PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL

DISSERTAÇÃO DE MESTRADO

DIVERSIDADE GENÉTICA E ESTRUTURA POPULACIONAL DO LOBO-GUARÁ (*CHRYSOCYON BRACHYURUS*)

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PORTO ALEGRE, 2009

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FACULDADE DE BIOCIÊNCIAS

PROGRAMA DE PÓS-GRADUAÇÃO EM ZOOLOGIA

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PORTO ALEGRE – RS – BRASIL

2009

Sumário

Resumo

O lobo-guará (*Chrysocyon brachyurus*) é o maior canídeo dentre as espécies Neotropicais. Sua distribuição, morfologia, ecologia e comportamento estão intimamente associados a ambientes campestres, especialmente ao Cerrado brasileiro. Este bioma de vegetação aberta é um ambiente vasto e complexo, sendo extremamente heterogêneo em sua composição vegetacional e apresentando muitos acidentes geográficos como rios e chapadas. Além disso, este é um dos biomas com maiores taxas de perda e fragmentação de habitat na América do Sul devido à atividade humana. Todos estes fatores podem levar elementos nativos da fauna e flora a apresentarem algum grau de estruturação populacional, dependendo também de características da biologia de cada espécie. Nesse caso, uma investigação de tais padrões se justifica, uma vez que para fins de conservação é de suma importância conhecer eventuais subdivisões geográficas históricas de uma espécie e/ou identificar processos de isolamento populacional causados pela ação humana. Uma vez que o lobo-guará apresenta inúmeras relações tróficas e ecológicas dentro do Cerrado pode-se dizer que esta é uma espécie-chave para esse ambiente, e um estudo acerca da sua estruturação populacional é assim importante para fins de conservação da espécie e do Cerrado como um todo. Nesse sentido, marcadores moleculares têm sido amplamente utilizados, e os métodos de análise associados têm-se aprimorado e permitido inferências cada vez mais amplas e robustas. Assim, este trabalho se propôs a estudar a estrutura populacional do lobo-guará e discutir suas causas à luz da história natural da espécie, para isso utilizando marcadores moleculares de evolução rápida, que assim permitam inferências mesmo sobre processos demográficos recentes. Para tal, amostras de tecido de 144 indivíduos de lobo-guará foram obtidas através de captura de animais de vida livre, animais de cativeiro com procedência conhecida e indivíduos atropelados. Foram amostradas quatro populações, além de indivíduos de diversos pontos distintos, resultando em uma ampla cobertura da distribuição da espécie. As amostram foram genotipadas para 14 locos de DNA microssatélite, originalmente descritos para o cão doméstico e escolhidos através de testes de eficiência e polimorfismo para utilização no lobo-guará. Foram conduzidos testes de variabilidade genética, F_{st} e R_{st} entre populações, análises de estruturação através do programa STRUCTURE e análises demográficas testando eventos Gargalo de Garrafa, expansão populacional e número efetivo da espécie (N_e). Os níveis de variabilidade foram significativamente altos (H_e média = 0,75) quando comparados a outras espécies de canídeos. Além disso, não se observou nenhum indício de subdivisão populacional, resultado que sugere que o lobo-guará se comporta como uma população praticamente panmítica na maior parte da sua distribuição. As análises demográficas corroboram estudos anteriores baseados em DNAmt que sugerem um evento de expansão populacional, e os valores de N_e estimados são altos. Em conjunto, tais resultados são compatíveis com um cenário em que a espécie passou por um crescimento populacional nos últimos milhares de anos, mantendo-se grande desde então, e sem apresentar subdivisões geográficas. A falta de estruturação populacional pode em parte ser explicada pela dieta generalista e grande capacidade de dispersão do lobo-guará, sendo a matriz antropizada permeável em algum grau até o presente momento para os indivíduos. Contudo, não se pode desconsiderar a perda de habitat e fragmentação do Cerrado como uma ameaça ao lobo-guará. O processo de isolamento pode ser muito recente para ser detectado até mesmo por microssatélites, e o alto N_e das populações pode estar tornando a detecção difícil. Caso este processo continue e se intensifique, os presentes resultados podem servir como base para comparação com futuros estudos genéticos da espécie, possibilitando a detecção e monitoramento de uma possível perda de variabilidade e diferenciação geográfica induzida pelo homem.

Abstract

The maned wolf (*Chrysocyon brachyurus*) is the largest species among the Neotropical canids. Its distribution, morphology, ecology and behavior are closely associated to open vegetation environments, especially the Brazilian Cerrado. This savanna-like biome is a large and complex environment, presenting highly heterogeneous vegetational composition and many geographic elements, such as rivers and mountains. Moreover, this is one of the most human-disturbed biomes in South America, undergoing a high rate of habitat loss and fragmentation. These natural and human-induced factors can lead to different degrees of population structuring of native fauna and flora, depending on ecological and behavioral characteristics of each species. A broad investigation of such patterns is justified, once it is very important in terms of conservation to know possible historical geographic subdivisions of a species, and/or to identify processes of population isolation caused by humans. Once the maned wolf presents many ecological interactions with other Cerrado-dwelling species, it can be considered a keystone species in this ecosystem, which highlights the need to characterize its population structure in the context of adequately conserving and managing the Cerrado biota. For such purposes, molecular markers are a widely utilized tool, and the associated analytical methods are becoming increasingly broad and robust. Thus, this study aimed to investigate the population structure of the maned wolf and to analyze it in the context of the species' natural history, through the utilization of fast evolving molecular markers that allow inferences on even recent demographic processes. To do that, tissue samples of 144 maned wolves were obtained through the capture of free living animals, captive animals with known geographic origin and road-killed individuals. Four local populations were sampled in addition to individual collection from many geographic points, resulting in a broad coverage of the species' distribution. The individuals were genotyped for 14 microsatellite loci, originally developed for the domestic dog and selected for use in this species after an initial screening for amplification efficiency and polymorphism. Analyses were performed to assess genetic variability, population pairwise F_{st} and R_{st}, structure analyses with the software STRUCTURE, and demographic tests to check for bottleneck events, population expansion and effective number of individuals (N_e). The diversity levels verified were quite high (mean He = 0.75) when compared to other canid species. Furthermore, no clear-cut population subdivision was detected, leading to the conclusion that the maned wolf exists as an almost panmictic population throughout a large portion of its distribution. The results of the demographic analyses corroborated previous analyses based on mtDNA data that had inferred a population expansion in this species, and N_e estimated numbers were high. Jointly, these results are compatible with a scenario where maned wolves underwent a population growth in the last few thousand years, maintaining a large number of individuals since then and not presenting any geographic subdivision. Lack of population structure can be, at least in part, explained by the generalist dietary habits and the great dispersal capability of the maned wolf, also implying that so far the anthropic habitat matrix has been permeable to a certain degree for the individuals of this species. However, habitat loss and fragmentation cannot be discarded as a threat for the maned wolf. The isolation process can be too recent to be detected even with microsatellites, and the still large effective size of regional populations may also delay the ability to detect ongoing processes of genetic fragmentation. In case such process continues and becomes even more intense, our present data set should provide a baseline for comparison with future genetic assessments of the maned wolf, allowing the detection of population-level trends of loss of variation and human-induced geographic differentiation.

Apresentação

Esta dissertação é um dos requisitos exigidos para obtenção do título de Mestre pelo Programa de Pós-Graduação em Zoologia, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul.

Os resultados aqui apresentados foram gerados ao longo dos meus últimos dois anos de estudos, realizados no Laboratório de Biologia Genômica e Molecular, vinculado a esta universidade, sob orientação do Professor Eduardo Eizirik.

O trabalho está sendo apresentado sob forma de artigo científico, segundo as normas da revista *Molecular Ecology*, para o qual o mesmo será posteriormente submetido (categoria *Original article*). As normas de submissão para autores estão disponíveis no sítio "http://www.wiley.com/bw/submit.asp?ref=0962-1083&site=1". Acompanha o artigo um resumo estendido do trabalho (versões em português e inglês, ver acima). Tabelas e figuras podem ser encontradas após o texto principal do artigo.

Artigo Científico

"High microsatellite diversity and remarkable lack of population structure in the maned wolf (*Chrysocyon brachyurus*) (Carnivora, Canidae)"

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A ser submetido ao periódico científico *Molecular Ecology*

Molecular Ecology

Revised Manuscript, 15 May 2009.

Original Article

High microsatellite diversity and remarkable lack of population structure in the maned wolf (*Chrysocyon brachyurus*) (Carnivora, Canidae)

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Keywords: maned wolf, microsatellites, demography, evolution, population structure, conservation

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Running Title: Population history of the maned wolf

Abstract

The maned wolf is the largest South American canid, and its distribution and habits are closely associated with the Brazilian Cerrado. As this is a highly heterogeneous environment, as well as one of most disturbed Neotropical Biomes (suffering from intense habitat loss and fragmentation), the population structure and history of this keystone species was investigated through the use of microsatellite markers. Fourteen tetranucleotide loci were genotyped for samples from most of the species' range, revealing a high level of genetic diversity (mean H_e = 0.75). Surprisingly, no evidence of historical or current geographic partitioning was observed. All the analyses pointed to the existence of an almost panmictic population spanning the main portion of the maned wolf distribution, which could be explained by some ecological and behavioral traits of this species. Furthermore, demographic analyses support a scenario of a population expansion in the last few thousand years, whose genetic signature can still be detected in the patterns of microsatellite variation.

