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

FILOGEOGRAFIA, HISTÓRIA DEMOGRÁFICA E DIVERSIDADE MOLECULAR DE DUAS ESPÉCIES NEOTROPICAIS DA FAMÍLIA PROCYONIDAE (MAMMALIA, CARNIVORA): *Nasua nasua* E *Procyon cancrivorus*

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DEDICATÓRIA

"As criaturas que habitam esta terra em que vivemos, sejam eles seres humanos ou animais, estão aqui para contribuir, cada uma com sua maneira peculiar, para a beleza e prosperidade do mundo."

Sua Santidade, o Dalai Lama

Dedico a todos aqueles que acreditam e vivem no respeito

por todas as formas de vida

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SUMÁRIO

RESUMO

Estudos filogeográficos comparados são úteis na compreensão de processos históricos compartilhados que afetam faunas regionais, bem como na identificação de padrões espécie-específicos que podem influenciar suas atuais características genéticas. Neste estudo, foram realizadas análises filogeográficas de dois carnívoros Neotropicais de médio porte, o quati de focinho marrom (*Nasua nasua*) e o mão pelada (*Procyon cancrivorus*), usando marcadores mitocondriais e microssatélites, afim de caracterizar e comparar seus padrões de diversidade genética e compreender sua história evolutiva. Adicionalmente, descreve-se o isolamento e a caracterização de oito loci polimórficos de microssatélites para *Nasua nasua*.

Ambas as espécies são bastante comuns na natureza e estão presentes em uma ampla variedade de habitats, sendo simpátricas ao longo da maior parte de sua distribuição. No entanto, diferentes padrões filogeográficos e de diversidade genética foram encontrados para *N. nasua* e *P. cancrivorus*: análises de DNA mitocondrial mostraram níveis de diversidade até dez vezes superiores para *N. nasua* com relação a *P. cancrivorus*. Adicionalmente, os mesmos marcadores revelaram a existência de 6 filogrupos reciprocamente monofiléticos para *N. nasua*, os quais também são suportados como populações distintas pelas análises de microssatélites. De maneira distinta, as análises de DNA mitocondrial para *P. cancrivorus* indicam a existência de três unidades populacionais; no entanto, a magnitude desta diferenciação foi muito menos evidente do que a observada em *N. nasua*. Além disso, os dados de microssatélites não suportaram a existência de qualquer subdivisão genética para *P. cancrivorus*, sugerindo que persiste uma completa conectividade entre todas as áreas amostradas. Estes resultados demonstram que estas espécies apresentam uma historia evolutiva bastante distinta, a qual pelo menos em parte pode ser atribuída a diferenças na estrutura social e no padrão de dispersão das mesmas. Tais resultados destacam a complexidade evolutiva da biota Neotropical e ressaltam a necessidade de análises multi-espécies empregando conjuntos de dados comparáveis, de forma que padrões comuns e contrastantes possam ser adequadamente investigados.

ABSTRACT

Phylogeography, demographic history and molecular diversity of two Neotropical species of family Procyonidae (Mammalia, Carnivora): *Nasua nasua* and *Procyon cancrivorus*.

Comparative phylogeographic analyses are useful to shed light on common historical processes affecting regional faunas, as well as to identify species-specific life history features that may influence their genetic legacy. Here we performed phylogeographic analysis of two medium-sized Neotropical carnivores, the brown-nosed coati (*Nasua nasua*) and the crab-eating raccoon (*Procyon cancrivorus*), using mitochondrial DNA and microsatellite markers, in order to characterize and compare their patterns of genetic diversity and underlying evolutionary history. We also describe the isolation and characterization of eight polymorphic microsatellite loci for brown-nosed coatis (*N. nasua*).

Both species are farily common in the wild and present in a wide variety of habitats, being sympatric throughout most of their ranges. However, different phylogeographic and diversity patterns were found for both markers: mitochondrial DNA analyses showed levels of diversity that were up to ten-fold higher for *N. nasua* relative to *P. cancrivorus*. Six reciprocally monophyletic mtDNA phylogroups were recognized for *N. nasua*, which were also supported as distinct populations by the microsatellite analyses. In contrast, the mtDNA data set for *P. cancrivorus* indicated the existence of three recognizable population units, but the magnitude of their differentiation was much less pronounced than that observed in *N. nasua*. Moreover, the microsatellite data did not support any genetic subdivision in this species, suggesting that full connectivity is maintain throughout all sampled areas. These results demonstrate that these species have very distinct evolutionary histories, which may at least in part be a consequence of differences in social structure and dispersal patterns. These results highlight the evolutionary complexity of the Neotropical biota, and underscore the need for multi-species analyses employing comparable data sets so that common and contrasting patterns can be adequately investigated.

APRESENTAÇÃO

O presente trabalho, intitulado "Filogeografia, história demográfica e diversidade molecular de duas espécies neotropicais da família Procyonidae (Mammalia, Carnivora): *Nasua nasua* e *Procyon cancrivorus* foi desenvolvido como parte dos requisitos necessários para a obtenção do título de Mestre junto ao Programa de Pós-Graduação em Zoologia da Pontifícia Universidade Católica do Rio Grande do Sul.

Este trabalho teve como principais objetivos (i) caracterizar a estrutura genética de duas espécies de procionídeos neotropicais, *Nasua nasua* (quati de focinho marrom) e *Procyon cancrivorus* (mão-pelada), (ii) inferir a história demográfica destas, comparando-a com outras espécies neotropicais a fim de investigar a ocorrência de padrões filogeográficos compartilhados (iii), além de integrar os dados moleculares obtidos com informações já disponíveis sobre estas espécies, a fim de contribuir para um melhor conhecimento de sua biologia, embasando estratégias para sua conservação a longo prazo na natureza.

Esta dissertação é apresentada no formato de dois artigos científicos: uma *Primer Note*, descrevendo o isolamento e caracterização de oito *loci* de microssatélites para *N. nasua* a ser submetido ao periódico *Molecular Ecology Resources* (apresentado no Apêndice) e o artigo principal, tratando de filogeografia comparada, a ser submetido ao periódico *Molecular Ecology*.

Comparative phylogeographic patterns reveal contrasting demographic histories in two Neotropical procyonids (*Nasua nasua* and *Procyon cancrivorus*)

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Running title: Contrasting genetic patterns in procyonids

Abstract

Comparative phylogeographic analyses are useful to shed light on common historical processes affecting regional faunas, as well as to identify species-specific life history features that may influence their genetic legacy. Here we performed phylogeographic analysis of two medium-sized Neotropical carnivores, the brown-nosed coati (*Nasua nasua*) and the crab-eating raccoon (*Procyon cancrivorus*), using mitochondrial DNA and microsatellite markers, in order to characterize and compare their patterns of genetic diversity and underlying evolutionary history. Both species are farily common in the wild and present in a wide variety of habitats, being sympatric throughout most of their ranges. Mitochondrial DNA analyses showed levels of diversity that were up to ten-fold higher for *N. nasua* relative to *P. cancrivorus*. Six reciprocally monophyletic mtDNA phylogroups were recognized for *N. nasua*, which were also supported as distinct populations by the microsatellite analyses. In contrast, the mtDNA data set for *P. cancrivorus* indicated the existence of three recognizable population units, but the magnitude of their differentiation was much less pronounced than that observed in *N. nasua*. Moreover, the microsatellite data did not support any genetic subdivision in this species, suggesting that full connectivity is maintained throughout all sampled areas. These results demonstrate that these species have very distinct evolutionary histories, which may at least in part be a consequence of differences in social structure and dispersal patterns. These results highlight the evolutionary complexity of the Neotropical biota, and underscore the need for multi-species analyses employing comparable data sets so that common and contrasting patterns can be adequately investigated.

Introduction

Several studies concerning different organisms attempted to understand the processes that have shaped the current species distribution and genetic structure found in the Neotropical region (Costa 2003, Hubert & Renno 2006, Carnaval & Bates 2007, Grazziotin *et al.* 2007, Martins *et al.* 2007). Although some common patterns can be identified among the studies, they all agreed that there is no single model of vicariance or climatic change that could explain the Neotropical complexity.

The use of common and widespread species in phylogeographic studies may be very useful due to the possibility of going beyond species-specific biogeographic patterns to pursue comparative analysis of regional or continental biotas. Some intra-specific studies concerning common vertebrates have been conducted in the Neotropical region (e.g. Wüstter *et al.* 2005, Noonan & Wray 2006, Grazziotin *et al.* 2007), but still very have few have focused on carnivores (e.g. Eizirik *et al.* 1998, 2001, Tchaicka *et al.* 2007, Trinca *et al.* 2007). Among the Brazilian Carnivora, some of the least studied species belong to the families Procyonidae, Mustelidae and Mephitidae (Oliveira 2006), so that in many cases basic aspects of their biology, ecology and geographic distributions remain to be clarified.

The family Procyonidae comprises six genera (*Potos, Procyon, Nasua, Nasuella, Bassaricyon, Bassariscus*) and fourteen recognized species (Wozencraft 2005), distributed from Canada to Argentina. The inter-generic relationships of the family have been subject of several phylogenetic studies based on morphological and molecular characters (Decker & Wozencraft 1991, Baskin 2004, Fulton & Strobeck 2007, Koepfli *et al.* 2007). Contrasting patterns were

recovered depending on the type of information used. According to Koepfli *et al.* (2007), *Potos* is the sister lineage to the clade containing the remaining genera (divergence time estimates: 21.6 – 24 mya [95% confidence intervals, CI = 12.1 – 36.0 mya]), which is divided into two subgroups: *Nasua* plus *Bassarycion* and *Procyon* plus *Bassariscus* (divergence time estimates: 18.3 – 20.7 mya, CI = 10.3 – 30.9 mya). Within *Nasua* and *Procyon*, the divergence time estimates are respectively 7 – 8 mya (CI = 3.7 – 12.9 mya) and 5 – 5.7 mya (CI = 2.6 – 9.2 mya), predating the closure of the Panamanian land bridge.

The brown-nosed coati (*Nasua nasua*) is a diurnal, highly social mesocarnivore (3.5 – 6.0 kg) that is distributed in South America, from Colombia and Venezuela to Uruguay and northern Argentina (Redford & Eisenberg 1992, Gompper & Decker 1998, Nowak 1999). It is found primarily in forested habitats, ranging from tropical rainforest and gallery forest to chaco, cerrado and dry scrub environments (Gompper & Decker 1998, Emmons 1990). Coatis forage both arboreally and terrestrially, and their diet includes primarily fruits, invertebrates and occasionally small vertebrates (Redford & Eisenberg 1992, Nowak 1999). Females and immature males form permanent groups while males are solitary, joining the groups only during the mating season. After this period, they seem to be excluded from the groups by adult females, apparently to avoid aggression against the juveniles (Russel 1981, Redford & Eisenberg 1992). Females leave the groups to give birth to young, which are born in an arboreal nest after seventy-seven days of gestation (Nowak 1999).

The crab-eating raccoon (*Procyon cancrivorus*) is a nocturnal, medium-sized carnivore (3 – 8 kg), which is distributed from Central America (southern Costa Rica and eastern Panama) throughout South America to northeastern Argentina and Uruguay. In Costa Rica and Panama, its

range overlaps with that of the northern raccoon (*Procyon lotor*), but the latter is mainly found in mangrove swamps, and the crab-eating raccoon is found mostly near inland rivers (Eisenberg 1989, Emmons 1990). Although this species occurs in diverse environments, it seems to be somewhat restricted to waterside habitats (Redford & Eisenberg 1992, Emmons 1990). *Procyon cancrivorus* is a generalized-omnivore, and its diet includes fruits, invertebrates and small vertebrates, depending on resource availability (Bisbal 1986, Santos & Hartz 1999, Gatti *et al.* 2006). They tend to forage alone, except for the female-offspring unit (Redford & Eisenberg 1992).

In this study, we aimed to characterize the genetic structure, phylogeographic patterns and demographic history of *N. nasua* and *P. cancrivorus*, using both mitochondrial and microsatellite markers. Our objective was to test if these two sympatric carnivores possess similar evolutionary histories, or if their patterns are species-specific and may be correlated to known biological differences between them. By comparing their phylogeographic structure and demographic history, we aimed to look for common or contrasting patterns that may enhance our understanding of the evolutionary dynamics of Neotropical mammals.

Material and Methods

Sample collection and laboratory techniques

Biological samples (blood and tissue) were collected from 90 *N. nasua* and 44 *Procyon cancrivorus* individuals (Tables 1 and 2, respectively) across the range of each species (Figure 1 and Figure 2, respectively). Blood samples were obtained from wild animals captured for ecological

117 studies and captive individuals of known origin, and preserved in a salt saturated solution (100mM
118 Tris, 100mM EDTA, 2% SDS). Tissue samples were collected from road-killed specimens and
119 preserved in 96% ethanol. Genomic DNA extraction was performed using a standard phenol-
120 chloroform protocol (Sambrook *et al.* 1989). All DNA samples were quantified in a 1% agarose gel
121 stained with GelRed® (Biotium Inc.) using the LowMass DNA Ladder (Invitrogen) as a
122 concentration standard, and diluted to a final working concentration of 10 ng/ μL.
123

124 ***Mitochondrial sequencing***

125

126 Three different mtDNA fragments were amplified with the polymerase chain reaction
127 (PCR): (i) the 5' portion of the *NADH dehydrogenase subunit 5* (*ND5*) gene using primers
128 described by Trigo *et al.* (2008); (ii) the 5' portion of the control region using the forward primer
129 MTLPRO2 described by Tchaika *et al.* (2007) and the reverse primer LonCR-R2 described by
130 Trinca *et al.* (2007); and (iii) the complete cytochrome *b* (*cyt-b*) gene using primers described by
131 Irwin *et al.* (1991) [L14724, L15162 and H15915] and Koepfli & Wayne (1998) [H15494]. The cyt-
132 b segment was divided in two sub-segments of approximately 750 base pairs (bp) each, with an
133 overlap of nearly 300 bp. PCR reactions were carried out in a PTC-100 thermocycler (MJ Research)
134 in a 20 μL volume, including 1X PCR Buffer (Invitrogen), 0.2 mM of each dNTP, 2 mM $MgCl_2$,
135 0.2 μM of each primer, 0.25 U Platinum® *Taq* Polymerase (Invitrogen), and 10-20 ng of genomic
136 DNA. Thermocycling conditions for the *ND5* and control region segments consisted of an initial
137 denaturing step at 94ºC for 3' followed by 40 cycles of 45'' denaturing at 94ºC, 45'' annealing at
138 65ºC and 1'30'' extension at 72ºC, and a final extension step at 72ºC for 3'. PCR conditions for
139 cytochrome *b* started with an initial denaturing step for 3' at 94ºC, 10 touchdown cycles [45''
140 denaturing at 94ºC, 45'' annealing at 60-51ºC and 1'30'' extension at 72ºC], followed by 30

141 additional cycles with annealing at 50ºC and a final extension at 72ºC for 3'. PCR products were
142 checked in an agarose gel stained with GelRed, purified with PEG8000, and then quantified with a
143 second analysis in an agarose gel. Both strands of each PCR product were sequenced using the
144 DYEnamic ET Dye Terminator Sequencing Kit (GE Healthcare), and analyzed in a MegaBACE
145 1000 automated sequencer (GEHealthcare).
146
147 ***Microsatellite genotyping***
148
149 Eight microsatellite loci were employed for each species. For *N. nasua*, we used the primers
150 developed for this species, previously described by Tsuchiya-Jerep *et al.* (in preparation, see
151 Apendix 1); for *P. cancrivorus*, we employed primers previously described for *P. lotor* by
152 Cullingham *et al.* (2006) [PLO3-71, PLO3-86, PLO3-117, PLO-M3, PLO-M15 and PLO-M17] and
153 by Fike *et al.* (2007) [PLOT-08 and PLOT-10]. All forward primers contained an M13 tail on their
154 5' end (Boutin-Ganache *et al.* 2001). PCR reactions were performed in a PTC-100 thermocycler
155 (MJ Research) in a 10µL volume, including 1X PCR Buffer (Invitrogen), 0.2 mM of each dNTP, 2
156 mM $MgCl_2$, 0.2 µM of each the reverse and the fluorescent M13 primer (FAM, NED or HEX),
157 0.013 µM of the forward primer, 0.25 U Platinum® *Taq* Polymerase (Invitrogen), 0.3% Trehalose
158 and 10-20 ng of genomic DNA. Amplification conditions were as follows: initial denaturing step at
159 94ºC for 3', 10 touchdown cycles [94ºC for 45'', annealing at 65-56ºC (-1ºC/cycle) for 45'' and
160 72ºC for 1.5'], 30 additional cycles with annealing at 55ºC, and a final extension at 72ºC for 30'.
161 PCR products were diluted 1:10, pooled in multiplex panels (Table 3), and genotyped in a
162 MegaBACE1000 automated sequencer (GE Healthcare), using the software Genetic Profiler 2.2 and
163 the internal size standard ETRox-550.
164

mtDNA sequence analysis

Forward and reverse sequences were assembled using the Phred/Phrap/Consed software package (Ewing *et al.* 1998, Ewing & Green 1998, Gordon *et al.* 1998) and consensus sequences were inspected by eye using CHROMAS (Technelysium) and then aligned using the CLUSTAL W algorithm implemented in MEGA 4.0 (Tamura *et al.* 2007). Alignments were manually checked and edited, and only unambiguous sequences were used for analysis.

