot-70	Muscle	M	21°44' 11"S - 48°10' 13"W	Araraquara
Pco dot-80	Feces (3)	F	21°37' 01"S - 47°48' 27"W	Luís Antônio
Pco dot-85	Feces	F	21°35' 47"S - 47°48' 12"W	Luís Antônio
Pco dot-99	Muscle	F	22°21' 07"S - 47°54' 55"W	Itirapina
Pco dot-101	Feces (2)	F	22°24' 48"S - 47°30' 39"W	Rio Claro
Pco dot-106	Feces	M	21°17' 14"S - 48°16' 45"W	Jaboticabal
Pco dot-107	Feces (2)	F	21°46' 19"S - 47°54' 31"W	São Carlos
Pco dot-111	Feces (2)	F	21°34' 42"S - 47°44' 12"W	Luís Antônio
Pco dot-112	Feces (2)	F	22°18' 16"S - 48°01' 15"W	Brotas
Pco dot-115	Feces	F	22°13' 17"S - 47°54' 21"W	Itirapina

Table 2. Observed allele frequencies at each locus for pumas sampled in the northeastern region of São Paulo state, Brazil.

Puma population in the northeast of São Paulo state, Brazil (n=37)		
Locus Pco A208	Allele	
(n=35)	205	0.086
	207	0.300
	209	0.086
	213	0.257
	217	0.186
	219	0.071
	221	0.014
Locus Pco A216	Allele	
(n=31)	255	0.226
	259	0.065
	261	0.306
	263	0.226
	265	0.016
	267	0.081
	269	0.081
Locus Pco A339	Allele	
(n=37)	278	0.014
	282	0.068
	284	0.095
	286	0.243
	288	0.027
	290	0.270
	292	0.027
	296	0.081
	298	0.176
Locus Pco B010	Allele	
(n=36)	219	0.014
	221	0.056
	223	0.028
	225	0.083
	229	0.056
	231	0.014
	233	0.056
	235	0.403
	237	0.097
	239	0.014
	245	0.111
	247	0.069
Locus Pco B210	Allele	
(n=36)	179	0.014
	181	0.056
	183	0.153
	185	0.319
	187	0.069

	189	0.069
	191	0.028
	193	0.014
	195	0.097
	197	0.125
	199	0.056
Locus Pco B003	Allele	
(n=35)	295	0.014
	297	0.071
	299	0.114
	303	0.043
	305	0.086
	307	0.286
	309	0.043
	317	0.029
	319	0.271
	321	0.029
	327	0.014
Locus Pco C108	Allele	
(n=27)	138	0.019
	142	0.056
	146	0.407
	150	0.333
	154	0.130
	162	0.019
	170	0.019
	178	0.019

Table 3. Measures of diversity and probability of identity ($P_{(ID)sib}$) for seven microsatellite loci on pumas from the northeastern region of São Paulo state, Brazil. (2) dinucleotide-repeat locus (4) tetranucleotide-repeat locus (N) sample size (A) number of alleles (AR) allele richness (H_O) observed heterozygosity (H_E) expected heterozygosity.

Locus	Range size (bp)	N	A	AR	$P_{(ID)sib}$	H_O	H_E
Pco A208	205-221 (2)	35	7	6.77	0.3740	0.80	0.80
Pco A216	255-269 (2)	31	7	6.87	0.3759	0.74	0.79
Pco A339	278-298 (2)	37	9	8.58	0.3570	0.83	0.82
Pco B010	219-247 (2)	36	12	11.18	0.3678	0.72	0.80
Pco B210 [a]	179-199 (2)	36	11	10.43	0.3449	0.94	0.84
Pco B003	295-327 (2)	35	11	10.42	0.3576	0.88	0.82
Pco C108	138-178 (4)	27	8	8.00	0.4336	0.88	0.71
Mean/Total	-	37	10.0	8.89	9.873×10^{-4}	0.82	0.79

(a) locus excluded from analyses due to linkage disequilibrium with the loci Pco B003 and A216.