Introduction

The population structure and natural history of organisms that are closely associated to one Biome or to a specific habitat can be shaped by past and present processes that affect the characteristics, range and continuity of this environment. Biomes occupying large areas are often subdivided into smaller domains, consisting of slightly different environments or separated by natural barriers such as rivers and mountains. Furthermore, anthropogenic activities are currently responsible for recent habitat loss and fragmentation, which may also lead to subdivisions of formerly continuous environments.

This scenario of natural and/or anthropogenic discontinuity might be expected for the Brazilian Cerrado, one of the largest South American Biomes (Silva & Bates 2005). This

savanna-like environment occupies most of the central region of Brazil, and harbors high levels of biodiversity (Myers *et al.* 2000; Klink & Machado 2005). Because of its large area, the Cerrado may contain biotic subdivisions, once it presents large rivers and other such geographic elements. Moreover, it is composed by a mosaic of well defined vegetational formations, ranging from totally open fields to forested areas (Silva & Bates 2005). Finally, the Cerrado is one of the environments in South America that is most impacted by human activities, as it is now widely utilized for agriculture and cattle ranching (Klink & Machado 2005). Some studies reveal that no more than 20% of the original biome remains intact to the present day (Myers *et al.* 2000), with the rate of habitat loss being very high (1.5% of the total area of the Cerrado per year – Machado *et al.* 2004).

All of these aspects can shape the distribution and spatial structure of this Biome, thus affecting the geographic range and population structure of its associated fauna and flora. Therefore, studying the population structure of its elements can shed light on natural processes leading to environmental disjunction, as well as on human-induced fragmentation. The latter issue is of great conservation concern, once it can lead to the isolation of Cerrado-dwelling species in small remnant populations. The consequences of this process are well documented by population genetics (Frankham *et al.* 2002), and to know if this is happening is crucial for the design of adequate management plans and conservation efforts.

The maned wolf (*Chrysocyon brachyurus*) is one of the most conspicuous components of the Cerrado, and presents a geographic distribution that largely coincides with the range of this Biome (Figure 1). It is the largest Neotropical canid, ranging from 20 to 30 kg of total body weight and from 95 to 115 cm in body length (Rodden *et al.* 2004). The morphology of the species is highly distinctive and well adapted to the life on savannas and prairies, as can be noticed by the presence of long legs, large ears and slender body. Behavioral traits, such as

73 the pacing gait, also denote the species' adaptations to this kind of environment (Dietz 1985).
74 In addition to its stronghold in the Cerrado, this species also occurs in some patches of the
75 South American Pampas (in Brazil, Uruguay and Argentina), Paraguayan Chaco and the
76 savannas of northern Bolivia, all of which are being formations (Rodden *et al.* 2004). The
77 dietary habits of this species also reveal close association to several fruits and small animals
78 that are typical of the Cerrado. Several studies have reported that the most important item of
79 the maned wolf diet is the fruit of *Solanum lycocarpum*, which may account for as much as
80 30-50% of the ingested food. Its diet also includes many other fruits and small animals such
81 as birds, lizards, snakes, rodents, marsupials and arthropods (Dietz 1984; Juarez & Marinho-
82 Filho 2002; Santos *et al.* 2003; Jácomo *et al.* 2004; for a review see Rodden *et al.* 2004).
83 Regarding the species' social structure, many issues remain to be understood. Since
84 the analysis of maned wolf ecology by Dietz (1984), few studies have been published
85 expanding those observations with larger sample sizes, or further refining our understanding
86 of the species' biology. Current knowledge suggests that the species is monogamous,
87 breeding once a year and raising usually 2-5 cubs (Dietz 1985). Pups and juveniles are
88 thought to spend their first year with parents, dispersing in the second year as they reach
89 sexual maturity (Rodden *et al.* 2004). However, details on patterns of parental care and
90 dispersal events are poorly known. Except for the mating season, individuals are solitary,
91 sharing large living areas apparently only with mates (Dietz 1984). However, tolerance to
92 home range overlap with pups and adjacent pairs has not yet been sufficiently investigated.
93 The territorialism and the large size of the living areas (*ca*. 27 km2 [Dietz 1984]) lead
94 to the prediction that long-term viable populations of maned wolf need large continuous areas
95 of habitat. Once the Cerrado is facing a rapid process of habitat loss and fragmentation, the
96 study of the species' population structure is very important to verify (i) if there are

97 geographically defined populations prior to any structure caused by current habitat
98 fragmentation, and (ii) if the current discontinuous distribution of the Cerrado is already
99 causing populations to become isolated. As mentioned before, to know if such processes are
100 happening and to what degree is an important issue for conservation biology, further
101 considering that the maned wolf can be seen as a key species for the Cerrado due to the high
102 number of trophic and ecological interactions (Simberloff 1998).
103 However, it is important to be noted that many aspects of the maned wolf ecology and
104 behavior can affect the existence and degree of such possible population structure. Species
105 with large home ranges, such as most canids, tend to present long distance dispersal
106 (Macdonald 1980 *apud* Macdonald & Sillero-Zubiri 2004; Macdonald & Bacon 1982 *apud*
107 Macdonald & Sillero-Zubiri 2004), thus being expected to exhibit more gene flow than those
108 that barely disperse. The matrix permeability is also expected to be species-specific, being
109 related not only to dispersal capability, but also to availability of resources, such as food and
110 cover, in the matrix.
111 Finally, an issue related to the power of isolation detection, and not to the isolation
112 process itself, is the effective size (N_e) of each population. Reduction of gene flow between
113 large populations tends to take longer to be detectable, as genetic drift is less effective (Page
114 & Holmes 1998). In the case of the maned wolf, estimates of the number of living individuals
115 are relatively large – *ca.* 20,000 – 30,000 (Paula *et al.* 2007), suggesting that N_e might also be
116 quite large. Nevertheless, it is important to note that the species' distribution is considerably
117 broad, and functional populations can actually present low numbers of individuals due to low
118 demographic densities (Rodden *et al.* 2004). Moreover, a large number of individuals does
119 not necessarily imply a large effective size, since this estimation is more directly related to the

120 species genetic variability and determined by mating system and demographic history
121 (Frankham *et al.* 2002).

122 Further than only measuring genetic diversity (which is in itself an important indicator
123 of demographic aspects such as N_e, and a significant parameter in the context of population
124 long-term viability [Franklin & Frankham 1998; Lynch & Lande 1998]), the utilization of
125 molecular markers can be a useful approach to investigate if historical or current subdivision
126 processes affecting the Cerrado have influenced the population structure of the maned wolf.
127 In this context, molecular approaches would help to answer questions such as the occurrence,
128 degree and timing of population isolation episodes, presence of migrants among areas, and
129 identification of spatial barriers to the movement of individuals. The species' evolutionary
130 history can also be investigated, once past demographic fluctuations can be inferred by such
131 tools (Page & Holmes 1998).

132 In a recent molecular survey, P. Prates *et al.* (in preparation) have analyzed
133 mitochondrial DNA (mtDNA) sequences of maned wolves sampled throughout most of their
134 range, and found very low levels of genetic diversity, with no evidence of geographic
135 structuring. In addition, no molecular diversity was found in a sample of nuclear introns.
136 Their analyses suggested that the species suffered a population bottleneck followed by a
137 significant demographic expansion process *ca.* 15-20 thousand years ago (kya).

138 However, the exact dimension of this growth and the resulting genetic diversity –
139 especially as measured by rapidly evolving markers – is still a question to be answered, so as
140 to shed light on the maned wolf recent demographic history. Fast evolving loci (*e.g.*
141 microsatellites) can also eliminate the problem of low information content present in mtDNA
142 and nuclear sequences, thus helping to address the current population structure of the maned
143 wolf. The high mutation rates that are typical of microsatellites allow the identification of

144 even recent or slight processes of isolation (Ellegren 2004), so that even the impact of the
145 current environmental fragmentation caused by human activities might be detected using
146 these markers.

147 Given that the low genetic diversity observed so far with sequence-based markers
148 might have hampered the detection of population structuring in this species, the objective of
149 this study was to investigate this issue using microsatellite markers. In addition to measuring
150 current levels of microsatellite diversity, and inferring aspects of the demographic history of
151 the maned wolf, we thus set out to test whether fast-evolving markers could detect the
152 existence of recent population structure in this species, attempting to verify the presence of
153 even subtle patterns of geographic differentiation that might be caused by environmental
154 discontinuities or human-induced habitat fragmentation.