For both individual and concatenated mtDNA segments, basic statistics of DNA diversity, including nucleotide (π) and haplotype (*H*d) diversities and neutrality tests (Tajima's *D* [Tajima 1989] and Fu's *Fs* [Fu 1997]), were estimated using DNASP (Rozas *et al.* 2003). We also constructed a haplotype network using the median-joining method implemented in the program NETWORK 4.5 (Bandelt *et al.* 1999). All subsequent analyses were performed using only the concatenated mtDNA dataset. To determine the appropriate model of sequence evolution, the Akaike Information Criterion (AIC) implemented in MODELTEST ver. 3.7 (Posada & Buckley 2004) was used and the selected model was employed in Bayesian inference (BI) and maximum likelihood (ML) phylogenetic reconstruction. Neighbor-joining (NJ) and maximum likelihood trees were estimated using PAUP*4.0b10 (Swofford 1998), maximum parsimony (MP) using WINCLADA (Nixon 2002) and NONA (Goloboff 1999), and Bayesian inference was performed in MRBAYES (Huelsenbeck & Ronquist 2001). For MP, we used the parsimony ratchet method (Nixon 1999) with 200 iterations, 5 sequential runs and random reweighting of 10% of characters; statistical confidence was estimated by bootstrap resampling with 1000 replications, using a heuristic search with TBR (tree-bisection-reconnection) branch-swapping. For the ML analyses, optimal phylogenies were inferred with NNI branch-swapping starting from an NJ tree; nodal support was

assessed by 100 bootstrap replications using the NNI (nearest-neighbor-interchange) heuristic search option. Bayesian inference was performed using 1,000,000 steps of the Markov Chain Monte Carlo (MCMC) algorithm (with trees sampled every 100 generations), and the posterior probabilities were calculated discarding the initial 100,000 iterations as burn-in, after the stabilization of log-likelihood values. *Nasua narica* and *Procyon lotor* were used as outgroups for all phylogenetic analyses of *N. nasua* and *P. cancrivorus* haplotypes, respectively.

An Analysis of Molecular Variance (AMOVA) (Excoffier *et al.* 1992) was carried out using ARLEQUIN 3.11 (Excoffier *et al.* 2005). To define which geographic subdivision best reflects the genetic structure of each species, several scenarios were tested. Initially, samples were divided into populations, according to their geographical origin, main vegetational domains (based on Josse *et al.* 2003 and www2.ibge.gov.br/downloads/mapa_murais/biomas_pdf.zip and) and phylogeographic information (obtained from mtDNA phylogenies and microsatellite-based analyses performed in this study); sampling points with only one individual were merged with the closest locality, based on geographic and/or phylogenetic information. Using ARLEQUIN, we calculated the overall F_{ST} and the pairwise F_{ST}'s among the populations incorporating a distance matrix (various distance models were explored, including *p*-distances and the model of sequence evolution defined by MODELTEST, when possible). If the pairwise comparisons found non-significant F_{ST} values for any pair of populations, this specific pair was merged as a joint population. The procedure was then repeated and if the overall F_{ST} value increased, this new configuration was accepted and we verified if there was any other population pair that could be merged. We repeated these steps until all values of pairwise difference became significant and the overall F_{ST} was maximized. If we found more than one population pair with non-significant values of difference, we first united the pair with the highest *P* value (and thus the smallest F_{ST}). We also performed a hierarchical AMOVA

incorporating two levels in the population structure, by testing different schemes based on the single-level scenarios that had led to the highest F_{ST} values. Mismatch distribution analyses (Harpending 1994) were also performed using ARLEQUIN. The correlation between genetic and geographic distances among the sampling sites (and thus the occurrence of isolation by distance) was assessed using a Mantel test (Mantel 1967) with 100,000 permutations in the program ALLELES IN SPACE (AIS) (Miller 2005).

Microsatellite data set

For most analyses, the microsatellite dataset was based on allelic size, except for those performed using ARLEQUIN 3.11 (see below). The conversion among the different input file formats was made using CONVERT 1.31 (Glaubitz 2004). Diversity indices, including number of alleles per locus, observed and expected heterozygosities, were calculated using both CERVUS 3.0.3 (Kalinowski *et al.* 2007) and ARLEQUIN; CERVUS was also employed to test for departures from Hardy-Weinberg equilibrium and ARLEQUIN was used to test for Linkage Disequilibrium. Possible genotyping errors and the presence of null alleles were assessed with MICROCHECKER (Van Oosterhout *et al.* 2004).

To infer the number of populations and to assign individuals to these putative populations, we employed the Bayesian approach implemented in the software STRUCTURE 2.2 (Pritchard *et al.* 2000): *K* values ranged from 1 to 10, and each run comprised 500,000 MCMC iterations, after an initial burn-in of 200,000, using an ancestry model that allows for admixture and correlated allele frequencies. Five independent analyses were performed for each *K* value; if the posterior probability values did not show stability among the different runs, we increased two-fold the length of the burn-

in and the sampling portion of the MCMC, and ran five additional simulations for that specific *K*. We analyzed the mean posterior probabilities [Ln(P|D)] for each *K*, and accepted the value that provided the best fit to the data (Pritchard *et al.* 2000).

An AMOVA and related calculations of fixation indices (F_{ST} and an analog of Slatkin's R_{ST} [Slatkin 1995]) were performed using ARLEQUIN. The population structure to be tested was defined based on the results obtained from the program STRUCTURE and also using the best geographic division found for the *mt*DNA dataset. In addition, Mantel tests were performed using AIS, to assess for the presence of an isolation-by-distance pattern, indicated by a correlation between geographic and genetic distances.

Results

mtDNA dataset

We obtained a total of 2,125 bp of sequence for *Nasua nasua* and 2,166 bp for *Procyon cancrivorus*. Both species were sequenced for 697 bp of the *ND5* gene and 1140 bp of the *cyt-b* gene; 288 bp and 329 bp of the mtDNA control region (CR) were sequenced for *N. nasua* and *P. cancrivorus*, respectively. For *N. nasua*, nucleotide diversity (π) in individual segments varied from 0.0175 to 0.0195 and haplotype diversity (*H*d) from 0.783 to 0.868, while *P. cancrivorus* showed nucleotide diversity values varying from 0.00187 to 0.00575 and haplotype diversity ranging from 0.762 to 0.832 (Table 4).

The haplotype networks for the three individual segments are shown in Figure 3, while Tables 5-10 provide a detailed description of these mtDNA regions for each species. Although the absolute number of haplotypes for each segment was nearly equal for both *N. nasua* and *P. cancrivorus*, there was a large difference in the number of mutational steps presented by each species (Table 4, Figure 3). Another interesting difference was that while P. cancrivorus showed no strong evidence of deep geographic structuring in its mtDNA networks, *N. nasua* exhibited a clear genetic structure, with most haplotypes being exclusive to specific geographic regions and/or to major vegetational domains. By comparing the three networks of each species, it was possible to establish some general patterns: for *N. nasua*, individuals from Acre, Pará, Alagoas, Ceará, Goiás and some from Mato Grosso do Sul states each possessed private haplotypes in all segments; MG and ES2 individuals shared a common haplotype for CR, but for *ND5* and *cyt*b each locality had its own haplotype; and individuals from Rio Grande do Sul, Paraná, São Paulo and Mato Grosso do Sul states (except those mentioned above) shared haplotypes in different combinations, depending on the segment analyzed. The general pattern found for *P. cancrivorus* was the presence of one or two more common haplotypes shared by individuals from different geographic regions. Although some individuals presented haplotypes restricted to specific geographic regions, this pattern was not consistently repeated among the three segments; the only exception were the individuals bPca024 and bPca 311 (from Maranhão and Alagoas states, respectively), which shared the same in all segments. When the three mtDNA segments were concatenated (Tables 11 and 12, Figure 4), an improved resolution of the relationships was achieved, and the overall patterns became more solid. For *N. nasua*, as the general network shape was very consistent among the three segments, there was a no significant change in the overall inference. However, for *P. cancrivorus*, some geographic subdivision emerged: private haplotypes were found in the Cerrado and, although still sharing

haplotypes with other ecoregions, the Pantanal and Pampas domains seemed to be more differentiated from the remaining populations.

The transversional model with allowance for a gamma distribution of rate variation among sites and a proportion of invariable sites (TVM+Γ+I) was the selected model of sequence evolution for *N. nasua* (I=0.4666 and α=0.7366). Using MP (parsimony ratchet), twelve best trees with 499 steps were found. In general, topology estimates from NJ, MP, ML and BI were very similar (**Figure 5**): the most prominent patterns were maintained among the reconstruction methods, and the differences were restricted to branches with shallow divergence. The deepest division was found between the clade formed by individuals from Pantanal, Bolivia and Acre (haplotypes Nn-T2 and Cb-12) and all the remaining specimens, followed by a North/ South subdivision. In the northern clade, eastern Amazonia plus Caatinga (Nn-T14, T15, T16, T17 and T18) were separated from the northern Atlantic forest (Nn-T11 and T12). The southern clade comprised three main subdivisions: (i) Nn-T8 (São Paulo state) plus Nn-N7 (Espirito Santo state); (ii) Nn-T9 (Minas Gerais state) plus Nn-T13 (Goiás state) and Nn-T10 (Espirito Santo state); (iii) and the remaining South Atlantic forest and Pantanal haplotypes. The internal branches within this latter clade were weakly supported and were collapsed in ML, MP and NJ analyses, reflecting the shallow divergence among these haplotypes.

For *P. cancrivorus*, the Tamura-Nei model (Tamura & Nei 1993) with gamma-distributed (Γ) rates across sites (α=0.3873) provided the best fit for the data set. Using MP analyses, 63 best trees (with 261 mutational steps) were recovered. The four different methods resulted in similar relationships (Figure 6); the majority of clades did not receive strong support and when a strict consensus rule was applied, almost all of them collapsed. The most evident geographic association

found is also weakly supported: all haplotypes from Pantanal area (Pc-T9, T17, T18, T19, T20 and Cb12) with exception to Pc-CR10 and Pc-T1, were all present in only one clade; however, Cerrado (Pc-Cb11) and South Atlantic Forest (Pc-T7) individuals were also in this same clade. Except for the groupings of Pc-T13 (MA) plus Pc-T14 (AL), and Pc-T1 (RS1) plus Pc-N4 (bPca05, RS2), the overall nodal support was weak.

The AMOVA results are shown in Tables 14 and 15 for *N. nasua* and *P. cancrivorus*, respectively. For *N. nasua*, the results indicated that most of the genetic variability can be explained by the presence of five populations: (i) Brazilian South (RS and PR states) plus São Paulo and Mato Grosso do Sul states, comprising the southern part of the Atlantic Forest and Pantanal domains; (ii) Espírito Santo, Minas Gerais and Goiás states, comprising the central Atlantic Forest and Cerrado domains; (iii) Alagoas state, comprising the northern portion of the Atlantic forest; (iv) Pará and Ceará States, comprising the Eastern Amazonian forest and Caatinga biomes; and (v) Bolivia plus Acre state (Brazil), also composed primarily by Amazonian forest domains. Espirito Santo state can also be separated from Minas Gerais and Goiás states, resulting in six significantly differentiated populations. When only geographic information was considered, even under the highest overall F_{ST} value (0.618) there were still pairs of populations with non-significant pairwise P values; if these populations were merged, the overall F_{ST} decreased (0.582). By coupling geographic and genetic information, the maximum observed F_{ST} value was higher (0.645), and the number of population was set to five. The results of the AMOVA incorporating two levels showed that six populations divided into four groups is the scenario that best reflects the *N. nasua* genetic structure. For *P. cancrivorus*, the AMOVA results indicated that the scheme that could best explain its genetic variability was the presence of three populations (Table 15): (i) Mato Grosso and Mato Grosso do Sul states, comprising the Pantanal and Cerrado domains; (ii) São Paulo state, comprising the

Cerrado domain; and (iii) the Brazilian southern region plus Espirito Santo State and the northern and northeastern regions (Maranhão, Alagoas, Paraíba and Pará states), comprising Atlantic Forest and Amazonian domains. The AMOVA with two levels was calculated using three different schemes: six populations grouped into four and five groups, and five populations grouped into four groups (Table 13). For all these scenarios, there was an increase in the F_{ST} values; however, the only combination that resulted in significant values of differentiation among groups was the one comprising six populations and four groups (F_{CT} = 0.560). The Mantel test results (Figure 7) indicated the presence of an isolation-by-distance component in the genetic variability of N. nasua (r = 0.388, P = 0.000), but not for *P. cancrivorus* (r = -0.002, P = 0.476). The mismatch distribution of pairwise differences (Figure 8) showed contrasting patterns between *N. nasua* and *P. cancrivorus*. The former presented a random distribution of pairwise differences, non-significant values of Tajima's *D* (*D* = 0.1358, p = 0.648) but significant negative values of Fu's *Fs*, (Fs = -203675, p = 0.002) while the latter depicted a mismatch distribution pattern associated with populations that have suffered a sudden expansion, which is corroborated by significantly negative values for both Tajima's *D* and Fu's *Fs* tests (*D* = -1.624, p = 0.027; *Fs* = -25.233, p = 0.000).

Microsatellite dataset

Tables 16 and 17 show the microsatellite diversity indices for *Nasua nasua* and *Procyon cancrivorus*, respectively. The populations were defined based on the Bayesian clustering performed with STRUCTURE (see text below). Although we were not able to employ the same set of microsatellite loci for both species, some comparative observations could be made based on these results. The mean number of alleles per locus was very similar between *N. nasua* and *P. cancrivorus* (12 and 10.25, respectively) and the observed heterozygosity was slight higher for *P.*

cancrivorus, even though the number of individuals sampled for this species was half that used for *N. nasua*. Null alleles were detected in three loci for each species (PLO3-71, PLO-M17 and PLO3-117 for *P. cancrivorus*; for *N. nasua*, each locus was detected in a different population [NnSTR-A08 for the southern population, NnSTR-H07 for Center-west population and NnSTR-D03 for that of the Northeast]). Departures from Hardy-Weinberg Equilibrium (after Bonferroni correction, p = 0.00625) were found for two loci (PLO3-117 and PLO3-71) in *P. cancrivorus* and for one locus (NnSTR-D03) for the Center-west population of *N. nasua*. Linkage disequilibrium was found for six pairs of loci in *P. cancrivorus* (PLO3-71 and PLOT-10, PLOT-08 and PLO3-117; PLOT-10 and PLOT-08; PLOT-08 and PLO3-86; PLO-M17 and PLO3-117) and in three locus-population combinations of *N. nasua*: Center-west population (NnSTR-A08 and NnSTR-F02) and northeastern population (NnSTR-B09 and NnSTR-D03; NnSTR-E05 and NnSTR-H07). We repeated the Bayesian clustering analysis (STRUCTURE) for *P. cancrivorus* excluding PLOT-08, PLOT-10 and PLO3-117 loci to test if any the results were consistent without them. Since the same results were recovered, we decided to present the analyses with complete dataset of microsatellite markers. In the *N. nasua* case, the linkage disequilibrium was not consistently detected in all populations, and because of this, we decided to treat all loci as unlinked.