Linkage disequilibrium was detected between locus Pco B210 and the loci B003 and A216 (Bonferroni correction; P<0.00714), so we excluded locus Pco B210 from further analysis. Significant deviations from HWE equilibrium were observed in two loci (Pco A208 and B003) (Bonferroni correction; P<0.00714) (Table 4), and an excess of heterozygotes was detected (P=0.9029). The estimated total F_{IS} coefficient was significant (F_{IS} = -0.022; P=0.0083), not indicating endogamy in the studied population. By investigating spatial structure of genetic diversity, we estimated values of LnP(D) equal to -752.3, -780.3 and -800.9 for K=1, K=2 and K=3, respectively. These values indicated that there was no evidence of genetic structuring in the study area, *i.e.* the pumas sampled in the northeast of São Paulo state constitute a single population. By testing a recent bottleneck occurrence in this puma population, we found significant values only in estimates assuming the IAM Model (P=0.0468).

Table 4. Hardy-Weinberg equilibrium deviations for the six analyzed loci. (S.E.) standard error (a) significant deviation after Bonferroni correction (P<0.00714)

Locus	PHW	S.E.	F_{IS}
Pco A208 [a]	0.0047 [a]	0.0002	0.001
Pco A216	0.0122	0.0003	0.073
Pco A339	0.1049	0.0013	-0.014
Pco B010	0.0902	0.0025	0.104
Pco B003 [a]	0.0000 [a]	0.0000	-0.074
Pco C108	0.0107	0.0004	-0.249
Mean	-	-	-0.022

Discussion

The overall genetic diversity of pumas in the northeastern region of São Paulo state is high (H_O=0.82, H_E=0.79, mean of 10 alleles per locus). High levels of genetic diversity in pumas from South America were already reported by Menotti-Raymond and O'Brien (1995), Culver et al. (2000) and Ruiz-Garcia (2001). Albeit using different genetic markers, which limits direct comparison with our data, Ruiz-Garcia (2001) reported an average expected heterozygosity of 0.75 and a mean of 7.40 alleles per locus for pumas sampled in Colombia, Peru and Bolivia. Menotti-Raymond and O'Brien (1995) reported

an average observed heterozygosity of 0.713 and a mean of 5.57 alleles per locus across the species geographic range. To our knowledge, no similar studies had been carried out in Brazilian wild puma populations.

We found a higher number of alleles and mean heterozygosity than described by Kurushima et al. (2006). For the same loci, these authors described a mean of 5.85 alleles per locus and mean H_O=0.47 and H_E=0.57. In fact, it is already known that North American pumas have much lower genetic variability than South American specimens. Culver et al. (2000) described greater overall variability in South American puma subspecies for different measures (mitochondrial DNA haplotypes, mitochondrial DNA genetic diversity, average microsatellite heterozygosity and number of alleles per locus), especially for pumas from eastern South America, including areas to the South of the Amazon River in Brazil. The differences among the continents may be consequence of a recent period of recolonization of the North American continent following a Pleistocene glaciation (Culver et al., 2000). Regarding differences between analyzed microsatellite loci, among different areas of the western United States, mean genetic variability (H_O) ranged from 0.42-0.52 (Culver et al. 2000), 0.42-0.66 (Walker et al., 2000), 0.44 (Ernest et al., 2003), 0.47 (Sinclair et al., 2001), to 0.54 (Anderson Jr et al., 2004).