155

156 **Materials and Methods**

157 *Samples*

158 A total set of 144 tissue samples was utilized in this study. Sample collection was conducted
159 over the past ten years, and covers most of the species' range (Fig. 1). Three free living
160 populations were sampled more thoroughly, through the analysis of wild-caught individuals:
161 Serra da Canastra National Park, MG (SCNP, $n = 34$), Emas National Park, GO (ENP, $n = 27$)
162 and Northwestern São Paulo State (NWSP, $n = 23$) – captured for ecology and/or genetic
163 surveys. Additional samples were collected in different parts of the species' distribution (from
164 road-killed and captive individuals of known origin), including the Argentinean province of
165 Corrientes and the Brazilian states of Mato Grosso do Sul (MS), Mato Grosso (MT), São
166 Paulo (SP), Minas Gerais (MG), Rio de Janeiro (RJ), Goiás (GO) and Distrito Federal (DF)
167 (see Table 1). In addition to SCNP, ENP and NWSP, samples from Distrito Federal and

168 surrounding areas were considered to comprise a fourth definable population (referred to here
169 as DF, n = 13) because of their closely spaced collection points, even though they were
170 collected over a longer time span. In the case of dead animals, muscle or cartilage was
171 collected and preserved in ethanol. For live individuals, blood samples were obtained and
172 preserved in TES solution (100mM Tris, 100mM EDTA and 2% SDS). A phenol-chloroform
173 DNA extraction was conducted for all samples following the protocol of Sambrook & Russel
174 (2001), with slight modifications.
175
176 *Markers and PCR*
177 Fourteen microsatellite loci were utilized to characterize the genetic diversity of the maned
178 wolf (Table 2). Ten of them had been previously tested and optimized by Fontoura-Rodrigues
179 *et al.* (2008), and four others were selected based on additional efficiency and polymorphism
180 tests of the same set of tetranucleotide markers developed for the domestic dog (Francisco *et*
181 *al.* 1996). PCRs were conducted with a standardized protocol for all loci in a 10 μL volume,
182 using 0.2 μM reverse and M13 fluorescent-labeled primers (FAM, NED or HEX) and 0.013
183 μM forward M13-labeled primer (Boutin-Ganache *et al.* 2001), 2 mM $MgCl_2$, 0.2 mM each
184 dNTP, 0.5 U of *Taq* DNA Polymerase (Invitrogen) and 10-30 ng of the target DNA.
185 Reactions began with an initial denaturing step at 94ºC for 3 min, followed by ten cycles of
186 touchdown with denaturing at 94ºC for 45s, annealing at 60-51ºC for 45s and extension at
187 72ºC for 1.5 min. Thirty cycles with the same profile as above were then run, but maintaining
188 the annealing temperature at 50ºC. The last step was a final extension at 72°C for 30 min.
189 Products were run on a MEGABACE 1000 (GE-Healthcare) automated sequencer, with an
190 ET-ROX 550 internal size ladder (GE-Healthcare). The 14 loci were optimized to fit a three
191 multiplex set, according to their dye label and allele size range (Table 2). Fragment length

192 was determined using GENETIC PROFILER 2.0 (GE-Healthcare). From the initial set of
193 samples, only those that achieved a minimum genotyping efficiency of *ca.* 66% (*i.e.* nine out
194 of 14 loci) were utilized in the final analyses.
195
196 *Genetic diversity analyses*
197 The entire set of genotypes was tested for Hardy-Weinberg equilibrium (HWE) (Hardy-
198 Weinberg Exact Tests [Guo & Thompson 1992]) and linkage equilibrium (LE) using
199 GENEPOP 3.4 (Raymond & Rousset 1995), with default parameters for the statistical
200 significance tests. Genetic diversity indices (expected heterozygosity, observed
201 heterozygosity, polymorphic information content, number of alleles and allelic size range)
202 and the probability of identity ($P_{(ID)}$) (Waits *et al.* 2001) per locus, as well as the estimated
203 frequency of null alleles, were calculated using CERVUS 3.0.3 (Marshall *et al.* 1998). $P_{(ID)}$ is
204 defined as the chance of two individuals presenting the same genotypes at all loci, given the
205 sample size and observed levels of diversity. It is estimated for each locus, but can be easily
206 combined for a set of loci. The lower the combined result, the greater is the power of the
207 marker set to perform analyses. An identity test, to check for the existence of individuals with
208 fully matching genotypes, was also run using CERVUS.
209
210 *Population differentiation and structure analyses*
211 To investigate the presence of long-term demographic structure (*i.e.* spanning thousands of
212 years, and caused by geographic barriers or environmental discontinuities) or recent isolation
213 due to habitat fragmentation, population differentiation and structure tests were performed.
214 Pairwise values of F_{st} and R_{st} (Reynolds *et al.* 1983; Slatkin 1995) were estimated for the four
215 assumed populations (SCNP, ENP, NWSP and DF; see Fig. 1) with ARLEQUIN 3.11

(Excoffier *et al.* 2005) using 10,000 permutations for the significance test. A population structure analysis was also performed using the Bayesian clustering approach implemented in STRUCTURE 2.2 (Pritchard *et al.* 2000), which assesses how many populations (*k*) best explain the data. First, only the individuals belonging to the four well-delimited local populations were included in the analysis. In a second set of runs, all genotyped individuals were included. The parameters of the STRUCTURE analysis were 50,000 burn-in steps plus 1,000,000 MCMC iterations for sampling, under the admixture model. For each value of *k*, ten runs were performed. Finally, we checked for the existence and degree of isolation by distance using a Mantel Test (Mantel 1967), carried out with the program AIS (Miller 2005), employing 1,000 replicates for the significance test.

Demographic analyses

Demographic analyses were conducted to investigate the species' population history over the past several thousand years. Genetic diversity is not only affected by population structure, but also by demographic processes such as population growth, bottleneck events and other types of fluctuations in the number of individuals over many generations. The inference of demographic parameters affected by such historical processes allows the investigation of the underlying causes of the variability observed today. All demographic tests were conducted without using loci *2137* and *2119*, as they likely presented a switch from a tetranucleotide to a dinucleotide repeat motif (see the 'Results' section for details). If that is the case, both loci would not fit any of the simplified mutation models available for analysis, and could thus bias the results.

To investigate whether drastic population size changes have occurred in the recent past, expansion and bottleneck tests were performed. To check for population expansion, two

240 analyses from Reich & Goldstein (1998) were employed: frequency distribution of allele
241 lengths at homologous loci (Fig. 2) and *g*-statistics. The former analysis is visual, based on
242 the prediction that populations that underwent a severe bottleneck and then expanded will
243 show a more smoothly peaked distribution of the allele length frequencies. Considering a
244 single-step mutation model (SMM) it is expected that all the alleles in the population that
245 descend from the single remaining copy will present increasingly low frequencies as they
246 differ from the ancestral size by more repeat units. The latter analysis is based on the
247 prediction that populations of constant size will present a great variance in the time of allele
248 bifurcation events from locus to locus. On the other hand, populations under growth
249 conditions tend to have a similar time for bifurcation events across loci. Thus, the test
250 compares the variance among loci of the variance in allele length distribution, relative to
251 values estimated for a constant size population. A low ratio between the first and the second
252 terms indicates population growth. Critical values of statistical significance for $\alpha = 0.05$ based
253 on simulations were drawn from Reich *et al.* (1999).

254 Bottleneck tests were conducted using the program BOTTLENECK 1.2 (Piry *et al.*
255 1999), whose principle is the prediction that, after a severe reduction of population size, allele
256 number is reduced faster than heterozygosity. Thus, heterozygosity expected at mutation-drift
257 equilibrium (H_{eq}) is calculated and compared with the expected heterozygosity (H_e) obtained
258 from the data. Three statistical tests are available to test whether or not there are more loci
259 than expected showing $H_e > H_{eq}$. The program also supports calculation under three
260 microsatellite mutation models: infinite allele model (IAM), single-step mutation model
261 (SMM) and a settable intermediate two-phase model (TPM) (for a review see Estoup *et al.*
262 2002). All statistical tests were performed under all three mutation models, setting the TPM

for a small deviation from the SMM as suggested by the authors (variance: 12; SMM proportion: 95%).

The effective population size (N_e) was estimated through the formula $\theta = 4N_e\mu$, where μ is the mutation rate per generation (Watterson 1975). Estimates of θ were performed in ARLEQUIN 3.11 for microsatellite data under the SMM, as described by the program authors. For μ, a range of 10^{-3} to 10^{-5} was utilized, once a high variation in mutation rate has been reported to occur among loci in various species of animals (Goldstein & Schlötterer, 1999).