For *Nasua nasua*, the Bayesian clustering analysis performed with STRUCTURE showed that the number of clusters that best explains the data was five [(Ln (P|D) = -1855] (Figures 4 and 8a and b), corresponding to the following subdivision: (i) Rio Grande do Sul, Paraná and São Paulo states representing the southern Atlantic Forest domain, hereafter called “South” (pink); (ii) Minas Gerais, Espirito Santo and Goiás states, corresponding to the central part of the Atlantic forest plus Cerrado and designated “Southeast” (yellow); (iii) Mato Grosso do Sul state, corresponding to the Pantanal domain, named here “Center-west” (blue); (iv) Pará state, comprising the Amazonian

forest domain, designated the "North" population (red); (v) and Ceará and Alagoas states, designated the "Northeast" population (green), and comprising the northern part of the Atlantic forest and Caatinga domains. The Mantel test (r = 0.680) (Figure 9) showed a strong and significant (P = 0.000) correlation between geographic and genetic distances. For *P. cancrivorus*, the Bayesian clustering analysis showed no evidence of subdivisions and the best-fitting value of K was one (Figure 8c and d). Remarkably, the result of the Mantel test (r = 0.0387; P = 0.214) further indicated the absence of even an isolation-by-distance pattern of population structure, given the absence of correlation between genetic and geographic distances (Figure 9).

The AMOVA results (Table 18) for *N. nasua* were very similar in the three schemes tested, with the overall F_{ST} being a little higher when the population subdivision suggested by the STRUCTURE software was applied. Differently, the highest R_{ST} value was found following the population structure recommended by mtDNA analysis. The pairwise comparisons among populations (R_{ST}) (Table 19) showed significant results for all comparisons. The absence of subdivision for *P. cancrivorus* was corroborated by the low and non-significant F_{ST} value (0.035) found when individuals were divided into the three populations defined with the mtDNA dataset, and by the contained pairwise comparisons (R_{ST}, data not shown) which yielded only non-significant P values.

Discussion

Genetic diversity

403 The mtDNA nucleotide diversity indices (π) estimated for *Nasua nasua* was about ten times
404 higher than those found for *Procyon cancrivorus* (except for the control region, which was
405 approximately three times greater for *N. nasua* than for *P. cancrivorus*). The haplotype diversity,
406 meanwhile, was slightly higher for *P. cancrivorus*, considering the three concatenated segments
407 (Table 4). Comparing both species with other carnivores, we found that haplotype diversity was
408 very similar among species, but the levels of nucleotide diversity presented some differences:
409 considering the mtDNA control region, *N. nasua* have diversity indices similar to *Cerdocyon thous*
410 (Tchaika *et al.* 2007), a Neotropical canid, higher than the Neotropical jaguar *Panthera onca*
411 (Eizirik *et al.* 2001) and smaller than *L. pardalis* and *L. wiedii* (Eizirik *et al.* 1998). On the other
412 hand, *P. cancrivorus* have diversity indices more similar to the *Cytochrome* b of *Gulo gulo*
413 (Tomasik & Cook 2005) and smaller than the mtDNA control region of the Neotropical otter *Lontra*
414 *longicaudis* (Trinca *et al.* 2007). Comparing the levels of diversity of *N. nasua* and *N. narica* (17
415 individuals from Belize, Panama, Mexico and United States) for the mtDNA control region, we
416 found that *N. narica* presented higher haplotype diversity (*H*d = 0.888), but smaller nucleotide
417 diversity (π = 0.0092) than *N. nasua* (MacFadden 2004). Comparing *P. cancrivorus and P. lotor*
418 (308 individuals, across the United States) also for the mtDNA control region, we found that both
419 diversity indices were higher for *P. lotor* (π = 0.013 and *H*d = 0.945) (Cullingham 2007).
420 Comparing the three segments at the intra-specific level, the most diverse segment for *N. nasua* was
421 *ND5*, providing the better resolution in the network trees. For *P. cancrivorus*, the CR was the
422 segment with the highest diversity; however, the resolution provided by it was not better than that
423 of the other fragments.
424
425 According to Grant & Bowen (1998), a high haplotype diversity coupled with low levels of
426 nucleotide diversity, such as the results found for *P. cancrivorus*, indicate a signal of demographic

expansion following a period of low population size. In the case of *P. cancrivorus*, this inference is also supported by the significantly negative results of Tajima's *D* and Fu's *Fs* tests, along with the shape of its mtDNA mismatch distribution (Figure 7). In contrast, high levels of both nucleotide and haplotype diversities may indicate (i) secondary contact between previously differentiated lineages or (ii) a history of large stable population size (Grant & Bowen 1998). In the case of *N. nasua*, both causes are compatible with our data, since (i) there was strong evidence for population differentiation in more than one population – actually, the AMOVA results indicated five populations significantly differentiated; (ii) there was no evidence that these populations have suffered from population decline. However, to assure these statements, we have to increase our sampling

The observed heterozygosity (*H*o) found using microsatellite markers were very similar for both species, although we have used a different set of loci for each of them. *Procyon cancrivorus* showed heterozygosity levels similar to those found for *P. lotor* (Cullingham 2007), comparing six loci used in common, and both *N. nasua* and *P. cancrivorus* exhibited levels of *H*o that were higher than those of *Potos flavus* (Kays *et al.* 2000) and *L. longicaudis* (Trinca 2007).

Comparing the Mantel tests results between *N. nasua* and *P.* cancrivorus (Figure 7 and 10), it became clear that there was a strong correlation between geographic and genetic distances for *N. nasua* but not for *P. cancrivorus*. Comparing the two *N. nasua* graphs, the one depicting the correlation for microsatellites showed a more homogeneous pattern, while we can note the presence of three "classes" of correlated distances for mtDNA: (i) individuals with geographic distances ranging from zero up to 2000 km and low genetic differentiation (bottom); (ii) intermediate levels of genetic difference and geographic distance ranging from 500 up to 3200 km (which is the maximum) (middle); (iii) highest levels of genetic differentiation and geographic distances ranging

451 from zero up to 2500 km. This latter class comprised comparisons involving some individuals from
452 the Pantanal and Bolivian Chaco domains (haplotypes Nn-Cb9, N2, CR2 and T2), which also
453 formed the most basal clade found in BI, MP, ML and NJ trees (Figure 5). It means that, in the
454 same area (geographic distance nearly equals to zero), we can found very divergent mtDNA
455 haplotypes, what resulted in a less pronounced r-value of Mantel test. When these individuals were
456 excluded from the mtDNA analysis, the differences among the populations become more
457 noticeable: the *r*-value increases from 0.388 to 0.757 and the F_{ST} goes from 0.645 to 0.760 (data not
458 shown).
459

460 ***Nasua nasua* versus *Procyon cancrivorus***

461

462 *Nasua nasua* exhibited a highly structured pattern of genetic diversity for both types of
463 markers. This species seems to have maintained an overall large population size for a long time, and
464 in general, the relationships among populations were well supported by the phylogenetic analyses
465 (Figure 6). On the other hand, *P. cancrivorus* presented low levels of population differentiation (or
466 even none, considering the microsatellites); it showed signs of a recent expansion in population size
467 and the phylogenetic relationships among clades were shallow and weakly supported (Figure 6).
468

469 The overall phylogeographic partitions found for *N. nasua* (considering both markers) were
470 the following: (i) Eastern Amazonia; (ii) Northern Atlantic forest (iii) Central Atlantic forest (iv)
471 Southern Atlantic forest (v) Pantanal; (vi) Bolivian Chaco plus Western Amazonia (only for
472 mtDNA). However, depending on marker considered, there were some changes in this general
473 pattern: the Caatinga population seemed to be more closely related to eastern Amazonia based on
474 the mtDNA data, but was more associated with the Northern Atlantic forest with microsatellites.

The Pantanal domain was inferred to be a distinct population with the microsatellite analyses, but it was not distinguishable from the southern Atlantic forest considering only the mtDNA. On the other hand, the Central Atlantic forest, which was subdivided into two populations based on the mtDNA, was considered to be a single population with the microsatellite data (**Figure 5**). The Bolivian Chaco plus Acre clade (which also includes some individuals from the Pantanal domain) was the most basal mtDNA lineage found for *N. nasua* in this study. A similar result was found by Eizirik *et al.* (1998) for the Neotropical cat *Leopardus wiedii*, in which the most basal lineage for this species in South America was also found in Bolivia. Trinca (2006) found a distinct mtDNA lineage for the Neotropical otter, *L. longicaudis*, in Bolivia, and Costa *et al.* (2000) recognized in this region a center of endemism. The paleoenvironmental changes that took place in this area since the Oligocene might be the cause of its distinctiveness (Sempere *et al.* 1990, Delsuc *et al.* 2004), and warrant additional efforts in terms of further characterization of phylogeographic patterns in multiple species.

The second major partition found for *N. nasua* was between northern (including the "North" and "Northeast" regions) and southern (including the "Center-West", "Southeast" and "South" regions) Brazil, although these regions did not correspond to a single population each (Figure 5). This North-South subdivision is in agreement with the pattern found by Tchaicka *et al.* (2007) for the crab-eating fox (*Cerdocyon thous*), a Neotropical canid sympatric with *N. nasua* in most of its range. The Northern Brazilian clade is subdivided into eastern Amazonia and northern Atlantic forest, with the Caatinga domain being more related to the former with mtDNA and to the latter with microsatellites. The origin of the Caatinga vegetation is still the subject of much debate, and this domain is considered to be highly related to both the Amazonian and Atlantic forest biomes (Borges-Nojosa & Caramaschi 2003, Prado 2003). The observed pattern may possibly indicate a

more effective historical connection with eastern Amazonia, but a more recent one with the Atlantic forest.

The southern clade is divided into Central Atlantic forest/Cerrado and Southern Atlantic forest/Pantanal clades based on the mtDNA; for microsatellites, the Pantanal domain was inferred to be a distinct population from the southern Atlantic forest. Considering that microsatellite markers are able to recover more recent events due to their higher mutation rates in comparison to the mtDNA (Goldstein & Schlötterer 1999, Brown *et al.* 1979), this subdivision may possibly reflect a more recent fragmentation of the Atlantic forest, which interrupted the gene flow between these two once contiguous domains. Another point relative to the Pantanal domain is that it seems to be an area of secondary contact between two very divergent mtDNA lineages (Figure 5, circles blue and green). The microsatellite results also supported this hypothesis, once this set of markers recognized only one population in that area.

For *Procyon cancrivorus*, the mtDNA and microsatellite markers showed different patterns: the mtDNA analyses identified three significantly differentiated populations (although the level of differentiation was much less prominent than those found for *N. nasua*), but the Bayesian clustering approach applied to the microsatellite data indicated only one panmictic population. The three phylogroups identified with mtDNA (Table 15) correspond to different habitats: (i) forests [Amazonian and Atlantic forests]; (ii) Cerrado; (iii) Pantanal + Cerrado. To explain the contrasting patterns between mtDNA and microsatellites, we can infer that females tend to be more philopatric (leading to more structured patterns of mtDNA diversity) and the males are responsible for mediation of gene flow among populations, explaining the absence of subdivision observed with the biparentally inherited nuclear markers.

Nasua nasua and *Procyon cancrivorus* are sympatric species distributed along a broad range; each has its own sister-species in the northern hemisphere, and both intrageneric splits were dated to before the complete closure of the Panamanian land bridge (Koepfli *et al.* 2007). However, their recent evolutionary history in South America seems to be very contrasting given the marked differences in their genetic structure, which cannot not be explained only by their current range, habitat and food preferences. An interesting avenue for future research is an investigation of whether these contrasting histories may be caused by differences in social structure and dispersal patterns in these species, which in turn might influence their response to common climatic and vegetational shifts in their pasts. Future analyses targeting this question would be important to shed light on the underlying processes shaping these different genetic structures.

Implications for conservation

Considering Moritz`s genetic criterion for recognizing 'Evolutionarily Significant Units' (ESUs), which assumes that "ESUs should be reciprocally monophyletic for mtDNA alleles and show significant divergence of allele frequencies at nuclear loci" (Moritz 1994), we advocate that each of the six mtDNA lineages found for *Nasua nasua* should be treated as a distinct ESU, being conserved and managed as a separate entity. Moreover, five of these phylogroups are also correlated to *N. nasua* subspecies previously described: *N. n. nasua* (in northeastern Brazil – maybe Caatinga and northern Atlantic forest); *N. n. solitaria* (in central Atlantic forest); *N. n. spadicea* (in southern Atlantic forest); *N. n. dorsalis* (in eastern Amazonia); *N. n. boliviensis* (In Bolivian Chaco). However, further work is required to understand the magnitude and causes of this marked genetic partitioning, including an assessment of morphological and ecological features, as well as an effort

547 to map the boundaries of these identified units. For *P. cancrivorus*, we did not identify major
548 evolutionary lineages, and according to this, it may be treated as a single population throughout the
549 sampled areas. However, additional work is still required to assess whether adaptive differences
550 might occur among biomes or regional populations, even though a recent history of expansion and
551 recurrent gene flow seem to homogenizing the genetic composition of this species across broad
552 geographic regions.
553

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729
730

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Figure Legends

Figure 1 Map depicting currently the recognized range for *Nasua nasua* (according to Patterson *et al.* 2007), identification of sampling locales (see Table 1 for details) and main vegetational domains (see legend). *Nasua nasua* illustration from Eisenberg 1989.

Figure 2 Map depicting currently the recognized range for *Procyon cancrivorus* (according to Patterson *et al.* 2007), identification of sampling locales (see Table 2 for details) and main vegetational domains (see legend). *Procyon cancrivorus* illustration from Eisenberg 1989.

Figure 3 Haplotype network for *Nasua nasua* and *Procyon cancrivorus* mitochondrial segments. A, *N. nasua cytochrome b* gene; B, *N. nasua ND5* gene; C, *N. nasua* control region; D, *P. cancrivorus cytochrome b* gene; E, *P. cancrivorus ND5* gene; F, *P. cancrivorus* control region. The number of differences among the haplotypes is represented by lines across branches, each one corresponding to one mutation.

Figure 4 Haplotype network for *N. nasua* (left) and *P. cancrivorus* (right) based on the concatenated mitochondrial data set.

Figure 5 Bayesian phylogram depicting the intra-specific relationships for *Nasua nasua*. Values above branches indicate, from top to bottom, NJ, MP, ML and BI support for the adjacent node (see text for details). Labels are haplotype identification numbers (see Tables 5, 6, 7 and 11). Dotted lines indicate the populations identified using microsatellite markers. For mtDNA, Mato Grosso do Sul (Pantanal) and São Paulo (south Atlantic forest) states possess individuals which belongs to

763 different haplotype groups. For microsatellites, only one individual from São Paulo state is more
764 related to central Atlantic forest group, instead of the south Atlantic forest one.
765
766 **Figure 6** Bayesian phylogram depicting the intra-specific relationships for *P. cancrivorus*. Values
767 above branches indicate, from top to bottom, NJ, MP, ML and BI support for the adjacent node (see
768 text for details). Labels indicate haplotype identification numbers (see Tables 8, 9, 10 and 12).
769
770 **Figure 7** Graphs depicting the correlation between genetic and geographic distances for *N. nasua*
771 (top) and *P. cancrivorus* (bottom), using the concatenated mitochondrial data set.
772
773 **Figure 8** Observed and expected mismatch distributions for *N. nasua* (top) and *P. cancrivorus*
774 (bottom) using the concatenated mtDNA data set.
775
776 **Figure 9** Barplots (proportion of individual assignment to each population cluster) and graphs
777 depicting the variation in likelihood as a function of the number of assumed populations (k) based
778 on the Bayesian analysis performed with STRUCTURE for *N. nasua* (A and B) *P. cancrivorus* (C and
779 D). In **A**, each color represents one geographic region (see text for details): red = "North"; green =
780 "Northeast"; yellow = "Southeast"; blue = "Center-west"; pink = "South". In C, a barplot assuming
781 two population units is shown, so as to demonstrate the even allocation of all individuals to both
782 populations.
783
784 **Figure 10** Graphs depicting the correlation between genetic and geographic distances for *N. nasua*
785 (top) and *P. cancrivorus* (bottom), using the microsatellite data set.
786

Table 1 Brown-nosed coati *(Nasua nasua)* samples analyzed in this study.