Deviations from HWE proportions were observed in two of the analyzed loci (Pco A208 and Pco B003), and heterozygote excess was significant (P=0.9029). Since there was no evidence in our data of null alleles or Wahlund effect (pumas in our study area belong to a single subspecies; Culver et al., 2000), and the observed F_{IS} value were significative (F_{IS}=-0.022; P=0.0083), deviations from HWE may be related to a deficiency in the number of sampled animals or to effects of mating system or selection. In addiction, the observed heterozygote excess suggests the occurrence of a recent bottleneck in the studied population (P=0.0468). In this situation, under the IAM model, genetic diversity is higher than the expected in equilibrium (Cornuet and Luikart, 1996) and this excess in heterozygosity may be a consequence of a swift loss of rare alleles due to genetic drift during a bottleneck, which have a minor contribution to the expected heterozygosity (Pearse and Crandall, 2004).

We found evidence of a bottleneck only when applying the IAM model, but it is already known that microsatellites do not always follow a strict SMM model, and there has been considerable controversy over the best method when analyzing microsatellite allele frequency data (Pearse and Crandall, 2004). Despite of the differences among these methods, the observed evidence of a bottleneck on pumas in the northeast of São Paulo state may be considered to be in concordance with the human occupation history of the region, or a consequence of interferences on the puma breeding system. We observed a high number of related individuals in the area and this may also reinforce the hypothesis of a bottleneck event in this population. Some studies have shown an increase of genetic variability after a bottleneck (see reviews of Pearse and Crandall, 2004; Bouzat, 2010), but there may also be an associated decrease on fitness and phenotypic values.

The landscape of the studied area has been severely transformed, especially in the last century (Dean, 1996). The expansion of urban centers, extensive conversion of ranches and native forest fragments into sugarcane crops and eucalyptus or citrus plantations, and implementation of a large road network connecting municipalities have drastically reduced pumas' natural habitats, which in turn has probably reduced their population size. However, in previous studies we estimated a high density of pumas inhabiting the JES/VSP areas (Miotto et al., in prep.), suggesting that the number of animals in the northeastern region of the state would be relatively high or even increasing. Another possibility is that the observed evidence of a bottleneck could be related to changes in the pumas' mating, as bottleneck effects may occur without drastic population size reduction if there are few breeders of one sex in polygynous breeding systems (Luikart et al., 1998), such as the puma mating system (Logan and Sweanor, 2001).

In previous studies in the JES/VSP areas (Miotto et al., in prep.), we observed population dynamics where female pumas may more frequently be resident in larger natural vegetation fragments, while males tend to disperse throughout the landscape. Therefore males, by dispersing, may be more significantly responsible for the gene flow among core habitat areas, rendering them more susceptible

to be roadkilled or dead in conflicts with humans, and this may in turn affect the sex ratio of the population. Accordingly, in 11 examined samples of roadkilled pumas, ten samples were from males; also, 20 out of the 26 pumas sampled inside or surrounding the protected areas were females, and only six were males. Since pumas apparently give birth to male and female cubs in equal proportions (Logan and Sweanor, 2001), these findings suggest differences in the spatial distribution of adults or subadults males and females in this population (Miotto et al., in prep.). Higher numbers of females in North American puma populations were already described in literature (Lindzey et al., 1994; Logan and Sweanor, 2001; Ross and Jalkotzy, 1992), probably in consequence of higher mortality and emigration rates among males (Logan and Sweanor, 2001).

The primary goal of this study was to verify the existence of population substructure in pumas inhabiting the northeastern region of the São Paulo state. We corroborated the hypothesis of lack of genetic structuring, *i.e.* the pumas sampled in the area constitute a single population, and this has important implications for planning conservation strategies for the species in this human-dominated landscape. Considering the long generation time of the species and approximately 100 years of intensive fragmentation and loss of natural habitats (Dean, 1996), which could represent a relatively short time to detect population structuration, it seems reasonable that pumas still maintain some level of gene flow among the protected areas of the studied area. This species possesses generalist habits and is capable to disperse over long distances (Ruth et al., 1998; Logan and Sweanor, 2001), even through areas of intense human activity such as sugarcane crops and eucalyptus plantations (Miotto et al. 2007; Lyra-Jorge et al. 2008), and this certainly contributed to the observed absence of genetic structuring in the population.