Results

Genetic diversity indices

Genotypes of 144 maned wolves were obtained for 14 loci. Minimum genotyping efficiency for a single individual was nine loci (*i.e.* ~66%), and the mean proportion of individuals typed across all loci was 86%. Identity tests showed that there were no two individuals with matching genotypes at all loci. Estimated genetic diversity indices were notably high, with the expected heterozygosity (H_e) ranging from 0.43 to 0.93 (mean 0.75) and the number of alleles per locus ranging from three to 27 (mean 10.21). The overall $P_{(ID),}$ as expected for a highly variable data set, was very low: 1.4^{-16}. Per locus diversity indices, null allele frequency estimates and $P_{(ID)}$ are shown in Table 2. The full genotype data set is available upon request.

Although all 14 loci were described for the domestic dog as tetranucleotide microsatellites (Francisco *et al.* 1996), two of them (*2137* and *2119*) presented two base pair variation between alleles in the maned wolf. Furthermore, they presented the highest levels of diversity, which were also similar to each other. A possible explanation for this phenomenon is a past insertion/deletion event of 2 base pairs (bp) in the flanking sequence or inside the

287 repetitive region. From this ancestral allele others could arise by simple microsatellite
288 mutation steps (i.e. insertion/deletion of whole repeat units). Once the primers utilized on this
289 study are originally designed for the domestic dog, whose lineage diverged from that of the
290 maned wolf *ca.* 10 million years ago (Wayne *et al.* 1997; Lindblad-Toh *et al.* 2005), it is not
291 difficult to imagine that such a situation could happen. The implications of this switch (from
292 tetranucleotide for dinucleotide) in these loci are negligible if the analysis considers data
293 qualitatively. However, analyses that make assumptions based on the mutational process or
294 uses the coalescent theory could be biased by the fact that these loci may deviate substantially
295 to any of the assumed mutational models. Therefore, markers *2137* and *2119* were not used in
296 some analyses.
297
298 *Equilibrium tests*
299 The first round of HWE and LE tests was performed with all individuals, *i.e.* not dividing
300 samples into pre-defined populations. Even so, no significant disequilibrium was verified in
301 any test after sequential Bonferroni corrections ($\alpha = 0.05$) (Rice 1989). Therefore, no
302 subsequent equilibrium analyses were performed for each population separately, as would be
303 advisable if any disequilibrium had been observed. Null allele frequency estimates were close
304 to zero at most loci, with the exception of *2018*, *2054*, *2088* and *2100* (which had values
305 higher than 0.05). However, these values were still rather low, and the HWE inferred for these
306 loci also suggests that the existence of non-genotyped alleles is unlikely.
307
308 *Population structure*
309 F_{st} estimates among the four initial populations were very low, even in the cases where they
310 were statistically significant (Table 3). The highest observed F_{st} value was 0.025, between the

311 DF and NWSP populations. The pair SCNP-ENP presented an F_{st} of 0.01, while all other
312 pairwise values were not statistically different from zero. No R_{st} value was statistically
313 different from zero (see Table 3).

314 The best likelihood value for *k* (number of estimated populations) calculated with the
315 method implemented in STRUCTURE (Table 4) was 1 for both approaches – (i) considering
316 only the individuals from the four local populations and (ii) considering all individuals.
317 Furthermore, the lowest likelihood variance (which tends to be smaller for the best *k*) was also
318 observed when $k = 1$. A Mantel test performed with AIS resulted in a correlation between
319 genetic and geographic distances of 0.14. Although statistically significant, this value is quite
320 low, and suggests a weak influence of geographic distance on genetic structuring.

321

322 *Demographic analyses*

323 The shape of the allele frequency distributions at all loci indicated a general trend for a
324 unimodal pattern, except for some deviations at loci *2140*, *2158* and *2100*. For loci *2140* and
325 *2158* the shapes fit a multimodal distribution. For marker *2100*, one allele (177-bp) had a
326 frequency that was higher than expected for a unimodal distribution. However, the shape did
327 not seem to fit a bimodal distribution either. An unknown molecular mechanism may be
328 biasing the mutational events at this locus, generating a tendency to generate the 177-bp allele
329 and thus increasing its frequency. The value found for the *g*-test of Reich & Goldstein (1998)
330 was 0.1601. Following the table of cutoff values given in Reich *et al.* (1999) for $p = 0.05$ this
331 result is statistically significant (*i.e.* lower than 0.19, which is the critical value for 12 loci and
332 a sample size of 80-160 individuals).

333 The BOTTLENECK results for the three types of statistical significance analysis were
334 congruent for the three mutation models. Under IAM, all tests pointed to a bottleneck event.

335 Under the SMM and TPM, no bottleneck was supported. As IAM and SSM are extreme
336 models, only the TPM results were considered. *P* values were 0.41 for the Sign Test, 0.34 for
337 the Standardized Differences Test and 0.25 for the Wilcoxon Test.

338 The value of θ_H as calculated by ARLEQUIN was 1.97 when averaged across all 12
339 loci used for this analysis. As a range of mutation rates was utilized, the resulting estimates of
340 N_e also ranged widely, from 492 to 49,250. However, considering the most utilized rate
341 among mammals, of *ca.* 10^{-4} (Paetkau *et al*. 1998; Rooney *et al.* 1999; Abdelkrim *et al.* 2005),
342 we estimated an N_e of 4,925 for this species.

343

344 **Discussion**

345 *Microsatellite diversity and population history*

346 The maned wolf presented very high genetic diversity at microsatellite markers based on a
347 broad sampling of its geographic distribution. This result is quite remarkable when contrasted
348 to the finding that this species exhibits extremely low diversity at the mtDNA and nuclear
349 introns, indicating the occurrence of a past bottleneck event in this species that drove its
350 genetic variability to near zero (P. Prates *et al.*, in preparation). Observed and estimated levels
351 of heterozygosity are similar to or higher than those recorded for other canids, even when
352 compared to species or populations for which no recent drastic demographic changes have
353 been reported (Girman *et al.* 2001; Klukowska *et al.* 2003; for a review see Wayne *et al.*
354 2004). This result is in agreement with previous genetic assessments of the maned wolf (Lion
355 2007; Salim *et al.* 2007), which have also reported microsatellite diversity levels that are
356 comparable to those observed in other canids. Therefore, it can be assumed that this species
357 recovered normal levels of microsatellite diversity in a rather short period of time, an
358 inference that is similar to that reported for cheetahs (Driscoll *et al.* 2002).

359 The present results of maned wolf heterozygosity levels are in total agreement with
360 our previous assessment (Fontoura-Rodrigues *et al.* 2008), which employed only nine loci and
361 20 individuals from a single population. The mean heterozygosity found in that study (0.729)
362 is almost the same as our present estimate for the same nine loci (0.723), now genotyped for
363 144 individuals, and still very close of our overall mean (0.75). These very similar values
364 indicate that (i) sampling a single population in our previous study yielded diversity estimates
365 that mirror those of the species as a whole, illustrating the effect of a virtual absence of
366 population structure; and (ii) the nine loci selected in our previous study as standardized
367 markers for Neotropical canids did capture a similar amount of genetic information as this
368 larger data set, highlighting their potential for future comparative assessments in multiple
369 species. If this observation is upheld by additional scrutiny, it will be interesting to further
370 investigate the rather high diversity levels that were also observed in that study for two other
371 species of Neotropical canids (*Lycalopex gymnocercus* and *L. vetulus*; mean H_e of 0.79 and
372 0.81, respectively).
373 The high variability observed in maned wolves is not only a good indicator of genetic
374 health, but also suggests a post-bottleneck population expansion, followed by the maintenance
375 of a large population size since then. The significant result of the *g*-test provides statistical
376 support for this expansion hypothesis, a scenario that is also compatible with the general trend
377 presented by the allele frequency distribution graphs (Fig. 2). Nine out of 12 graphs show an
378 unequivocal unimodal shape, which has been suggested to be a signal of population expansion
379 (Reich & Goldstein 1998). Even in the loci that did not present such a clear shape, either (i) a
380 trend suggestive of it could be discerned, or (ii) few peaks were present, which is still
381 compatible with a scenario of population bottleneck and expansion where a few ancestral
382 alleles survived. No bottleneck was identified here, indicating that the demographic reduction

prior to the inferred expansion could not be detected with microsatellites, implying that this signal has been 'erased' by the subsequent population growth. Also, it indicates that since this recent episode of population growth the number of individuals has remained fairly large, with no evidence of further dramatic reductions. Maintenance of large historical population size is also supported by the estimated values of N_e. Even the lowest estimate (492) corresponds to a relatively large effective size, approaching the critical long-term N_e of 500-5,000 suggested by Franklin & Frankham (1998) to be desirable for maintaining the evolutionary potential of populations. Assuming the microsatellite mutation rate most commonly utilized for mammals, and consequently estimating an N_e of *ca*. 5,000 individuals, it can be concluded that the effective population size of the maned wolf may be appreciable large, even when compared to the estimated current census size (N_c) of the species (20,000 – 30,000), given that N_e/N_c ratios are usually low in animals (Frankham *et al.* 2002). This relatively high N_e/N_c ratio may be due to the maintenance in the last few thousand years of a rather large, almost panmictic population (see below) over a broad area, and also to recent human-induced reductions in the census size, which likely have not yet had any effect on the effective size.