Ecoregion	Geographic Origin (Sampling site)	Samples	Institution/ contact
Southern Atlantic Forest	P. N. Iguaçu, Paraná State (PR) S Brazil	bNna02*	Instituto Pró-Carnívoros
	Rio Grande do Sul State (RS1) S Brazil	bNna03*	Vanessa Fortes, Everton Behr and Marilise Krügel
	Rio Grande do Sul State (RS2) S Brazil	bNna04*, bNna05*, bNna06*	Júlio César Menezes de Sá
	Rio Grande do Sul State (RS3) S Brazil	bNna61†,‡,§	Felipe Peters
	São Paulo State (SP) S Brazil	bNnaSPA*, bNnaSPB*, bNnaSPC*, bNnaSPD*, bNnaSPE* bNnaSPF†,¶,‡,§	Ligia Motta
	P. N. Iguazu, Argentina (AR)	bNnaARG1‡, bNnaARG2†,¶,‡, bNnaARG3†,¶,‡, bNnaARG4‡, bNnaARG5†,¶,‡, bNnaARG6†,¶,‡, bNnaARG7†,¶,‡, bNnaARG8†,¶,‡	Ben Hirsch
Central Atlantic Forest	Espírito Santo State (ES1) SE Brazil	bNna14£,†,‡,§	Rodosol/ Andreas Kierbusch
	Espírito Santo State (ES2) SE Brazil	bNna302*, bNna303*, bNna304*, bNna305*	CENAP-IBAMA
	Minas Gerais State (MG) SE Brazil	bNna51*, bNna52*, bNna53*, bNna54*, bNna55*, bNna56*, bNna57*, bNna58*, bNna59*, bNna60*	Nadja Hemétrio and Fabrício Rodrigues dos Santos
Northern Atlantic Forest	Alagoas State (AL1) NE Brazil	bNna307*, bNna308*, bNna309*, bNna311*	CENAP - IBAMA
	Alagoas State (AL2) NE Brazil	bNna310*	CENAP – IBAMA
Cerrado	P. N. Emas Goiás State (GO) Central Brazil	bNna01*	Museu Nacional
Pantanal	Mato Grosso do Sul State (MS) SW Brasil	bNna07*, bNna08*, bNna09£,¶,‡,§, bNna10*, bNna11*, bNna12*, bNna13†,¶,‡, bNna16*, bNna17*, bNna18*, bNna19£,†,‡,§, bNna20‡,§, bNna22£,†,‡,§, Nna023£,†,¶,§, bNna24*, bNna25£,†,‡,§, bNna26£,†,¶,§, bNna27£,¶,‡,§, bNna28£,¶,§, bNna29*, bNna30£,†,¶,§, bNna31*, bNna32£,†,¶,§, bNna33*, bNna34£,†,¶,§, bNna35£,†,¶,§, bNna36†,¶,‡,§, bNna37£,†,¶,§, bNna38£,†,¶,§, bNna39£,†,¶,§, bNna40£,†,¶,§, bNna41£,†,¶,§, bNna42*, bNna43*, bNna44£,†,¶,§, bNna45£,†,¶,§, bNna46£,†,¶,§, bNna47£,†,¶,§, bNna48£,†,¶,§, bNna49£,†,¶,§,	Guilherme Mourão, Rita de Cássia Bianchi, Fabiana Rocha and Natalie Olifers
Caatinga	Ceará State (CE) NE Brazil	bNna21*	Marco Renato Mattos
Eastern Amazônia	Pará State (PA) N Brazil	bNnaPAA*, bNnaPAB*, bNnaPAC*, bNnaPAD*, bNnaPAE*, bNnaPAF*, bNnaPAG*, bNnaPAH*	Ligia Motta
Western Amazônia	Acre State (AC) N Brazil	bNnaAC †,¶,‡	Museum of Vertebrate Zoology (MVZ195089)
Bolivian Chaco	San Rámon, Santa Cruz Bolivia	bNnaBol †,¶,‡	Museum of Southwestern Biology (MSB12987)
Outgroup *Nasua narica*	Barro Colorado Island Panama	bNnr07 †,¶,‡	UCLA

* samples typed for the three mtDNA segments and microsatellites

£ samples typed for the mtDNA control region

† samples typed for the first segment of the cytochrome *b* gene

¶ samples typed for the second segment of the cytochrome *b* gene

‡ samples typed for the *ND5* gene

§ samples typed for microsatellites

Table 2 Crab-eating raccoon (*Procyon cancrivorus)* samples analyzed in the present study.

Ecoregion	Geographic Origin (Sampling site)	Samples	Institution/ contact
Pampas (Southern Grasslands)	Rio Grande do Sul State (RS1) S Brazil	bPca07*	Instituto Pró-Carnívoros
	Rio Grande do Sul State (RS2) S Brazil	bPca09*	Instituto Pró-Carnívoros
	Rio Grande do Sul State (RS3) S Brazil	bPca15*	Paulo Chaves Barcelos
	Rio Grande do Sul State (RS4) S Brazil	bPca16*	Marcus Lisenfield and Rodrigo Magalhães
	Rio Grande do Sul State (RS5) S Brazil	bPca17*	Fundação Zoobotânica
	Rio Grande do Sul State (RS6) S Brazil	bPca29*	Carlos Benhur Kasper
	Rio Grande do Sul State (RS7) S Brazil	bPca33£,‡,§	Felipe Peters
Southern Atlantic Forest	Rio Grande do Sul State (RS8) S Brazil	bPca01£,‡,§	Joceleia Koenemann
	Rio Grande do Sul State (RS9) S Brazil	bPca02*	Thales Freitas and Juliana Silva
	Rio Grande do Sul State (RS10) S Brazil	bPca03*, bPca05*	Carla Kotzian, Alberto Senra and Diego Hoffmann; Instituto Pró-Carnívoros
	Rio Grande do Sul State (RS11) S Brazil	bPca04*	Instituto Pró-Carnívoros
	Rio Grande do Sul State (RS12) S Brazil	bPca06*	Instituto Pró-Carnívoros
	Rio Grande do Sul State (RS13) S Brazil	bPca31*	Carlos Benhur Kasper and Marina Piccoli
	Rio Grande do Sul State (RS14) S Brazil	bPca34*	Felipe Peters
	Santa Catarina State (SC1) S Brazil	bPca22§	Fernanda Trierveiler
	Santa Catarina State (SC2) S Brazil	bPca25*	Felipe Grazziotin, Adrian Garda
	Paraná State (PR) S Brazil	bPca26*	Felipe Grazziotin and Adrian Garda
Central Atlantic Forest	Minas Gerais State (MG) SE Brazil	bPca32£,§	Fernando Jerep, Tiago Carvalho and Christian Cramer
	Espirito Santo State (ES1) SE Brazil	bPca19*	Rodosol/ Andreas Kierbusch
	Espirito Santo State (ES2) SE Brazil	bPca18*	Rodosol/ Andreas Kierbusch

Table 2 *Continued.*

Central Atlantic Forest (continued)	Espirito Santo State (ES3) SE Brazil	bPca20*	Rodosol/ Andreas Kierbusch
	Espirito Santo State (ES4) SE Brazil	bPca21*	Rodosol/ Andreas Kierbusch
Northern Atlantic Forest	Paraíba State (PB) NE Brazil	bPca308*	CENAP-IBAMA
	Alagoas State (AL) NE Brazil	bPca311*, bPca312*	CENAP-IBAMA
Cerrado	São Paulo State (SP1) SE Brazil	bPca301*, bPca302*	CENAP-IBAMA
	São Paulo State (SP2) SE Brazil	bPca303*	CENAP-IBAMA
	São Paulo State (SP3) SE Brazil	bPca14*	Juliana Griese
	Mato Grosso do Sul State (MS1) SW Brazil	bPca27*	Felipe Grazziotin and Adrian Garda
Pantanal	Mato Grosso do Sul State (MS2) SW Brazil	bPca28*	Rita Bianchi
	Mato Grosso do Sul State (MS3) Central Brazil	bPca35£,‡,§	Guilherme Mourão and Fabiana Rocha
	Mato Grosso State (MT1) SW Brazil	bPca10*, bPca12*	Instituto Pró-Carnívoros
	Mato Grosso State (MT2) SW Brazil	bPca13*	Instituto Pró-Carnívoros
	Mato Grosso State (MT3) SW Brazil	bPca11*	Instituto Pró-Carnívoros
	Mato Grosso State (MT4) SW Brazil	304*, bPca305*, bPca306*, bPca307£,¶,‡,§, bPca309£,¶,‡,§	Instituto Pró-Carnívoros
Eastern Amazônia	Maranhão State (MA) NE Brazil	bPca24*	Tadeu Gomes de Oliveira
	Para State (PA) N Brazil	bPca23*	Tadeu Gomes de Oliveira
Outgroup *Procyon lotor*	Genbank	Plo9126 Plo7804	Accession numbers: NC009126 AB297804

* samples typed for the three mtDNA segments and microsatellites

£ samples typed for the mtDNA control region

† samples typed for the first segment of the cytochrome *b* gene

¶ samples typed for the second segment of the cytochrome *b* gene

‡ samples typed for the *ND5* gene

§ samples typed for microsatellites.

Table 3 Microsatellite loci used in this study, including the genotyping multiplex panels employed for *Nasua nasua* and *Procyon cancrivorus*.

	Multiplex panel	Microsatellite Loci	Dye	Repeat size (jn bp)
Nasua nasua				
	N1	NnSTR-D03	FAM	2
		NnSTR-E05	FAM	2
		NnSTR-H03	HEX	2
		NnSTR-H07	NED	2
	N2	NnSTR-A08	FAM	2
		NnSTR-B09	HEX	2
		NnSTR-F02	NED	2
		NnSTR-F03	NED	2
Procyon cancrivorus				
	P1	PLO3-71	NED	4
		PLO-M15	FAM	4
		PLOT-10	HEX	4
		PLO-M3	NED	4
		PLOT-08	HEX	4
	P2	PLO-M17	FAM	4
		PLO3-86*	HEX	2
		PLO3-117*	NED	2

* Loci originally described as containing tetranucleotide repeats.

Table 4 Mitochondrial DNA diversity estimates for *Nasua nasua* and *Procyon cancrivorus* using segments of *ND5* and *cytochrome b* genes and the control region.

Species	Segments	*L*	*N*	*h*	*V*	*S*	*P*	π (SD)	k	*Hd* (SD)
Nasua nasua	*ND5*	697 (679)	71	16	86	84	60	0.01949 (± 0.00001)	13.2350	0.864 (± 0.0005)
	Cytochrome b	1140 (1090)	80	13	119	106	96	0.01771 (± 0.00253)	19.3082	0.783 (± 0.00153)
	CR	288 (287)	77	15	24	24	20	0.01748 (± 0.00182)	5.0161	0.790 (± 0.00133)
	Concatenated*	2125 (2107)	50	18	197	189	174	0.02080 (± 0.00288)	43.8351	0.909 (± 0.00044)
Procyon cancrivorus	*ND5*	697 (686)	42	12	14	14	9	0.00239 (± 0.00060)	1.6376	0.829 (± 0.00212)
	Cytochrome b	1140 (1138)	34	10	10	10	8	0.00187 (± 0.00014)	2.1244	0.832 (± 0.00161)
	CR	329 (323)	43	11	13	13	8	0.00575 (± 0.00035)	1.8560	0.762 (± 0.00181)
	Concatenated*	2166 (2148)	34	20	34	39	19	0.00272 (± 0.00026)	5.8396	0.938 (± 0.00071)

* Only samples without missing data were used for this analysis.

L, sequence length; numbers in parentheses indicate the segments lengths after exclusion of all sites containing gaps or missing information

N, number of sequences

h, number of haplotypes

V, variable sites

S, segregating sites

P, parsimony-informative sites

π, nucleotide diversity

k, average number of nucleotide differences

Hd, haplotype diversity

SD, standard deviation

Table 5 List of haplotypes of the *cytochrome b* gene for *Nasua nasua*, including the individuals that bear each haplotype, the absolute frequency in total sample (Fr) and geographic distribution of each haplotype.

Haplotype	Individuals	Fr	Ecoregion
Nn-Cb1	bNna02,03,06,07,08,10,011,012,16,23,26,29,30,31,32,34,35,36, 37,39,40,41,43,44,45,46,47,48,49,SPA,SPB,SPD,SPE,SPF	34	Southern Atlantic Forest, Pantanal
Nn-Cb2	bNna04,05	2	Southern Atlantic Forest
Nn-Cb3	bNnaSPC	1	Southern Atlantic Forest
Nn-Cb4	bNnaARG2,ARG3,ARG5,ARG6,ARG7,ARG8	6	Southern Atlantic Forest
Nn-Cb5	bNna302,303,304,305	4	Central Atlantic Forest
Nn-Cb6	bNna01,51,52,53,54,55,56,57,58,59,60	11	Central Atlantic Forest, Cerrado
Nn-Cb7	bNna307,308,309,311	5	Northern Atlantic Forest
Nn-Cb8	bNna310	1	Northern Atlantic Forest
Nn-Cb9	bNnaBol,17,18,24,33,38,42	7	Pantanal, Bolivian Chaco
Nn-Cb10	bNnaPAA,PAB,PAC,PAE,PAF,PAG,PAH	7	Eastern Amazônia
Nn-Cb11	bNnaPAD	1	Eastern Amazônia
Nn-Cb12	bNnaAC	1	Western Amazônia
Nn-Cb13	bNna21	1	Caatinga
Nn-Cb14*	bNna014	1	Central Atlantic Forest

* Samples that possess distinct haplotypes when we consider only the second part of the cytochrome b gene (not show in the network).

Table 6 List of the *ND5* haplotypes for *Nasua nasua*, including the individuals that bear each haplotype, the absolute frequency in the total sample (Fr) and the geographic distribution of each haplotype.

Haplotype	Individuals	Fr	Ecoregion
Nn-N1	bNna07,08,09,10,11,12,13,16,19,20,22,25,27,29,31,36,43	17	Pantanal
Nn-N2	bNna17,18,24,33,42,Bol	6	Pantanal, Bolivian Chaco
Nn-N3	bNna02,03,04,06,SPA,SPD,SPE,SPF,ARG1,ARG2,ARG3,ARG4,ARG5, ARG6,ARG7,ARG8	16	Southern Atlantic Forest
Nn-N4	bNna05	1	Southern Atlantic Forest
Nn-N5	bNna61	1	Southern Atlantic Forest
Nn-N6	bNnaSPC	1	Southern Atlantic Forest
Nn-N7	bNna14	1	Central Atlantic Forest
Nn-N8	bNna51,52,53,54,55,56,57,58,59,60	10	Central Atlantic Forest
Nn-N9	bNna302,303,304,305	4	Central Atlantic Forest
Nn-N10	bNna01	1	Cerrado
Nn-N11	bNna307 ,308,309,310,311	5	Northern Atlantic Forest
Nn-N12	bNnaPAB ,PAC,PAG,PAH	4	Eastern Amazonia
Nn-N13	bNnaPAD	1	Eastern Amazonia
Nn-N14	bNnaPAE	1	Eastern Amazonia
Nn-N15	bNna21	1	Caatinga
Nn-N16	bNnaAC	1	Western Amazonia

Table 7 List of haplotypes of the mitochondrial DNA control region for *Nasua nasua*, including the individuals that bear each haplotype, the absolute frequency in the total sample (Fr) and the geographic distribution of each haplotype

Haplotype	Individuals	Fr	Ecorregion
Nn-CR1	bNna04,05,07,08,09,10,11,12,16,19,22,23,25,26,27,29,30,31,32, 34,35,37,39,40,43,44,45,46,47,48,49	21	Southern Atlantic Forest, Pantanal
Nn-CR2	bNna17,18,24,28,33,38,42	7	Pantanal
Nn-CR3	bNna41	1	Pantanal
Nn-CR4	bNna02,SPA ,SPB,SPC,SPD,SPE	6	Southern Atlantic Forest
Nn-CR5	bNna03	1	Southern Atlantic Forest
Nn-CR6	bNna06	1	Southern Atlantic Forest
Nn-CR7	bNna14	1	Central Atlantic Forest
Nn-CR8	bNna51,52,53,54,55,56,57,58,59,60,302,303,304,305	14	Central Atlantic Forest
Nn-CR9	bNna307,308,309,311	4	Northern Atlantic Forest
Nn-CR10	bNna310	1	Northern Atlantic Forest
Nn-CR11	bNnaPAA,PAC,PAE,PAF,PAG,PAH	6	Eastern Amazonia
Nn-CR12	bNnaPAB	1	Eastern Amazônia
Nn-CR13	bNnaPAD	1	Eastern Amazônia
Nn-CR14	bNna01	1	Cerrado
Nn-CR15	bNna21	1	Caatinga

Table 8 List of *cytochrome b* haplotypes for *Procyon cancrivorus*, including the individuals that bear each haplotype, along with the absolute frequency in the total sample (Fr) and the geographic distribution of each haplotype

Haplotype	Individuals	Fr	Ecoregion
Pc-Cb1	bPca04,07,09,15,16,17,23,29,34	10	Eastern Amazonia, Southern Atlantic Forest, Pampas
Pc-Cb2	bPca02,03,06,10,25,28,304,306,307,308	10	Southern Atlantic Forest, Northern Atlantic Forest, Pantanal
Pc-Cb3	bPca05,26	1	Southern Atlantic Forest
Pc-Cb4	bPca12,13	2	Pantanal
Pc-Cb5	bPca305	1	Pantanal
Pc-Cb6	bPca14,21,301,312	4	Central Atlantic Forest, Northern Atlantic Forest, Cerrado
Pc-Cb7	bPca19	1	Central Atlantic Forest
Pc-Cb8	bPca24,311	2	Northern Atlantic Forest, Eastern Amazonia
Pc-Cb9	bPca31	1	Southern Atlantic Forest
Pc-Cb10	bPca302,303	2	Cerrado
Pc-Cb11*	bPca27	1	Cerrado
Pc-Cb12*	bPca309	1	Pantanal

* Samples that possess distinct haplotypes when only the second segment of cytochrome b were considered (not show in the network).