By analyzing relatedness in our dataset, we observed pairs of individuals sampled in distinct areas (for example in JES/VSP and FEENA) exhibiting significant PO relationships, which suggests successful movements among the protected areas. In contrast, Haag (2009) observed genetic structuring among jaguar subpopulations in natural vegetation fragments less than 100 km distant from each other in a

recently fragmented area of the Upper Paraná Atlantic Forest, Brazil. Differently from pumas, that study indicated the importance of high quality patches for jaguar dispersion movements and that, in more specialist large carnivore species, genetic structuring may be detected over relatively small distances and shortly after the beginning of the fragmentation process.

In North America, Anderson et al. (2004) have reported the occurrence of a panmitic megapopulation of pumas surrounding the Wyoming Basin, with high levels of gene flow among five adjacent populations. Sinclair et al. (2001) also reported high gene flow across puma populations in Utah, and Culver et al. (2000) suggested that North American pumas should be classified as a single subspecies due to the absence of genetic structuring. In contrast, genetic structuring was reported by Ernest et al. (2003) in California, where the expansion of urban centers has severely reduced and isolated natural habitats for pumas, an evidence of the impacts of human activities on this species' persistence.

Considering the lack of genetic structuring observed in this study, in the northeastern region of the São Paulo state conservation strategies should be focused on the maintenance of puma movements among core areas, especially by mitigating human activities barriers to their dispersion, and consequently, avoiding problems associated to small and isolated populations such as endogamy, genetic drift and susceptibility to future stochastic events. The increasing number of puma-human conflicts and roadkilling events in the area will probably jeopardize effective puma dispersion in the region over the years. For wide-ranging carnivores, populational structuration in consequence of roads was already reported in the literature. Riley et al. (2006) observed that even though some animals successfully crossed roads in California, the contribution of the dispersers was not high enough to maintain non-structured populations of wolfs and bobcats among the sides of the studied highways.

Looking into local media reports of pumas rescued near urban areas or predating livestock in the studied area, we recorded more than 10 reports in the last 10 years. Hunting is prohibited in Brazil, and despite being certainly high, the number of pumas being killed by ranchers are officially unknown.

Verdade and Campos (2004) reported that in 1997, seven pumas were killed by ranchers in a single property after livestock depredation in the São Paulo state. Unfortunately, puma livestock depredations often are not officially evaluated and prevented (Verdade and Campos, 2004). From the ranchers standpoint, each encounter with pumas represents a threat and the easiest solution is to kill the animal. In fact, conservation of large carnivores requires heavy governmental investments on personnel, time and resources to patrol areas against hunting (Treves and Karanth, 2003). Investments are also required from ranchers, for example, to maintain their livestock away from areas of natural vegetation or maintaining them in night shelters. Conflicts tend to increase as human population and farming frontiers expand (Treves and Karanth 2003), turning human persecution a rising threat to carnivore population viability (Rabinowitz, 1986).

Also, large protected areas in the northeastern region of the São Paulo state are scarce and distant from one another, a context with important consequences on the dispersion and prey availability for wide-ranging carnivore species. Connectivity could be improved by maintaining smaller habitat patches in private properties, for example, in sugarcane and pasture areas. This is already established by the Brazilian legislation, but is not followed by almost none of the landowners in the studied area. Some studies have already demonstrated the importance of maintaining habitat patches in different land use areas for conservation of some birds and mammal species (Chiarello, 2000; Haines et al., 2006). Since the core areas are not large enough to provide all habitat requirements for large carnivores, these patches would act as stepping stones creating a functional network linking distinct core areas. Moreover, they would increase gene flow among adjacent puma subpopulations. Increasing the number of protected core areas would prevent poaching and illegal hunting, and guarantee prey availability and habitat requirements. Identifying critical points of roadkilling and establishing crossing structures on highways may affect not only the number of deaths and facilitate dispersion in the population, but also prevent sex ratio deviations that may increase the risk of cryptic genetic bottlenecks from sex-biased roadkill mortality.