Population structure

Population structure analyses are all consistent with a scenario of no geographic partitions or population fragmentation due to old or recent barriers. Our genotypic data set is in HW and linkage equilibrium, which not only shows that it can be utilized as a single unit for all analyses, but also indicates a lack of detectable population divisions. If that was not the case, probably some degree of HW disequilibrium due to the Wahlund effect would have been observed (Hartl & Clark 1997). The Bayesian algorithm implemented in STRUCTURE, which is based on the attempt to minimize HW and linkage disequilibrium by subdividing

407 populations, also indicated that a single demographic unit was the best-fitting scenario for our
408 data set.

409 Furthermore, pairwise F_{st} and R_{st} estimates for local populations were also concordant
410 with the inference of high levels of gene flow among them (see Table 3). No R_{st} value was
411 statistically significant, which was also the case for four out of six for F_{st} estimates. The only
412 two significant F_{st} values were 2% for DF *vs.* NWSP, and 1% for ENP *vs.* SCNP. Although
413 significantly different from zero, these are very low values, with probably little biological
414 significance. It may also be noted that at least two of these four populations (ENP and SCNP)
415 were intensively sampled during a narrow window of time (no more than five years) and on a
416 limited geographic scale. So, it is possible that some of the sampled individuals are related to
417 each other, which might lead to a decrease in within-population diversity relative to random
418 expectations. Even so, there was no sign of restriction of gene flow between populations that
419 are on average 550 km distant from each other (ranging from 250 km [NWSP-SCNP] to 685
420 km [ENP-SCNP]).

421 Finally, the Mantel test also corroborated this general picture. The correlation between
422 geographic and genetic distances of *ca.* 14% indicates that there is a pattern of isolation by
423 distance in this species, but that even this effect is rather weak in terms of generating
424 population differentiation.

425 Based on these convergent results, we hypothesize a scenario in which maned wolves
426 behave as an almost panmictic population throughout most of its distribution. However, some
427 areas near the edge of the species' distribution have not been sampled, and others (such as
428 Argentina) were represented by few individuals. Thus it can not be definitively stated that
429 there are no geographic partitions at any point of the maned wolf range until populations can
430 be sampled throughout its entire distribution. Nevertheless, it may be concluded that the lack

431 of population structure is valid for the majority of its range, throughout the Brazilian Cerrado.
432 Since knowledge on even basic aspects of the maned wolf's biology (*e.g.* its exact distribution
433 and population status) is mostly lacking from its areas of occurrence in Argentina, Paraguay,
434 Uruguay, Bolivia and southern Brazil (see Fig. 1), genetic analyses extending these findings
435 to those regions will require the intensification of field-based efforts targeting this species. It
436 is reported that some isolated patches of habitat still persist in those areas and could support
437 maned wolf populations (Rodden *et al.* 2004), which must be investigated to verify if this
438 distribution disconnection might be causing population structuring.

439 The remarkable lack of population structure observed here, which is unusual even
440 among very vagile animals (Avise 2008), may be in part explained by some ecological and
441 behavioral traits of the maned wolf. The species is omnivorous, and further, feeds on a vast
442 number of different plant and small animal species. Its diet preferences seem to be functional
443 and not species-specific, in spite of the importance of *Solanum lycocarpum* in regions where
444 it occurs. Generalist species are recognized by their great plasticity (enabling them to live in
445 different kinds of environments), as well as tolerance to some level of anthropic disturbance
446 (Macdonald & Sillero-Zubiri 2004). Moreover, the species shows a high level of
447 territorialism, defending core living areas which are shared only with mates, of up to 100 km^2
448 (Rodden *et al.* 2004). Reported distances traveled by an individual on a single day within its
449 territory can reach a mean of 9 km and a maximum of 15 km (Melo *et al.* 2007). Although
450 little is known about the species' dispersal patterns, the territorialism and the great distances
451 that an individual can cover in a short period of time indicate that maned wolves can disperse
452 to far areas to establish territories.

453 Jointly, the high dispersal capability and generalist habits can help explain the species'
454 lack of geographic structure. Although the Cerrado exhibits a heterogeneous composition, our

455 data suggest that it represents a homogeneous environment for the maned wolf, whose
456 patterns of habitat use may imply continuous connectivity among distant areas. Indeed, the
457 species can occupy environments as diverse as tall grasslands, shrub habitats, woodland with
458 open canopy and damp fields (Rodden *et al.* 2004), all of which are typical and well-delimited
459 formations within the Cerrado (Silva & Bates 2005). In addition, recent reports of maned wolf
460 sightings or captures include disturbed areas formerly covered by dense Amazonian or
461 Atlantic rainforest (Paula *et al.* 2007; this study); moreover, individuals have been seen
462 hunting and resting on lands under cultivation for agriculture and pasture (Rodden *et al.*
463 2004). This set of observations reinforces the view that the maned wolf's habitat requirements
464 are quite flexible, allowing it to cross or even stably occupy a broad range of vegetational
465 landscapes.
466
467 *Conclusions and implications for conservation*
468 The maned wolf has a complex and not fully understood evolutionary history. Recent
469 molecular phylogenies of the family Canidae are all concordant in strongly placing
470 *Chrysocyon brachyurus* as the sister-species of the bush dog (*Speothos venaticus*) (Zrzavy &
471 Ricankova 2004; Bardeleben *et al.* 2005; Lindblad-Toh *et al.* 2005). This is a remarkable
472 finding point, since these species are very different regarding morphological, ecological and
473 behavioral traits. The bush dog is small, communal and lives mostly in forested areas
474 (Zuercher *et al.* 2004), while the maned wolf has a large size, solitary habits and occupies
475 open environments (Dietz 1984). The exact time and conditions that led their lineages to split,
476 as well as the evolutionary forces that drove these species to achieve such distinct
477 ecomorphological states, are interesting questions that remain to be answered.

478 The demographic history of the maned wolf subsequent to its divergence from the
479 bush dog is also quite unique. Current mtDNA and nuclear sequence data indicate that species
480 has recently expanded subsequent to being almost devoid of any sequence variation. Further
481 work is required to verify if an extreme population bottleneck in the Late Pleistocene is the
482 most likely explanation for this lack of variation at those markers. Although both mtDNA and
483 microsatellite markers bear evidence of a recent population expansion, the exact time and
484 magnitude of this demographic process should be further refined, allowing better inferences
485 on the underlying processes and their genetic consequences.

486 With respect to the current structure of maned wolf populations, our findings indicate
487 that this species is almost panmictic, with no significant geographic subdivisions. If this
488 observation is affirmed by the analysis of additional genomic loci, it may simplify the design
489 of conservation plans for this species, as it may represent a single management unit from a
490 genetic standpoint. Still, the possibility that local adaptation to specific environments affects
491 some genomic loci in this species should be considered, so that artificial translocations of
492 individuals across large areas should be discouraged. Nevertheless, given the species lack of
493 population structure and inferred ability to efficiently traverse varied types of habitat
494 matrices, our data provide an optimistic perspective for the long-term persistence of the
495 species in the Cerrado, in spite of current levels of anthropogenic disturbance. However, it is
496 probable that maned wolf individuals would not tolerate indefinite levels of habitat alteration
497 and isolation of preserved patches due to its close association to animal and plant species of
498 the original Cerrado, so that eventually the process of fragmentation should become an
499 important threat for the species. Even if this detrimental process is already ongoing, the
500 reduction of gene flow due to habitat disconnection may be such a recent process that it may
501 not yet be detectable, especially given the rather large N_e estimated for the species. As a large

502 N_e reduces the intensity of genetic drift, analyses that are based on the time-dependent
503 differentiation of allele frequencies and composition between isolated populations (such as
504 F_{st}) lose detection power, and might be not able to demonstrate the isolation even if the it is
505 actually ongoing. Although also recent, the human-induced fragmentation of the Atlantic
506 Forest is already resulting on drastic isolation of jaguar (*Panthera onca*) populations (Haag *et*
507 *al.*, in preparation), a process that is likely accelerated by the small size of remnant
508 populations and thus higher levels of genetic drift. The same process may be starting for
509 maned wolves, although their more generalist habits would make them less vulnerable to
510 complete isolation by an anthropogenic matrix, but still susceptible depending on the degree
511 of disturbance. Further studies are needed to better understand the maned wolf evolutionary
512 and natural history, and to monitor current gene flow across its distribution. In this sense, the
513 data collected in this study can serve as a baseline framework allowing future assessments of
514 spatial differentiation in the face of continuing habitat loss and fragmentation, so as to
515 monitor their effects on the structure and long-term viability of maned wolf populations.
516

517 **Acknowledgements**

518 We would like to thank all the people and institutions listed in Table 1, especially Flávio H.
519 G. Rodrigues, Rogério C. de Paula, Paulo Prates Jr., Marília B. Lion, Rodrigo Jorge, Joares
520 May Jr., Jean Pierre, Cristiana Prada, Paulo Sérgio R. de Mattos, Dênis Sana, Tathiana
521 Bagatini, Mauro Almeida, Nazaré Klautau, Leandro Silveira, Anah Jácomo, Frederico G.
522 Lemos, Museu Nacional (Brazil), UFES DNA Collection and Instituto Pró-Carnívoros for
523 providing biological samples. We are also grateful to Cladinara Roberts Sarturi, Sandro L.
524 Bonatto, Ricardo Kanitz and Nelson Fagundes for assisting with study design and

implementation. We especially thank Felipe Grazziotin for assistance with some aspects of data analysis and relevant discussions.