Table 9 List of the *ND5* haplotypes for *Procyon cancrivorus*, including the individuals that bear each haplotype, the absolute frequency in the total sample (Fr) and the geographic distribution of each haplotype

Haplotype	Individuals	Fr	Ecorregion
Pc-N1	bPca02,03,06,11,12,13,18,25,27,28,33,304,305,309,308	15	Southern Atlantic Forest, Central Atlantic Forest, Northern Atlantic Forest, Pantanal, Pampas
Pc-N2	bPca04,07,15,16,23,29,34	7	Southern Atlantic Forest, Pampas, Eastern Amazonia
Pc-N3	bPca05	1	Southern Atlantic Forest
Pc-N4	bPca01	1	Southern Atlantic Forest
Pc-N5	bPca09	1	Pampas
Pc-N6	bPca26	1	Southern Atlantic Forest
Pc-N7	bPca17,35	2	Pampas, Pantanal
Pc-N8	bPca14,21,301,302,303,312	6	Central Atlantic Forest, Northern Atlantic Forest, Cerrado
Pc-N9	bPca19,306,307	3	Central Atlantic Forest, Pantanal
Pc-N10	bPca20,31	2	Central Atlantic Forest, Southern Atlantic Forest
Pc-N11	bPca10	1	Pantanal
Pc-N12	bPca24,311	2	Eastern Amazonia, Northern Atlantic Forest

Table 10 List of haplotypes of the mtDNA control region for *Procyon cancrivorus*, including the individuals that bear each haplotype, the absolute frequency in the total sample (Fr) and the geographic distribution of each haplotype

Haplotype	Individuals	Fr	Ecorregion
Pc-CR1	bPca04,07,09,15,16,17,18,19,21,23,34,302,303,312	14	Southern Atlantic Forest, Pampas, Central Atlantic Forest, Northern Atlantic Forest, Eastern Amazonia, Cerrado
Pc-CR2	bPca03,06,10,11,12,13,25,27,28,302,304,305,306, 307,308,309	16	Southern Atlantic Forest, Northern Atlantic Forest, Pantanal, Cerrado
Pc-CR3	bPca01,05	2	Southern Atlantic Forest
Pc-CR4	bPca26	1	Southern Atlantic Forest
Pc-CR5	bPca29	1	Pampas
Pc-CR6	bPca33	1	Pampas
Pc-CR7	bPca20,31	2	Central Atlantic Forest, Southern Atlantic Forest
Pc-CR8	bPca32	1	Central Atlantic Forest
Pc-CR9	bPca24,311	2	Northern Atlantic Forest, Eastern Amazonia
Pc-CR10	bPca35	1	Pantanal
Pc-CR11	bPca14,301	2	Cerrado

Table 11 List of haplotypes identified in the concatenated mtDNA data set for *Nasua nasua*, including the individuals that bear each haplotype, the absolute frequency in the total sample (Fr) and the geographic distribution of each haplotype

Haplotype	Individuals	Fr	Ecorregion
Nn-T1	bNna07,08,10,11,12,16,29,31,43	9	Pantanal
Nn-T2	bNna17,18,24,33,42	5	Pantanal
Nn-T3	bNna02,SPA ,SPD,SPE	4	Southern Atlantic Forest
Nn-T4	bNna03	1	Southern Atlantic Forest
Nn-T5	bNna04	1	Southern Atlantic Forest
Nn-T6	bNna05	1	Southern Atlantic Forest
Nn-T7	bNna06	1	Southern Atlantic Forest
Nn-T8	bNnaSPC	1	Southern Atlantic Forest
Nn-T9	bNna51,52,53,54,55,56,57,58,59,60	10	Central Atlantic Forest
Nn-T10	bNna302,303,304,305	4	Central Atlantic Forest
Nn-T11	bNna307,308,309,311	4	Northern Atlantic Forest
Nn-T12	bNna310	1	Northern Atlantic Forest
Nn-T13	bNna01	1	Cerrado
Nn-T14	bNnaPAB	1	Eastern Amazônia
Nn-T15	bNnaPAC ,PAG,PAH	3	Eastern Amazônia
Nn-T16	bNnaPAD	1	Eastern Amazônia
Nn-T17	bNnaPAE	1	Eastern Amazônia
Nn-T18	bNna21	1	Caatinga

Table 12 List of haplotypes identified in the concatenated mtDNA data set for *Procyon cancrivorus*, including the individuals that bear each haplotype, along with the absolute frequency in the total sample (Fr) and the geographic distribution of each haplotype.

Haplotype	Individuals	Fr	Ecorregion
Pc-T1	bPca05	1	Southern Atlantic Forest
Pc-T2	bPca09	1	Pampas
Pc-T3	bPca16	1	Pampas
Pc-T4	bPca17	1	Pampas
Pc-T5	bPca26	1	Southern Atlantic Forest
Pc-T6	bPca29	1	Pampas
Pc-T7	bPca31	1	Southern Atlantic Forest
Pc-T8	bPca04,07,15,23,34	5	Pampas, Southern Atlantic Forest, Eastern Amazonia
Pc-T9	bPca02,03,06,25,28,304,308	7	Southern Atlantic Forest, Northern Atlantic Forest, Pantanal
Pc-T10	bPca014	1	Cerrado
Pc-T11	bPca019	1	Central Atlantic Forest
Pc-T12	bPca21,312	2	Central Atlantic Forest, Northern Atlantic Forest
Pc-T13	bPca24	1	Eastern Amazonia
Pc-T14	bPca311	1	Northern Atlantic Forest
Pc-T15	bPca301	1	Cerrado
Pc-T16	bPca302 ,303	2	Cerrado
Pc-T17	bPca10	1	Pantanal
Pc-T18	bPca12,13	2	Pantanal
Pc-T19	bPca305	1	Pantanal
Pc-T20	bPca306,307	2	Pantanal

Table 13 F_{ST} values calculated for different population groupings of *N. nasua*, calculated using the mtDNA concatenated data set. Sample sites are labeled according to the abbreviations defined in Table 1 and Figure 1. Values in bold indicate the highest F_{ST} values found for each specific scenario.

Scenario		Populations	F_{ST}
One-level AMOVA (Only geographic information)	1. Nine geographic groups (initial definition)	(RS) (PR+Arg) (MS) (SP) (ES) (GO+MG) (AL+CE) (PA) (Bol+AC)	0.574*
	2. Eight populations based on pairwise F_{ST} results [a]	(RS) (PR+Arg+MS) (SP) (ES) (GO+MG) (AL+CE) (PA) (Bol+AC)	0.590*
	3. Seven populations based on pairwise F_{ST} results [a]	(RS+PR+Arg+MS) (SP) (ES) (GO+MG) (AL+CE) (PA) (Bol+AC)	0.602*
	4. Six populations based on pairwise F_{ST} results [a]	(RS+PR+Arg+MS+SP) (ES) (GO+MG) (AL+CE) (PA) (Bol+AC)	**0.618***
	5. Five populations based on pairwise F_{ST} results [a]	(RS+PR+Arg+MS+SP) (ES+GO+MG) (AL+CE) (PA) (Bol+AC)	**0.618***
	6. Five populations based on pairwise F_{ST} results	(RS+PR+Arg+MS+SP) (ES) (GO+MG) (AL+CE+Bol+AC) (PA)	**0.582***
One-level AMOVA (Including phylogenetic information)	1. Nine geographic and phylogenetic groups (initial definition)	(RS) (PR+Arg) (MS) (SP) (ES) (GO+MG) (AL) (CE+PA) (Bol+AC)	0.602*
	2. Eight populations based on pairwise F_{ST} results	(RS) (PR+Arg+MS) (SP) (ES) (GO+MG) (AL) (CE+PA) (Bol+AC)	0.618*
	3. Seven populations based on pairwise F_{ST} results	(RS+PR+Arg+MS) (SP) (ES) (GO+MG) (AL) (CE+PA) (Bol+AC)	0.630*
	5. Five populations suggested by STRUCTURE	(RS+PR+Arg + SP) (MS+Bol) (ES+GO+MG) (AL+CE) (PA+AC)	0.546*
	6. Six populations based on pairwise F_{ST} results	(RS+PR+Arg+MS+SP) (ES) (GO+MG) (AL) (CE+PA) (Bol+AC)	**0.644***
	7. Five populations based on pairwise F_{ST} results	(RS+PR+Arg+MS+SP) (ES+GO+MG) (AL) (CE+PA) (Bol+AC)	**0.645***
Two-level AMOVA	1. Six populations divided into five groups, according to phylogenetic information.	[(RS+PR+Arg+MS+SP)] [(ES) (GO+MG)] [(AL)] [(CE+PA)] (Bol+AC)]	0.650* (F_{CT} = 0.602)
	2. Six populations divided into four groups, according to phylogenetic information.	[(RS+PR+Arg+MS+SP) (ES) (GO+MG)] [(AL)] [(CE+PA)] [(Bol+AC)]	**0.732*** **(F_{CT} = 0.560*)**
	3. Five populations divided into four groups, according to phylogenetic information	[(RS+PR+Arg+MS+SP) (ES+GO+MG)] [(AL)] [(CE+PA)] [(Bol+AC)]	0.729* (F_{CT} = 0.557)

[a] Despite the higher values of F_{ST}, the differences among the putative populations were not significant.

* Significant value.

Table 14 F_{ST} values calculated for different population groupings of *P. cancrivorus*, calculated using the mtDNA concatenated data set. Sample sites are labeled according to the abbreviations defined in Table 2 and Figure 1. Values in bold indicate the highest F_{ST} values found for each specific scenario.

Scenario		Populations/ Groups	F_{ST}
One-level AMOVA	1. Seven populations based on geographic and main vegetational domains information	(MT+MS) (RS1*) (RS2*+SC+PR) (SP) (ES) (PA+MA) (AL+PB)	0.235*
	2. Six populations based on pairwise F_{ST}	(MT+MS) (RS1) (RS2+SC+PR) (SP) (ES) (PA+MA+AL+PB)	0.248*
	3. Five populations based on pairwise F_{ST}	(MT+MS) (RS+SC+PR) (SP) (ES) (PA+MA+AL+PB)	0.273*
	4. Four populations based on pairwise F_{ST}	(MT+MS) (RS+SC+PR) (SP) (ES+PA+MA+AL+PB)	0.255*
	5. Four populations based on pairwise F_{ST}	(MT+MS) (SP) (ES) (RS+SC+PR+PA+MA+AL+PB)	0.274*
	6. Three populations based on pairwise F_{ST}	(MT+MS) (SP) (RS+SC+PR+ES+PA+MA+AL+PB)	**0.285***
Two-level AMOVA	1. Five populations divided in three groups, corresponding to general vegetational patterns.	[(MT+MS)] [(RS+SC+PR) (ES) (PA+MA+AL+PB)] [(SP)]	**0.314*** (F_{CT} = 0.208)
	2. Five populations divided in four groups, corresponding to general vegetational patterns.	[(MT+MS)] [(RS+SC+PR) (ES)] [(PA+MA+AL+PB)] [(SP)]	0.287* (F_{CT} = 0.183)
	3. Four populations divided in three groups, corresponding to general vegetational patterns.	[(MT+MS)] [(SP)] [(RS+SC+PR+PA+MA+AL+PB) (ES)]	0.298* (F_{CT} = 0.206)

* RS1 and RS2 refer to two vegetational domains: RS1, Pampas and RS2, Southern Atlantic Forest (See Figure 1 and Table 2 for details)

Table 15 Summary of genetic variation at eight microsatellite loci scored for *N. nasua* populations.

	NnSTR-A08					NnSTR-B09					NnSTR-D03					NnSTR-E05				
	N	A	H_O	H_E	F_{IS}	*N*	A	H_O	H_E	F_{IS}	*N*	A	H_O	H_E	F_{IS}	*N*	A	H_O	H_E	F_{IS}
South	12	5	0.417	0.710	0.097	12	3 (1)	0.417	0.518	-0.044	12	5	0.667	0.656	0.229	12	3	0.667	0.507	-0.001
Southeastern	16	6 (1)	0.812	0.774	-0.336	14	5 (1)	0.500	0.655	0.324	14	6 (1)	0.857	0.783	0.239	15	4	0.857	0.751	0.120
Center-west	38	8 (3)	0.846	0.728	-0.099	39	3	0.316	0.382	0.267	39	7	0.718	0.763	-0.045	39	6 (1)	0.282	0.281	-0.096
North	8	6 (2)	0.875	0.717	-0.263	8	5 (4)	0.750	0.667	0.044	8	4 (2)	0.750	0.742	-0.533	8	4 (2)	0.750	0.642	0.087
Northeastern	5	4 (1)	0.333	0.454	0.443	6	4 (1)	0.600	0.778	-0.367	6	6 (1)	0.333	0.818	0.037	6	4 (1)	0.667	0.757	0.283
Overall	79	14	0.741	0.864	-0.115	79	11	0.430	0.660	0.026	79	12	0.709	0.840	0.005	80	9	0.519	0.604	0.006

Table 15 (*continued*)

	NnSTR-F02					NnSTR-F03					NnSTR-H03					NnSTR-H07					Averaged over all loci				
	N	A	H_O	H_E	F_{IS}	*N*	A	H_O	H_E	F_{IS}	*N*	A	H_O	H_E	F_{IS}	*N*	A	H_O	H_E	F_{IS}	*N*	*a*	H_O	H_E	F_{IS}
S	11	5	0.583	0.659	-0.148	12	6	0.545	0.783	0.127	11	4 (1)	0.500	0.764	0.643	12	3	0.454	0.480	-0.066	12	4.250	0.531	0.635	0.082
SE	16	6	0.533	0.616	-0.172	16	8 (1)	0.688	0.774	-0.063	16	8 (3)	0.688	0.802	0.585	15	6	0.625	0.673	0.225	16	6.130	0.695	0.729	0.288
CW	37	6 (1)	0.744	0.790	0.240	39	5	0.676	0.746	-0.239	38	8 (3)	0.641	0.686	0.480	38	9 (2)	0.500	0.715	0.227	39	6.500	0.590	0.636	0.121
N	7	4 (2)	0.875	0.692	-0.190	8	5 (1)	0.714	0.670	-0.097	8	6 (1)	0.875	0.800	-0.201	8	3	1.000	0.667	0.067	8	4.630	0.823	0.699	-0.162
NE	6	4 (1)	0.667	0.773	-0.312	6	5	0.500	0.576	-0.245	6	3 (1)	0.667	0.590	-0.500	6	5 (1)	0.833	0.788	0.054	6	4.380	0.575	0.691	0.033
Overall	77	11	0.688	0.802	0.036	81	11	0.649	0.837	-0.119	79	17	0.654	0.859	0.498	79	11	0.595	0.776	0.218	81	12	0.623	0.780	

N, number of individuals;

a, average number of alleles

H_O, observed heterozygosity

H_E, expected heterozygosity

A, number of alleles per locus; number between parentheses indicates the number of privates alleles.

F_{IS}, Weir and Cockerham's (1984) analog of Wright's fixation index.

Table 16 Summary of genetic variation at eight microsatellite loci scored from *P. cancrivorus*.

	PLO3-86					PLO3-71					PLO3-117					PLO-M3				
	N	A	H_O	H_E	F_{IS}	*N*	A	H_O	H_E	F_{IS}	*N*	A	H_O	H_E	F_{IS}	*N*	A	H_O	H_E	F_{IS}
Only one population considered	30	17	0.889	0.905	0.108	39	8	0.533	0.847	0.475	37	18	0.410	0.932	0.024	41	8	0.649	0.771	0.060

Table 16 (*continued*)

PLO-M15					PLO-M17					PLOT-08					PLOT-10					Averaged over all loci				
N	A	H_O	H_E	F_{IS}	*N*	A	H_O	H_E	F_{IS}	*N*	A	H_O	H_E	F_{IS}	*N*	A	H_O	H_E	F_{IS}	*N*	*a*	H_O	H_E	F_{IS}
39	6	0.805	0.789	0.294	37	7	0.538	0.734	0.016	38	8	0.784	0.828	0.074	37	10	0.789	0.847	0.569	36	10.250	0.675	0.832	0.253

N, number of individuals

a, average number of alleles

H_O, observed heterozygosity

H_E, expected heterozygosity

A, number of alleles per locus.