In this scenario, to ensure the studied puma population persistence we propose that (1) landscape management in the study area should be focused on increasing habitat connectivity, creating protected areas and structures to allow highway crossing of pumas; and (2) educational actions should be undertaken to change community perception of large carnivores, and possibly the implementation of compensatory actions to ranchers.

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CONSIDERAÇÕES FINAIS

Apesar de ser um método caro e laborioso, a análise do DNA fecal por meio de marcadores moleculares se mostrou uma ferramenta poderosa para o estudo e monitoramento de grandes carnívoros. A partir da coleta de amostras fecais em campo, pudemos estudar uma população de onças-pardas sem que um único animal fosse capturado, ou até mesmo visualizado.

Mesmo não sendo considerada uma espécie ameaçada de extinção (IUCN 2010), cabe ressaltar que a persistência de populações de onças-pardas, assim como a de qualquer outro grande carnívoro, em paisagens de intensa atividade humana merece especial atenção e monitoramento ao longo do tempo. É inegável o sucesso atual das onças em um ambiente tão alterado como a região nordeste do estado de São Paulo; ao percorrer essa região do estado, torna-se evidente a grande extensão de terras de cultivo de cana-de-açúcar, eucalipto e citricultura em detrimento às áreas de vegetação natural. É certo que seus hábitos generalistas, com presas que vão de pequenos roedores a cervos, catetos e tamanduás (Ciocheti 2007), e sua grande capacidade de dispersão (Ruth *et al.* 1998, Beier 1995, Logan e Sweanor 2001) contribuem para o deslocamento dos indivíduos dessa espécie em praticamente todos os tipos de uso do solo da região, com exceção dos centros urbanos.

Ao mesmo tempo, o fato de essa população não ter sido estudada antes dificulta o entendimento de quão grande é o sucesso desses animais nessa paisagem. Duas situações podem ser consideradas: (1) é muito provável que o número de animais na região tenha sido afetado ao menos no início do processo de colonização humana do interior do estado, mas os resultados de densidade (Capítulo 2) e diversidade genética (Capítulo 3) estimados nesse trabalho são contraditórios em relação aos de uma população em perigo. Ao contrário, somados aos relatos informais de visualização feitos por moradores da região, de atropelamentos e de conflitos (Capítulo 1), esses fatores podem indicar que essa população está se expandindo; (2) ou diferentemente dessa situação, a grande concentração de animais em áreas de vegetação remanescente, o grande número de atropelamentos e aparecimentos de animais em centros urbanos podem indicar que a perda de hábitat está afetando

severamente essa população e que ela tende a declinar à medida que se intensificam as atividades humanas na região.

Nesse contexto, esse estudo torna-se um ponto de partida para o acompanhamento dessa população ao longo dos anos. As estimativas de densidade e variação genética aqui apresentadas poderão servir de base de comparação entre cenários diferentes, uma vez que a região tende a se expandir economicamente. Mesmo que algumas questões permaneçam por ora não respondidas, é certo que medidas que visam o incremento da conectividade e a mitigação de atropelamentos e conflitos entre onças e humanos, como as discutidas anteriormente em cada capítulo, são de extrema importância para a manutenção da espécie na região nordeste do estado de São Paulo.

Considerando que as onças extrapolam os limites das Unidades de Conservação e que se deslocam pela matriz mantendo fluxo gênico, informações relativas ao modo como os animais utilizam a paisagem podem contribuir para a conservação da espécie. Numa próxima etapa, procuraremos estimar possíveis rotas de dispersão entre as maiores áreas preservadas da região com base na configuração da paisagem. Esperamos assim, propor pontos-chave para a implementação de passagens de fauna em rodovias, bem como identificar fragmentos, que se preservados, venham a facilitar a dispersão dos indivíduos.

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