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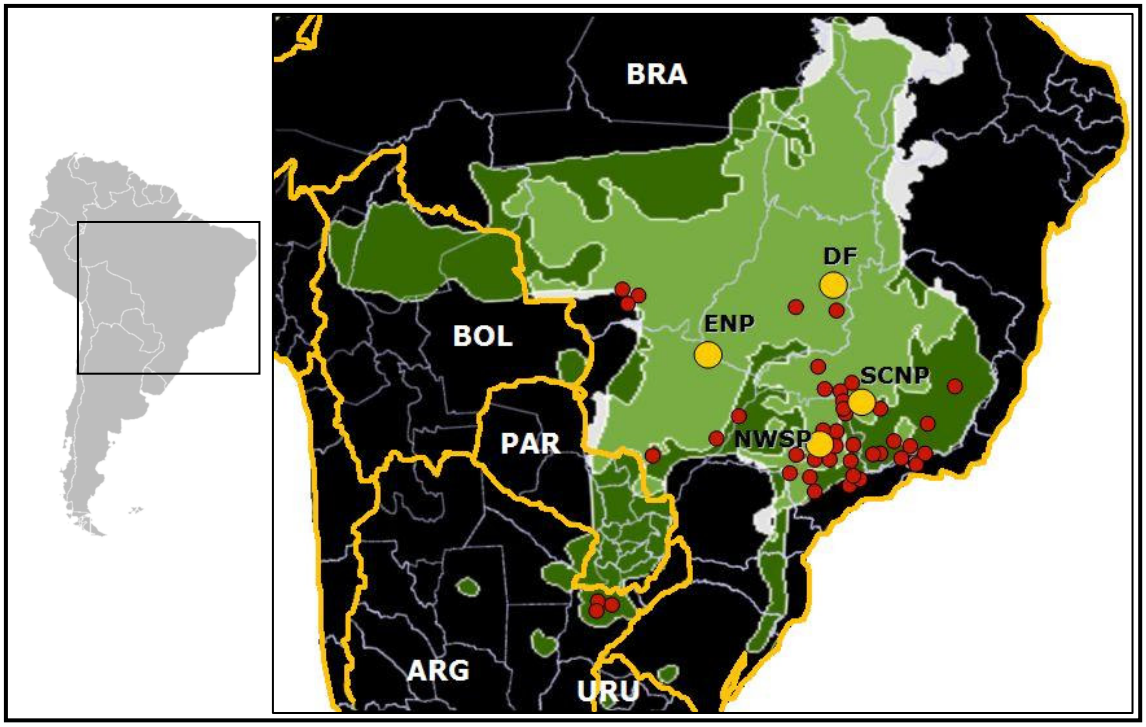

Figure 1. Geographic distribution of the maned wolf (in dark green) (Rodden *et al.* 2004) overlaid on that of the Cerrado biome (in white) (Silva & Bates 2005). The light green areas indicate the overlapping portions of both distributions. Red circles indicate individual collection points. Yellow circles indicate the four local populations analyzed in this study.

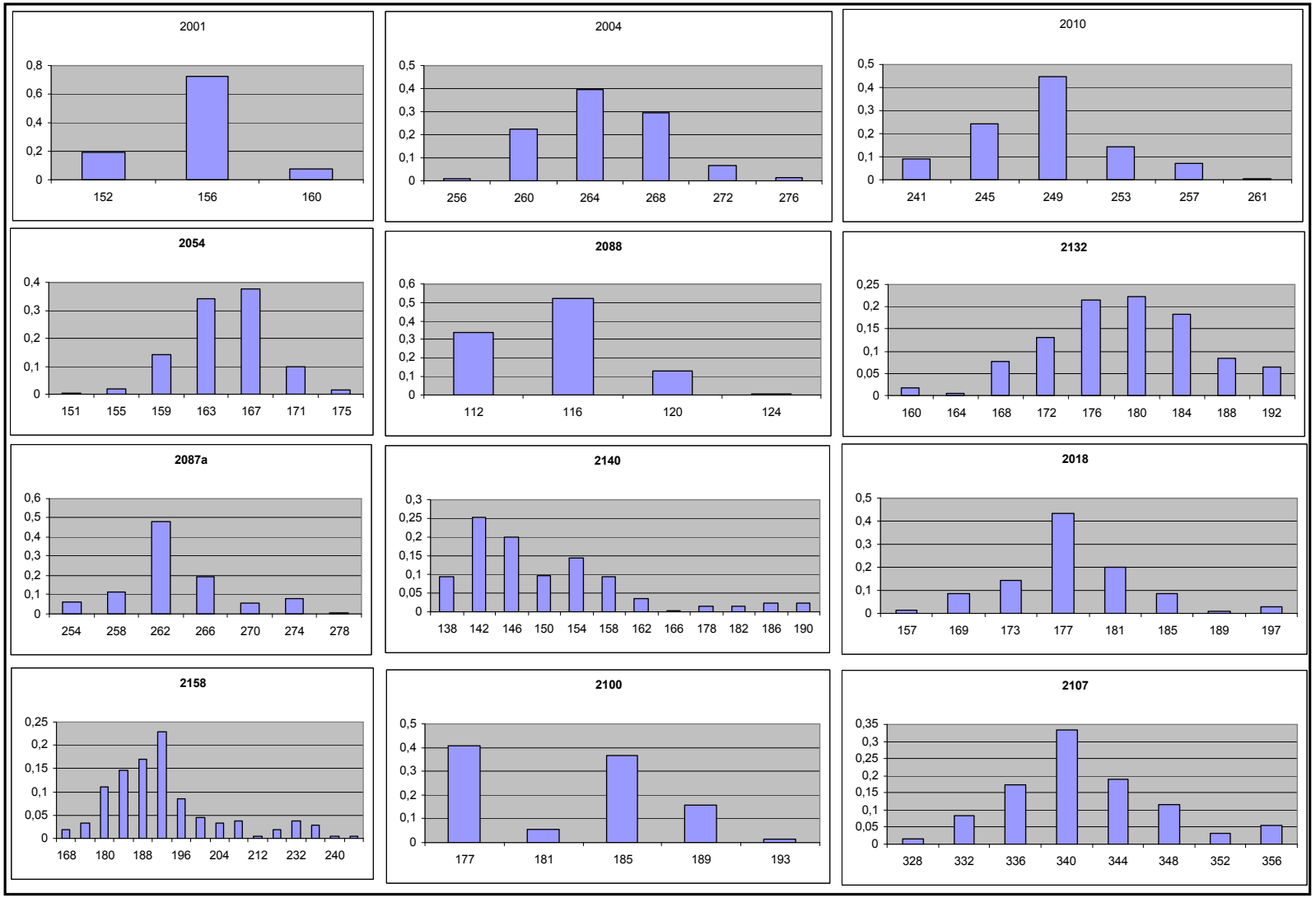

Figure 2. Distribution of allele frequencies for each microsatellite locus. Numbers defining the X-axis categories represent the PCR product size (in base pairs) for each allele.

Table 1. Individual ID, geographic origin, local population (when applicable), collector/contact information and sampling strategy for each of the samples analyzed in this study.