F_{IS}, Weir and Cockerham's (1984) analog of Wright's fixation index.

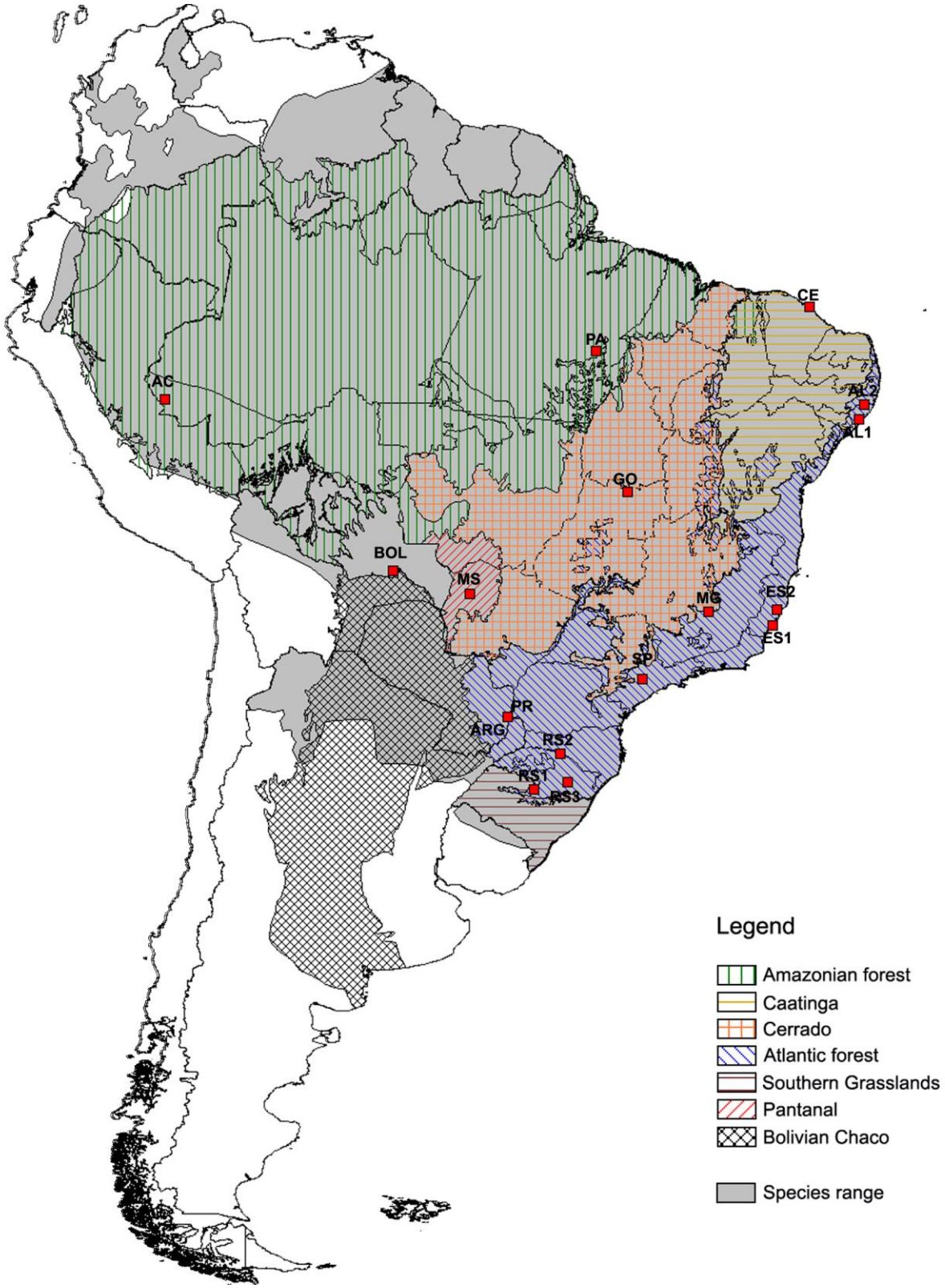
Table 17 F_{ST} and R_{ST} values calculated for different population groupings of *N. nasua* using eight microsatellite loci. Sample sites are labeled according to the abbreviations defined in Table 1 and Figure 1. Values in bold indicate the highest F_{ST} values found.

Scenario	Populations	F_{ST}	R_{ST}
1. Four populations suggested by mtDNA results	(RS+PR+ SP+MS) (ES+GO+MG) (AL+CE) (PA)	0.195*	0.332*
2. Five populations suggested by mtDNA results	(RS+PR+ SP+MS) (ES) (GO+MG) (AL+CE) (PA)	0.197*	**0.336***
3. Five populations suggested by STRUCTURE	(RS+PR+ SP) (MS) (ES+GO+MG) (AL+CE) (PA)	**0.202***	0.306*

*Significant values

Table 18 Pairwise R_{ST} estimates for *Nasua nasua* populations (below the diagonal) and corresponding significance level (above the diagonal). All values were statistically significant.

	South	Southeastern	Center-west	North	Northeastern
South	*	0.00020	0.00307	0.00000	0.00000
Southeastern	0.29670	*	0.00663	0.00000	0.01317
Center-west	0.09093	0.07813	*	0.00000	0.00040
North	0.70886	0.42420	0.52476	*	0.00040
Northeastern	0.58954	0.18573	0.25024	0.37379	*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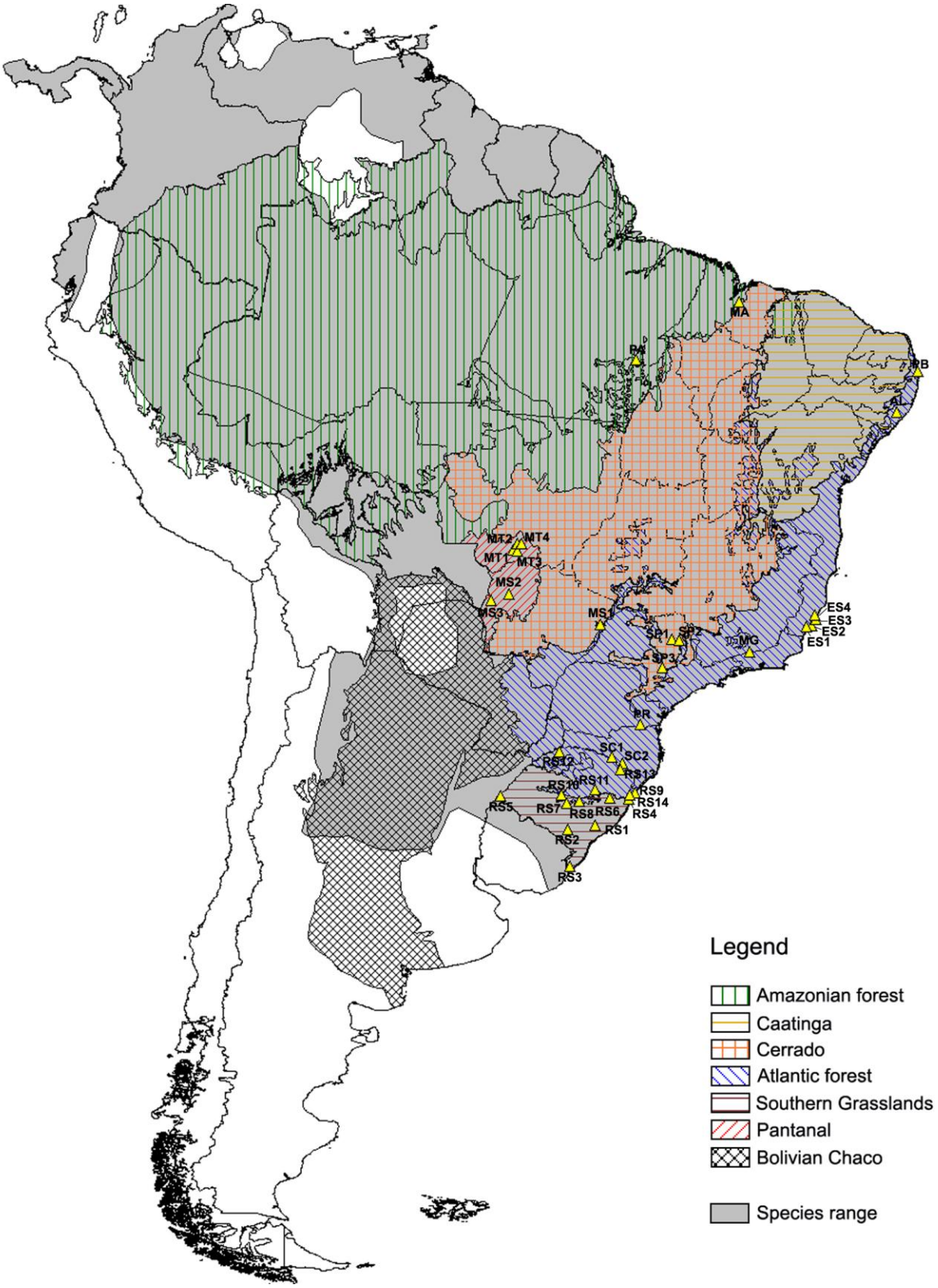
Caatinga
Cerrado
Atlantic forest
Southern Grasslands
Pantanal
Bolivian Chaco
Species range

Figure 1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SC2
RS12
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RS11
RS10
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RS7
RS8
RS6
RS4
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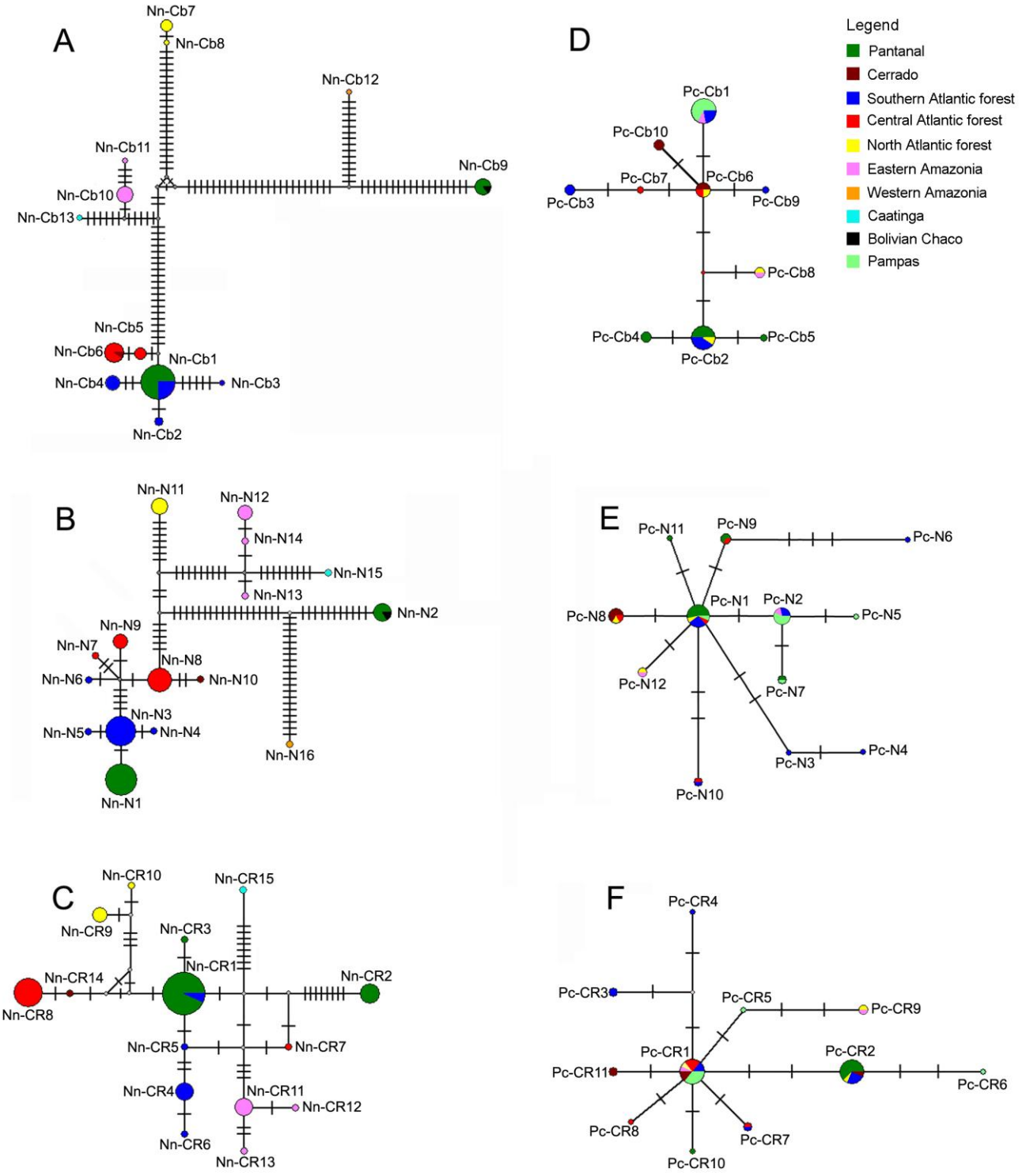
Caatinga
Cerrado
Atlantic forest
Southern Grasslands
Pantanal
Bolivian Chaco
Species range