ID	Geographic Origin	Population	Institution/Collector/Contact	Sampling Strategy
bCbr-004	Mato Grosso State, C Brazil	---	Pró-Carnívoros	wild-caught
bCbr-005	Mato Grosso State, C Brazil	---	Pró-Carnívoros	wild-caught
bCbr-006	Mato Grosso State, C Brazil	---	Pró-Carnívoros	wild-caught
bCbr-008	Minas Gerais State, SE Brazil	---	Joares May Jr	road-killed
bCbr-009	Distrito Federal State, C Brazil	DF	Pró-Carnívoros	road-killed
bCbr-010	Mato Grosso do Sul State, C Brazil	---	Dênis Sana	road-killed
bCbr-011	Mato Grosso do Sul State, C Brazil	---	UFES	road-killed
bCbr-012	Minas Gerais State, SE Brazil	---	Frederico Gemesio Lemos	road-killed
bCbr-014	São Paulo State, SE Brazil	NWSP	Paulo Sérgio Ribeiro de Mattos	wild-caught
bCbr-015	São Paulo State, SE Brazil	NWSP	Paulo Sérgio Ribeiro de Mattos	wild-caught
bCbr-016	São Paulo State, SE Brazil	NWSP	Paulo Sérgio Ribeiro de Mattos	wild-caught
bCbr-017	São Paulo State, SE Brazil	NWSP	Paulo Sérgio Ribeiro de Mattos	wild-caught
bCbr-018	São Paulo State, SE Brazil	NWSP	Paulo Sérgio Ribeiro de Mattos	wild-caught
bCbr-019	São Paulo State, SE Brazil	NWSP	Paulo Sérgio Ribeiro de Mattos	wild-caught
bCbr-021	São Paulo State, SE Brazil	NWSP	Paulo Sérgio Ribeiro de Mattos	wild-caught
bCbr-022	São Paulo State, SE Brazil	NWSP	Paulo Sérgio Ribeiro de Mattos	wild-caught
bCbr-023	São Paulo State, SE Brazil	NWSP	Paulo Sérgio Ribeiro de Mattos	wild-caught
bCbr-024	São Paulo State, SE Brazil	NWSP	Paulo Sérgio Ribeiro de Mattos	wild-caught
bCbr-025	São Paulo State, SE Brazil	NWSP	Paulo Sérgio Ribeiro de Mattos	wild-caught
bCbr-026	São Paulo State, SE Brazil	NWSP	Paulo Sérgio Ribeiro de Mattos	wild-caught
bCbr-029	São Paulo State, SE Brazil	NWSP	Paulo Sérgio Ribeiro de Mattos	wild-caught
bCbr-030	São Paulo State, SE Brazil	NWSP	Paulo Sérgio Ribeiro de Mattos	wild-caught
bCbr-031	São Paulo State, SE Brazil	NWSP	Paulo Sérgio Ribeiro de Mattos	wild-caught
bCbr-032	São Paulo State, SE Brazil	NWSP	Paulo Sérgio Ribeiro de Mattos	wild-caught
bCbr-033	São Paulo State, SE Brazil	NWSP	Paulo Sérgio Ribeiro de Mattos	wild-caught
bCbr-034	São Paulo State, SE Brazil	NWSP	Paulo Sérgio Ribeiro de Mattos	wild-caught
bCbr-036	São Paulo State, SE Brazil	NWSP	Paulo Sérgio Ribeiro de Mattos	wild-caught
bCbr-037	São Paulo State, SE Brazil	NWSP	Paulo Sérgio Ribeiro de Mattos	wild-caught
bCbr-038	São Paulo State, SE Brazil	NWSP	Paulo Sérgio Ribeiro de Mattos	wild-caught
bCbr-039	São Paulo State, SE Brazil	NWSP	Paulo Sérgio Ribeiro de Mattos	wild-caught
bCbr-040	São Paulo State, SE Brazil	NWSP	Paulo Sérgio Ribeiro de Mattos	wild-caught
bCbr-041	Rio de Janeiro State, SE Brazil	---	Museu Nacional	?
bCbr-301	Minas Gerais State, SE Brazil	SCNP	Pró-Carnívoros	wild-caught
bCbr-302	Minas Gerais State, SE Brazil	SCNP	Pró-Carnívoros	wild-caught
bCbr-303	Minas Gerais State, SE Brazil	SCNP	Pró-Carnívoros	wild-caught
bCbr-304	Minas Gerais State, SE Brazil	SCNP	Pró-Carnívoros	wild-caught
bCbr-305	Minas Gerais State, SE Brazil	SCNP	Pró-Carnívoros	wild-caught
bCbr-306	Minas Gerais State, SE Brazil	SCNP	Pró-Carnívoros	wild-caught
bCbr-307	Minas Gerais State, SE Brazil	SCNP	Pró-Carnívoros	wild-caught
bCbr-308	Minas Gerais State, SE Brazil	SCNP	Pró-Carnívoros	wild-caught
bCbr-309	Minas Gerais State, SE Brazil	SCNP	Pró-Carnívoros	wild-caught
bCbr-310	Minas Gerais State, SE Brazil	SCNP	Pró-Carnívoros	wild-caught
bCbr-311	São Paulo State, SE Brazil	---	Cristiana Prada	road-killed
bCbr-312	São Paulo State, SE Brazil	---	Cristiana Prada	road-killed
bCbr-313	São Paulo State, SE Brazil	---	Cristiana Prada	road-killed
bCbr-315	São Paulo State, SE Brazil	---	Cristiana Prada	road-killed
bCbr-317	São Paulo State, SE Brazil	---	Cristiana Prada	road-killed
bCbr-319	São Paulo State, SE Brazil	---	Cristiana Prada	road-killed
bCbr-320	São Paulo State, SE Brazil	---	Cristiana Prada	road-killed
bCbr-325	São Paulo State, SE Brazil	---	Mauro Almeida	wild-caught
bCbr-327	São Paulo State, SE Brazil	---	Pró-Carnívoros	wild-caught
bCbr-329	Goiás State, C Brazil	ENP	Leandro Silveira	wild-caught
bCbr-330	Goiás State, C Brazil	ENP	Leandro Silveira	wild-caught
bCbr-344	Goiás State, C Brazil	ENP	Leandro Silveira	wild-caught
bCbr-345	Goiás State, C Brazil	ENP	Leandro Silveira	wild-caught
bCbr-350	Goiás State, C Brazil	ENP	Leandro Silveira	wild-caught
bCbr-364	São Paulo State, SE Brazil	---	Rodrigo Jorge/Tathiana Bagatini	road-killed
bCbr-366	Minas Gerais State, SE Brazil	---	Joares May Jr/Jean Pierre	road-killed
bCbr-370	Minas Gerais State, SE Brazil	SCNP	Pró-Carnívoros	wild-caught
bCbr-371	Minas Gerais State, SE Brazil	SCNP	Pró-Carnívoros	wild-caught
bCbr-372	Minas Gerais State, SE Brazil	SCNP	Pró-Carnívoros	wild-caught
bCbr-373	Minas Gerais State, SE Brazil	SCNP	Pró-Carnívoros	wild-caught
bCbr-374	Minas Gerais State, SE Brazil	SCNP	Pró-Carnívoros	wild-caught
bCbr-375	Minas Gerais State, SE Brazil	SCNP	Pró-Carnívoros	wild-caught
bCbr-376	Minas Gerais State, SE Brazil	SCNP	Pró-Carnívoros	wild-caught
bCbr-377	Minas Gerais State, SE Brazil	SCNP	Pró-Carnívoros	wild-caught
bCbr-378	Minas Gerais State, SE Brazil	SCNP	Pró-Carnívoros	wild-caught
bCbr-379	Minas Gerais State, SE Brazil	SCNP	Pró-Carnívoros	wild-caught
bCbr-380	Minas Gerais State, SE Brazil	SCNP	Pró-Carnívoros	wild-caught
bCbr-381	Minas Gerais State, SE Brazil	SCNP	Pró-Carnívoros	wild-caught
bCbr-382	Minas Gerais State, SE Brazil	SCNP	Pró-Carnívoros	wild-caught