Figure 2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Pc-Cb8
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Caatinga
Bolivian Chaco
Pampas
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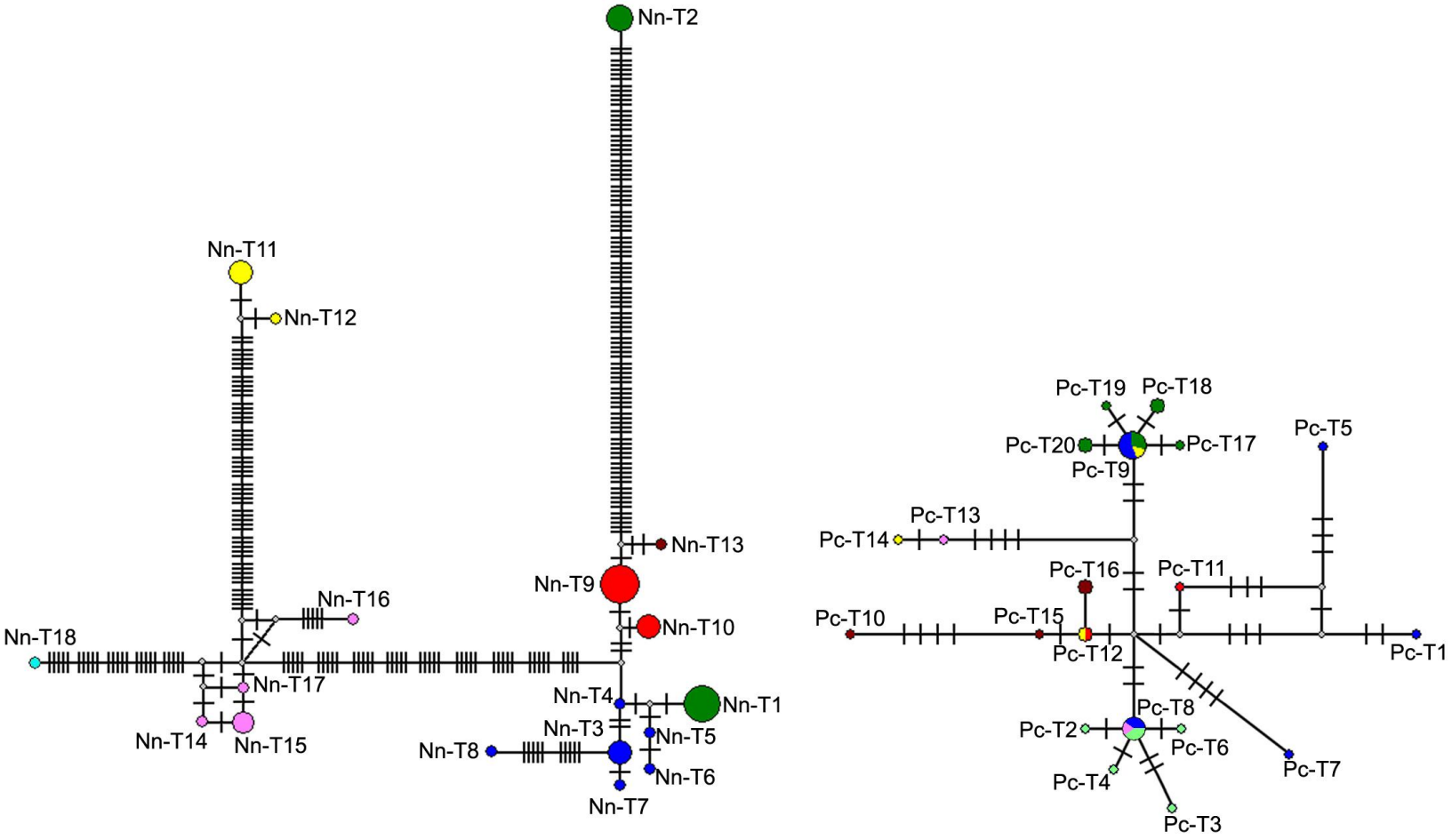
Figure 3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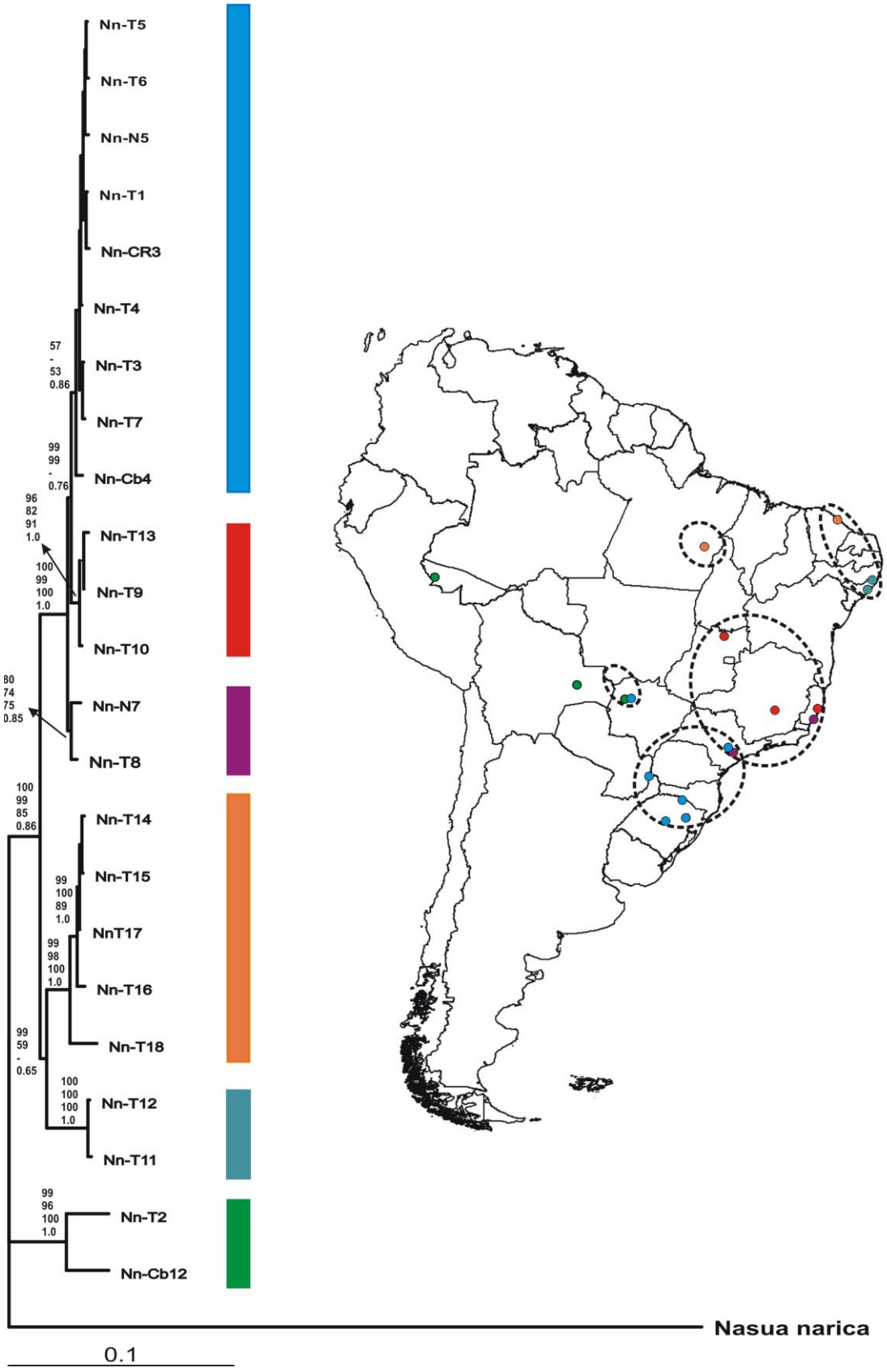
Pc-T3

Figure 4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Nasua narica
0.1

Figure 5

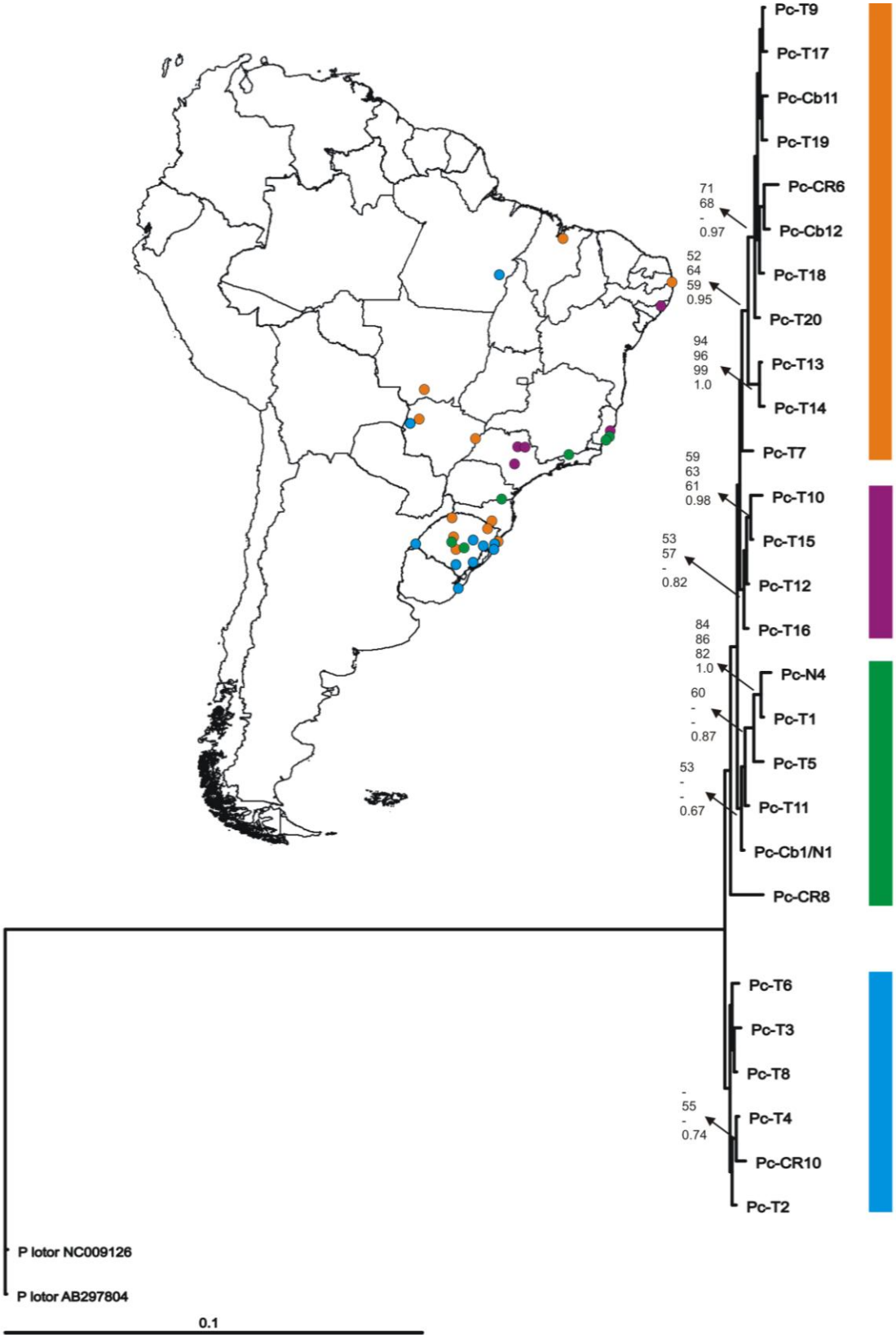

Pc-T9
Pc-T17
Pc-Cb11
Pc-T19
Pc-CR6
Pc-Cb12
Pc-T18
Pc-T20
Pc-T13
Pc-T14
Pc-T7
Pc-T10
Pc-T15
Pc-T12
Pc-T16
Pc-N4
Pc-T1
Pc-T5
Pc-T11
Pc-Cb1/N1
Pc-CR8
Pc-T6
Pc-T3
Pc-T8
Pc-T4
Pc-CR10
Pc-T2
71
68
-
0.97
52
64
59
0.95
94
96
99
1.0
59
63
61
0.98
53
57
-
0.82
84
86
82
1.0
60
-
-
0.87
53
-
-
0.67
-
55
-
0.74
P lotor NC009126
P lotor AB297804
0.1

Figure 6

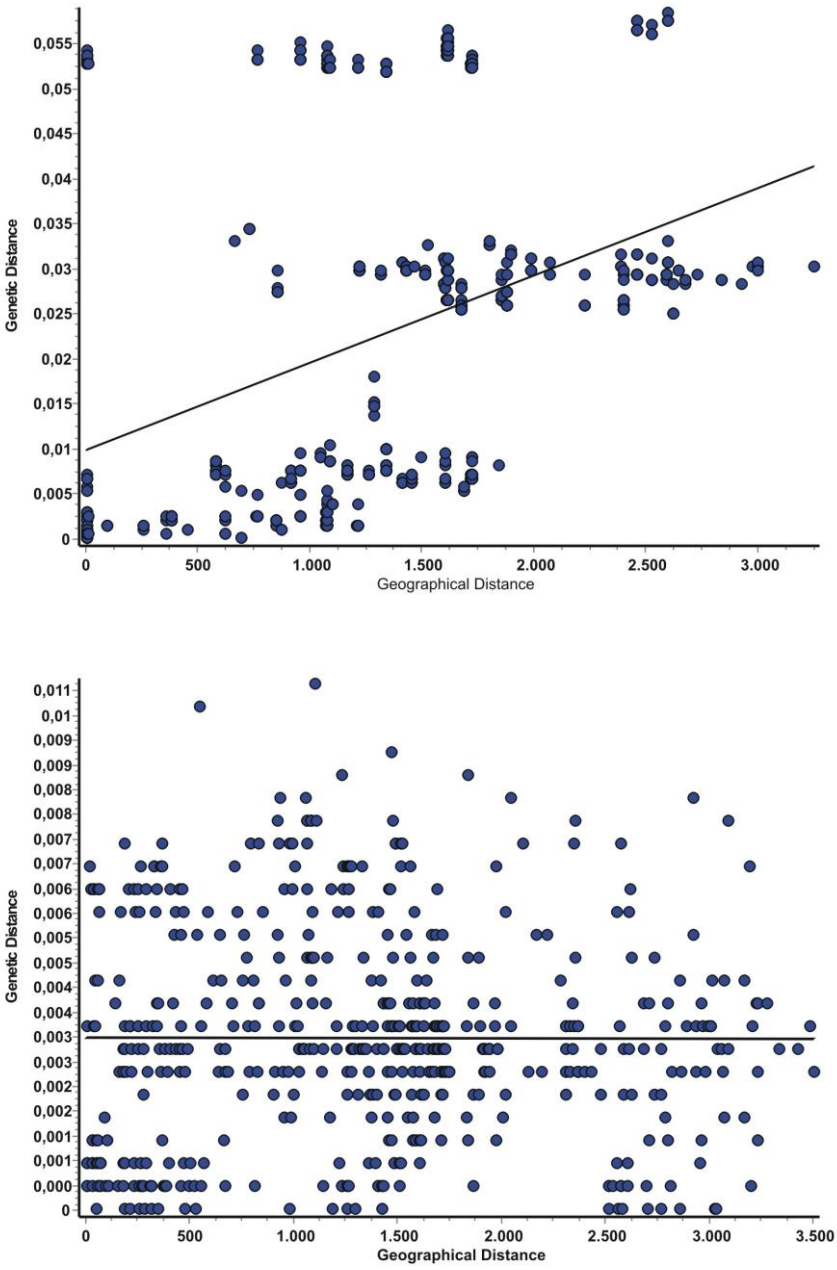
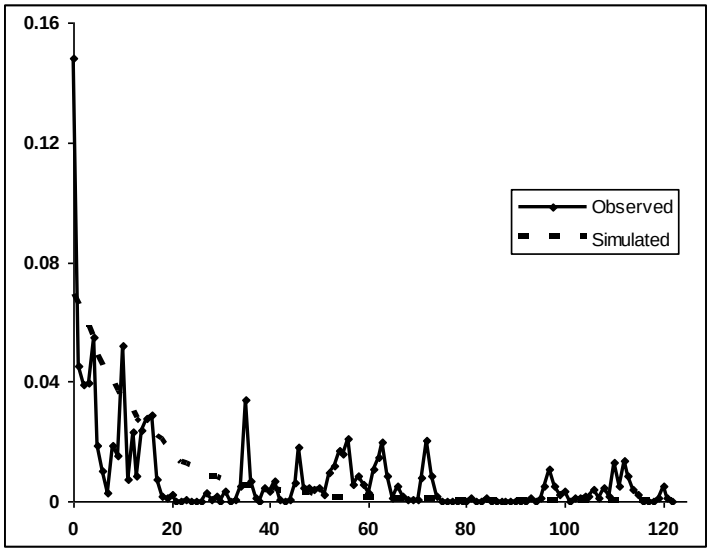

Genetic Distance
0,055
0,05
0,045
0,04
0,035
0,03
0,025
0,02
0,015
0,01
0,005
0
0
500
1.000
1.500
2.000
2.500
3.000
Geographical Distance
Genetic Distance
0,011
0,01
0,009
0,009
0,008
0,008
0,007
0,007
0,006
0,006
0,005
0,005
0,004
0,004
0,003
0,003
0,002
0,002
0,001
0,001
0,000
0
0
500
1.000
1.500
2.000
2.500
3.000
3.500
Geographical Distance