Table 1. Continued

ID	Geographic Origin	Population	Institution/Collector/Contact	Sampling Strategy
bCbr-383	Minas Gerais State, SE Brazil	SCNP	Pró-Carnívoros	wild-caught
bCbr-384	Minas Gerais State, SE Brazil	SCNP	Pró-Carnívoros	wild-caught
bCbr-388	Minas Gerais State, SE Brazil	SCNP	Pró-Carnívoros	wild-caught
bCbr-389	Minas Gerais State, SE Brazil	SCNP	Pró-Carnívoros	wild-caught
bCbr-390	Minas Gerais State, SE Brazil	SCNP	Pró-Carnívoros	wild-caught
bCbr-401	Minas Gerais State, SE Brazil	SCNP	Pró-Carnívoros	wild-caught
bCbr-402	Minas Gerais State, SE Brazil	SCNP	Pró-Carnívoros	wild-caught
bCbr-403	Minas Gerais State, SE Brazil	SCNP	Pró-Carnívoros	wild-caught
bCbr-404	Minas Gerais State, SE Brazil	SCNP	Pró-Carnívoros	wild-caught
bCbr-405	Minas Gerais State, SE Brazil	SCNP	Pró-Carnívoros	wild-caught
bCbr-406	Minas Gerais State, SE Brazil	SCNP	Pró-Carnívoros	wild-caught
bCbr-420	Minas Gerais State, SE Brazil	---	Rogério Cunha de Paula	wild-caught
bCbr-421	Minas Gerais State, SE Brazil	---	Rogério Cunha de Paula	wild-caught
bCbr-422	São Paulo State, SE Brazil	---	Rodrigo Jorge/Tathiana Bagatini	wild-caught
bCbr-425	Goiás State, C Brazil	ENP	Leandro Silveira/Anah Jácomo	wild-caught
bCbr-426	Goiás State, C Brazil	ENP	Leandro Silveira/Anah Jácomo	wild-caught
bCbr-427	Goiás State, C Brazil	ENP	Leandro Silveira/Anah Jácomo	wild-caught
bCbr-428	Goiás State, C Brazil	ENP	Leandro Silveira/Anah Jácomo	wild-caught
bCbr-429	Goiás State, C Brazil	ENP	Leandro Silveira/Anah Jácomo	wild-caught
bCbr-451	Rio de Janeiro State, SE Brazil	---	Rogério Cunha de Paula/Joares May Jr	captive
bCbr-452	Rio de Janeiro State, SE Brazil	---	Rogério Cunha de Paula/Joares May Jr	captive
bCbr-455	Goiás State, C Brazil	ENP	Leandro Silveira/Anah Jácomo	wild-caught
bCbr-456	Goiás State, C Brazil	ENP	Leandro Silveira/Anah Jácomo	wild-caught
bCbr-457	Goiás State, C Brazil	ENP	Leandro Silveira/Anah Jácomo	wild-caught
bCbr-458	Goiás State, C Brazil	ENP	Leandro Silveira/Anah Jácomo	wild-caught
bCbr-459	Goiás State, C Brazil	ENP	Leandro Silveira/Anah Jácomo	wild-caught
bCbr-460	Goiás State, C Brazil	ENP	Leandro Silveira/Anah Jácomo	wild-caught
bCbr-461	Goiás State, C Brazil	ENP	Leandro Silveira/Anah Jácomo	wild-caught
bCbr-462	Goiás State, C Brazil	ENP	Leandro Silveira/Anah Jácomo	wild-caught
bCbr-463	Goiás State, C Brazil	ENP	Leandro Silveira/Anah Jácomo	wild-caught
bCbr-464	Goiás State, C Brazil	ENP	Leandro Silveira/Anah Jácomo	wild-caught
bCbr-465	Goiás State, C Brazil	ENP	Leandro Silveira/Anah Jácomo	wild-caught
bCbr-466	Goiás State, C Brazil	ENP	Leandro Silveira/Anah Jácomo	wild-caught
bCbr-467	Goiás State, C Brazil	ENP	Leandro Silveira/Anah Jácomo	wild-caught
bCbr-468	Goiás State, C Brazil	ENP	Leandro Silveira/Anah Jácomo	wild-caught
bCbr-469	Goiás State, C Brazil	ENP	Leandro Silveira/Anah Jácomo	wild-caught
bCbr-470	Goiás State, C Brazil	ENP	Leandro Silveira/Anah Jácomo	wild-caught
bCbr-471	Goiás State, C Brazil	ENP	Leandro Silveira/Anah Jácomo	wild-caught
96/001	Minas Gerais State, SE Brazil	---	Zoológico de Varginha	captive
96/003	Minas Gerais State, SE Brazil	---	Zoológico de Varginha	captive
96/004	Minas Gerais State, SE Brazil	---	Zoológico de Varginha	captive
96/012	São Paulo State, SE Brazil	---	Zoológico de Curitiba	captive
96/014	Minas Gerais State, SE Brazil	---	Zoológico de Curitiba	captive
96/023	São Paulo State, SE Brazil	---	Zoológico de Uberaba	captive
96/025	Minas Gerais State, SE Brazil	---	Zoológico de Uberaba	captive
96/029	Minas Gerais State, SE Brazil	---	Zoológico de Araxá	captive
96/031	São Paulo State, SE Brazil	---	Zoológico de Sorocaba	captive
96/035	São Paulo State, SE Brazil	---	Zoológico de Bauru	captive
96/040	São Paulo State, SE Brazil	---	Zoológico de Bauru	captive
96/043	São Paulo State, SE Brazil	---	Zoológico de Bauru	captive
96/046	Goiás State, C Brazil	DF	Zoológico de Brasília	captive
96/047	Goiás State, C Brazil	---	Zoológico de Brasília	captive
96/049	Distrito Federal State, C Brazil	DF	Zoológico de Brasília	captive
96/051	Goiás State, C Brazil	DF	Zoológico de Brasília	captive
96/052	Goiás State, C Brazil	DF	Zoológico de Brasília	captive
LG-007	Minas Gerais State, SE Brazil	---	Zoológico de Uberlândia	captive
LG-009	Goiás State, C Brazil	---	Zoológico de Goiânia	captive
LG-028	Minas Gerais State, SE Brazil	---	Zoológico de Belo Horizonte	captive
LG-031	Minas Gerais State, SE Brazil	---	Zoológico de Belo Horizonte	captive
LG-032	Minas Gerais State, SE Brazil	---	Zoológico de Curitiba	captive
LG-033	São Paulo State, SE Brazil	---	Zoológico de Curitiba	captive
LG-034	São Paulo State, SE Brazil	---	Zoológico de Curitiba	captive
AE/LG-75	Distrito Federal State, C Brazil	DF	Flávio HG Rodrigues	wild-caught
AE/LG-82	Distrito Federal State, C Brazil	DF	Flávio HG Rodrigues	wild-caught
AE/LG-92	Distrito Federal State, C Brazil	DF	Flávio HG Rodrigues	wild-caught
ESECAE-4	Distrito Federal State, C Brazil	DF	Marília Lion	road-killed
ESECAE-7	Distrito Federal State, C Brazil	DF	Marília Lion	road-killed
Colorado	Distrito Federal State, C Brazil	DF	Marília Lion	road-killed
Marília	Distrito Federal State, C Brazil	DF	Marília Lion	wild-caught
Flavão	Distrito Federal State, C Brazil	DF	Marília Lion	wild-caught
AR-1	Corrientes Province, NE Argentina	---	Paulo Prates Jr	?
AR-2	Corrientes Province, NE Argentina	---	Paulo Prates Jr	?
AR-7	Corrientes Province, NE Argentina	---	Paulo Prates Jr	?

Table 2. Fluorescent labels and multiplex panel employed for each locus within the optimized three-panel multiplexing scheme used here. The number of alleles (A), allele range in base pairs, observed heterozygosity (H_o), expected heterozygosity (H_e), polymorphic information content (PIC), probability of identity ($P_{(ID)}$) and estimated frequency of null alleles (F(Null)) for each locus are also shown, followed by the overall mean across loci at the bottom.

Locus	Label	Multiplex	A	Range (bp)	H_o	H_e	PIC	$P_{(ID)}$	F(Null)
2001	Fam	1	3	152 - 160	0.435	0.431	0.382	0.373	-0.0071
2004	Fam	1	6	256 - 276	0.657	0.706	0.649	0.142	+0.0340
2010	Ned	1	6	241 - 261	0.698	0.709	0.665	0.128	+0.0110
2018	Hex	1	8	157 - 197	0.717	0.739	0.703	0.102	+0.0757
2054	Ned	1	7	151 - 175	0.615	0.713	0.660	0.134	+0.0657
2088	Ned	2	4	112 - 124	0.516	0.595	0.516	0.243	+0.0771
2132	Ned	2	9	160 - 192	0.715	0.839	0.815	0.048	+0.0230
2137	Fam	2	25	219 - 283	0.890	0.934	0.926	0.009	+0.0256
2140	Fam	2	12	138 - 190	0.805	0.848	0.827	0.042	+0.0082
2158	Hex	2	16	168 - 244	0.853	0.873	0.857	0.030	+0.0089
2087a	Fam	3	7	254 - 278	0.688	0.706	0.671	0.121	+0.0135
2100	Fam	3	5	177 - 193	0.652	0.676	0.612	0.168	+0.0630
2107	Fam	3	8	328 - 356	0.711	0.803	0.773	0.066	+0.0113
2119	Hex	3	27	232 - 312	0.914	0.937	0.928	0.009	+0.0096
mean			10.21	112 - 356*	0.704	0.75	0.713	1.47^{-16}**	

* Allele range considering all loci.

** Combined $P_{(ID)}$ across all loci.

Table 3. Pairwise F_{st} (below the diagonal) and R_{st} (above the diagonal) values estimated between local populations. Asterisks indicate statistical significance ($\alpha = 0.05$).

	NWSP	SCNP	DF	ENP
NWSP	-	-0.00953	0.03385	-0.00431
SCNP	-0.01446	-	-0.03441	-0.00579
DF	0.02557*	-0.01820	-	-0.00287
ENP	-0.00048	0.01042*	-0.00324	-

Table 4. Mean values (across 10 runs) of likelihood and likelihood variance for each assumed number of populations (*k*) calculated with the program STRUCTURE. Column A presents the values when considering all individuals, whereas column B depicts the results of analyses considering only the individuals belonging to the four assumed local populations.

	A		*B*	
K	**LnP(D)**	**Var[LnP(D)]**	**LnP(D)**	**Var[LnP(D)]**
1	-6034.03	61.64	-4099.63	56.02
2	-6202.86	626.17	-4361.31	747.6
3	-6571.13	1599.92	-4744.29	1674.13
4	-6476.91	1608.04	-4562.92	1491.63
5	-6496.6	1876.02	-4800.95	2056.71
6	-6424.34	1829.29	-4810.95	2159.05