Figure 7


0.16
0.12
0.08
0.04
0
0
20
40
60
80
100
120
Observed
Simulated

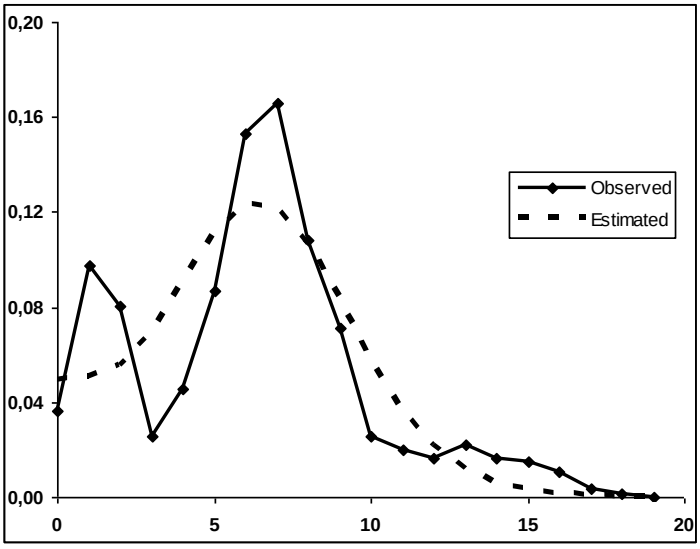

0,20
0,16
0,12
0,08
0,04
0,00
0
5
10
15
20
Observed
Estimated

Figure 8

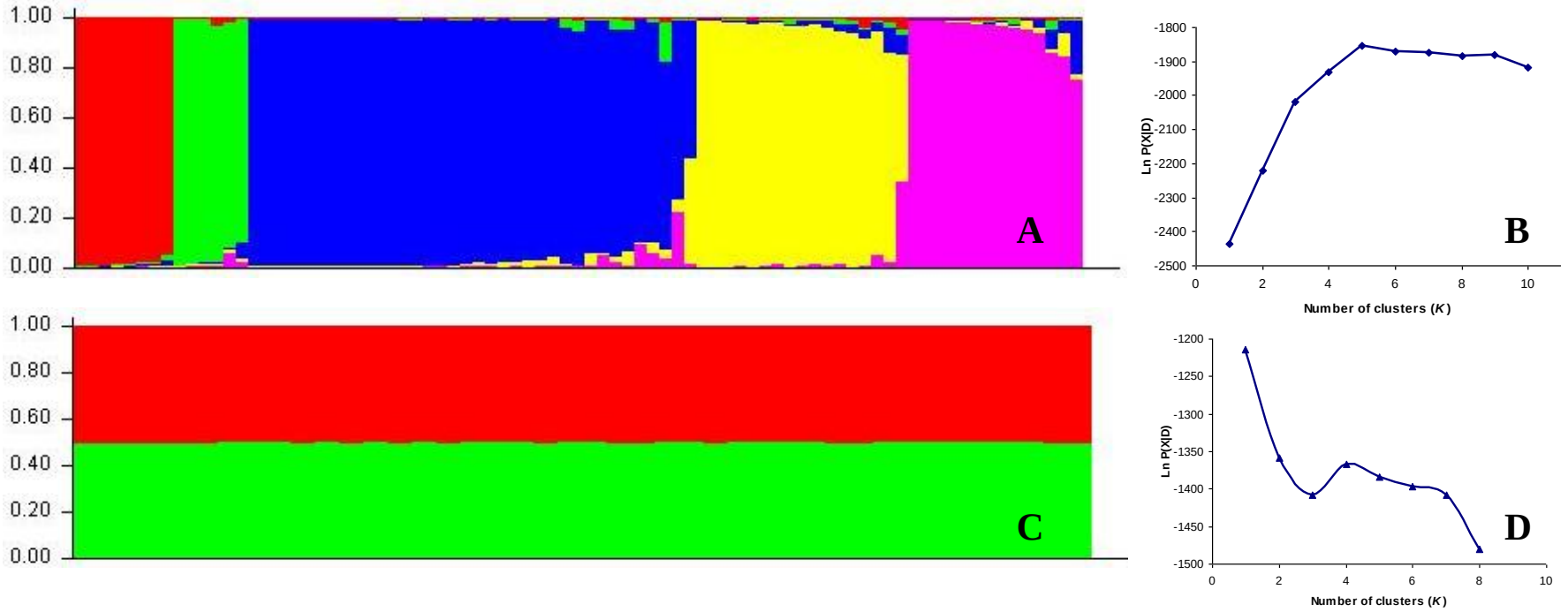
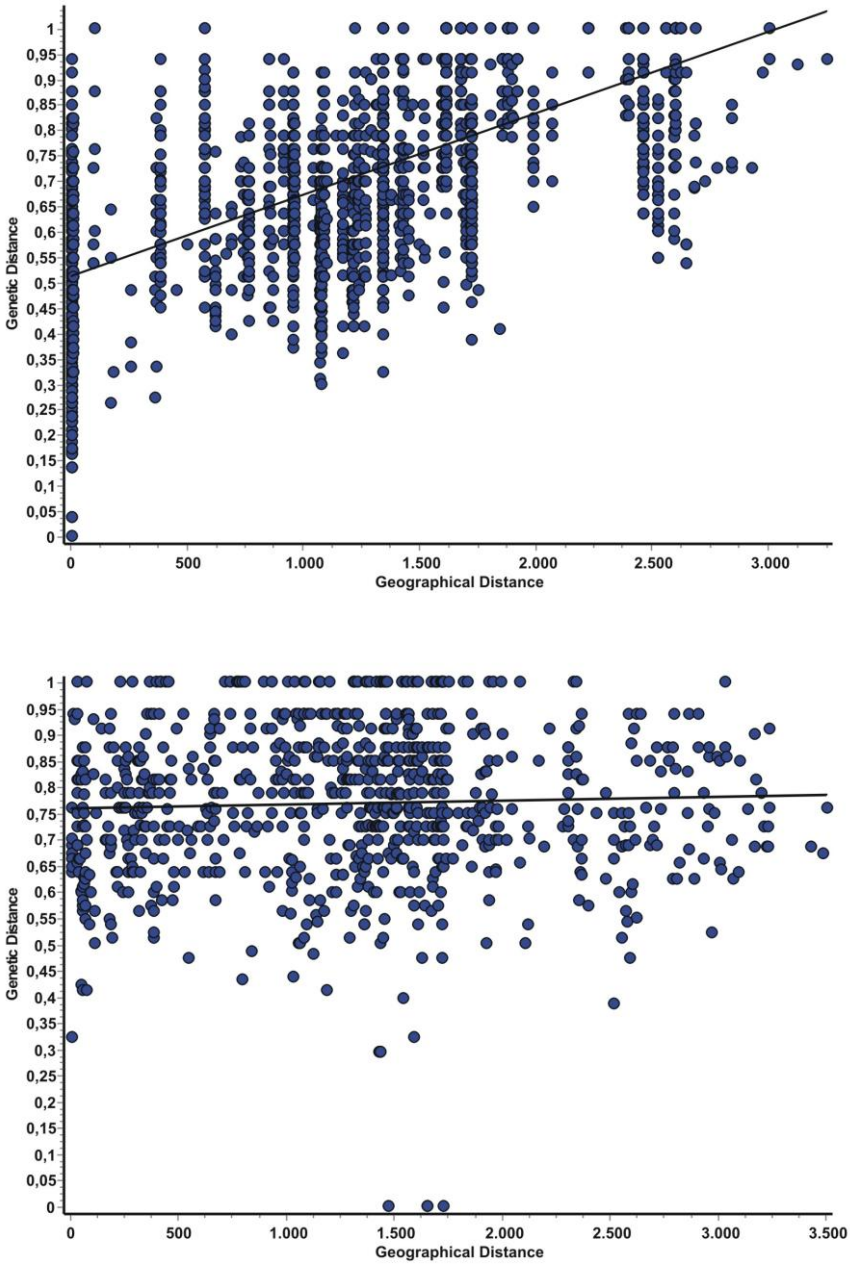

1.00
0.80
0.60
0.40
0.20
0.00
A
B
Ln P(X|D)
-1800
-1900
-2000
-2100
-2200
-2300
-2400
-2500
0
2
4
6
8
10
Number of clusters (K)
1.00
0.80
0.60
0.40
0.20
0.00
C
D
Ln P(X|D)
-1200
-1250
-1300
-1350
-1400
-1450
-1500
0
2
4
6
8
10
Number of clusters (K)

Figure 9


Genetic Distance
Geographical Distance
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0,9
0,85
0,8
0,75
0,7
0,65
0,6
0,55
0,5
0,45
0,4
0,35
0,3
0,25
0,2
0,15
0,1
0,05
0
0
500
1.000
1.500
2.000
2.500
3.000
3.500

Figure 10

1

APÊNDICE

Isolation and characterization of eight microsatellite loci in the Brown-nosed Coati, *Nasua nasua* (Mammalia, Carnivora, Procyonidae)

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Running title: Microsatellites in Brown-nosed Coati.

Abstract

We describe the isolation and characterization of eight polymorphic microsatellite loci for brown-nosed coatis (*Nasua nasua*). Two multiplexed panels were designed and employed to genotype 24 individuals from a single population in the southern Pantanal biome, Brazil. The allelic diversity ranged from two to seven alleles per locus, and the observed heterozygosity ranged from 0.250 to 0.792. One locus showed a departure from Hardy-Weinberg equilibrium due to an excess of heterozygotes, and no evidence of linkage disequilibrium was found. These markers should be useful for studies addressing population genetics, ecology, and social structure of this poorly known species as well as related procyonids.

The brown-nosed or South American coati (*Nasua nasua*) is a diurnal, highly social mesocarnivore belonging to the family Procyonidae (Eisenberg 1989; Gompper & Decker 1998). The species is distributed from Colombia and Venezuela to Uruguay and Northern Argentina, and is found in many vegetation types, although it prefers wooded areas (Gompper & Decker 1998; Nowak 1999; Redford & Eisenberg 1992). Coatis are omnivorous and forage on trees as well as on the ground. Their diet is composed predominantly of fruits and invertebrates (and occasionally small vertebrates), depending on local availability. A recent study in an Atlantic forest fragment showed that coatis are effective seed dispersers (Alves-Costa & Eterovick 2007). The social organization is thought to be similar to that of its Central American congener, *N. narica*: groups are formed by females and immature males, while adult males are solitary, joining the groups during the mating season (Beisiegel & Mantovani 2005; Gompper & Decker 1998).

Despite being a common, broadly distributed species, *Nasua nasua* remains among the least studied Neotropical carnivores (Oliveira 2006). The existing studies focus mainly on diet and behavioral ecology (Alves-Costa & Eterovick 2007; Alves-Costa *et al.* 2004; Beisiegel & Mantovani 2005; Blanco & Hirsch 2006), and until now there is no study addressing genetic aspects of this species. Since microsatellites are useful markers for most applications in population genetics and molecular ecology (Vali *et al.* 2008), the objective of this study was to identify and characterize multiple such loci for *N. nasua*, in order to provide new molecular tools that allow the development of in-depth studies targeting this species.

We constructed a microsatellite-enriched genomic library using a protocol modified from the one described by Billotte *et al.* (1999), starting from genomic DNA extracted using a standard phenol-chloroform method (Sambrook *et al.* 1989). Five µg of DNA were digested with *Rsa*I (Invitrogen), and *Rsa*21 (5′-CTCTTGCTTACGCGTGGACTA-3′) and *Rsa*25 (5′-

TAGTCCACGCGTAAGCAAGAGCACA-3′) linkers were ligated to the digested fragments. The library was enriched for dinucleotide repeats using $(CT)_8$ and $(GT)_8$ biotin-labeled probes and streptavidin-coated paramagnetic beads (Streptavidine MagneSphere Paramagnetic Particles, Promega). The selected fragments were amplified by PCR using *Rsa*21 primers, and the products were cloned into pGEM-T vectors (Promega). These plasmids were introduced into *Escherichia coli* XL-1 Blue strains, and transformed cells were grown onto agar plates containing 100 µg.ml–1 ampicilin and 50 µg.ml–1 X-galactosidase. We selected 95 positive colonies which were grown for 22 hours in a 96-well plate containing 100 µg/uL amplicilin and 1 mL of Circle Grow medium (QBio-Gene), followed by plasmid isolation as described by Sambrook *et al.* (1989). All positive clones were sequenced using SP6 primers, the DYEnamic ET Dye Terminator Sequencing Kit (GE Healthcare), and a MegaBACE 1000 (GE Healthcare) automated sequencer. We also employed T7 primers for sequencing the reverse strand of selected clones to increase the reliability of primer design.

Microsatellite repeats were found in 55 clones (ca. 60% of the total), of which 27 were perfect (STRs without interruptions): 25 bore simple repeats (only one motif) and two contained compound repeats (more than one motif). We selected 9 clones (based on repeat number and availability of reliable flank sequences) for primer design, which was performed using the program PRIMER 3 (Rozen & Skaletsky 2000) was employed for this purpose. All forward primers received an M13 tail at their 5’ end for flexible dye-labeling (Boutin-Ganache *et al.* 2001). PCR reactions were carried out in a PTC-100 thermocycler (MJ Research) in 10µL volume, including 1X PCR Buffer (Invitrogen), 0.2 mM of each dNTP, 2 mM MgCl2, 0.2 µM each of the reverse and the fluorescent M13 primer (FAM, NED or HEX), 0.013 µM of the forward primer, 0.25 U Platinum® *Taq* Polymerase (Invitrogen), 0.3% Trehalose and 10-20 ng of genomic DNA. The amplification profile consisted of an

initial denaturing step at 94ºC for 3 min, 10 touchdown cycles [94ºC for 45 s, annealing at 65-56ºC (-1ºC/cycle) for 45 s and 72ºC for 1 min 30 s], 30 additional cycles with annealing at 55ºC, and a final extension at 72ºC for 30 min. PCR products were diluted 1:10 and then genotyped with a MegaBACE1000 (GE Healthcare) automated sequencer, using the software Genetic Profiler 2.2 and the internal size standard ETRox-550.

Initially, we genotyped five specimens from different geographic regions (data not shown) to assess amplification success, product size range and overall polymorphism. Eight loci showed positive amplification and some level of polymorphism. Based on the allelic size range, two multiplex panels were designed (Table 1). Twenty-four coati samples from a single population in the Pantanal biome (160 km east of Corumbá, MS, Brazil) were genotyped following the same PCR and genotyping conditions described above. The observed and expected heterozygosities (*H*o and *H*e, respectively) were calculated using CERVUS 3.0.3 (Kalinowski *et al.* 2007) and the presence of null alleles was assessed with MICROCHECKER (van Oosterhout *et al.* 2004). GENEPOP 3.4 (Raymond & Rousset 1995) and ARLEQUIN 3.11 (Excoffier *et al.* 2005) were used to test for departures from Hardy-Weinberg equilibrium Linkage equilibrium (a Bonferroni correction was used for both tests).

Allelic diversity ranged from two to seven alleles per locus, and the observed heterozygosity varied from 0.250 to 0.792 (Table 1). There was no evidence of null alleles at any of the loci. We found no deviation from Hardy-Weinberg equilibrium using ARLEQUIN 3.11 (Excoffier *et al.* 2005), but GENEPOP 3.4 (Raymond & Rousset 1995) detected HW disequilibrium due to excess of heterozygotes at locus NnSTR-F02. We tested 28 combinations of loci for linkage disequilibrium and found no significant value. These results indicate that the markers described here are informative and reliable for population-level studies, and will likely be very useful to investigate the genetic structure, behavioral ecology

and evolutionary history of coatis, opening up new research avenues aimed at understanding this poorly known species.

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159

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163

164 **Table 1** Main features and primer sequences for eight polymorphic microsatellite loci identified in the brown-nosed coati. See text for PCR conditions.
165 M13 tails added to the 5' end of forward primers are indicated in bold types. Number of alleles (*A*), expected (H_E) and observed (H_O) heterozygosities
166 were assessed based on 24 individuals from the Pantanal biome, Brazil. Occurrence of significant heterozygote excess is denoted by an asterisk adjacent
167 to the locus ID.
168

Multiplex	Locus		Primer Sequences	Repeat Motif	Dye	Size range	*A*	H_O	H_E
1	NnSTR-D03	F:	**CACGACGTTGTAAAACGAC** AGG CTT GAA TTT GTC CAG CTA	$(CA)_{14}$	FAM	275-293	7	0.792	0.735
		R:	CCA AGA ATC CTG TGG CAA A						
	NnSTR-E05	F:	**CACGACGTTGTAAAACGAC** CCC AAT CCT GAT AGC CCT TC	$(CA)_{18}$	FAM	134-174	4	0.292	0.301
		R:	TAT TTT TGT TGG GCC CGA GT						
	NnSTR-H03	F:	**CACGACGTTGTAAAACGAC** GCC CCT GAG CCA ATT CTT	$(TC)_{17}\_(AC)_{12}$	HEX	137-167	7	0.750	0.723
		R:	TTC TCC TGT ATT AGG GTT CTC CA						
	NnSTR-H07	F:	**CACGACGTTGTAAAACGAC** GAA GTC AAT AAG GCA GCC AAA	$(TG)_{18}$	NED	179-197	7	0.542	0.683
		R:	TGC CTG ACT GAT CCT TGT CA						
2	NnSTR-A08	F:	**CACGACGTTGTAAAACGAC** CCT TCA TTC CAA CTG TAA ATG ACT	$(TG)_{17}$	FAM	223-245	5	0.792	0.704
		R:	TCC CTA CAA ATG GAA AAA GGA A						
	NnSTR-B09	F:	**CACGACGTTGTAAAACGAC** GCT TTT GCT GGC CAT AGT TT	$(TG)_{19}$	HEX	232-234	2	0.250	0.337
		R:	TCA CTA ATT ACA ACT AAA AAC CCT GA						
	NnSTR-F02*	F:	**CACGACGTTGTAAAACGAC** CAT TTG AGT GAA AAT CCA GTG A	$(TG)_{15}$	NED	220-234	6	0.792	0.772
		R:	GCT CTT GAT AAA GCA AGC ACA A						
	NnSTR-F03	F:	**CACGACGTTGTAAAACGAC** TTG TGT CTG AAA TGG CCG TA	$(CG)_9\_(CA)_{16}$	NED	132-140	5	0.708	0.735
		R:	GCG TCT ATG TTG ATT TGA GGT G						