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FILOGEOGRAFIA DO BUGIO RUIVO, *Alouatta guariba* (PRIMATES, ATELIDAE)

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SUMÁRIO

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RESUMO

Nós examinamos a diversidade genética do DNA mitocondrial (região controle e gene citocromo b) e locos de microssatélites do primata do Novo Mundo, endêmico da Mata Atlântica, *Alouatta guariba*, para descobrir a sua estruturação genética e história evolutiva bem como seu status taxonômico. A filogenia mitocondrial mostra uma profunda divergência entre o clado A (sul de Santa Catarina) e a parte norte da distribuição e um segundo grupo divergente entre o clado B (Rio de Janeiro), mais central, e o clado C(Espírito Santo) mais ao norte, embora a população de São Paulo apresente haplótipos nos três clados com 16 de 102 indivíduos presentes no clado A, 11 de 16 no clado B e 14 de 32 no clado C. O tempo de divergência estimado entre os clados A e B/C foi de aproximadamente 750 mil anos atrás e entre os clados B e C foi de aproximadamente 600 mil anos atrás. Em concordância com os resultados do DNA mitocondrial, os dados de microssatélites mostram um claro isolamento das áreas sul e central+norte. Portanto, nossos dados consistentemente refutam a hipótese de uma subespécie ou espécie ao norte separada da central+sul (*A. g. clamitans*). Entretanto embora os dois grupos isolados identificados aqui certamente mereçam apropriadas estratégias de conservação, a ausência de: completa concordância entre dos dados de DNA mitocondrial e microssatélites, recíproca monofilia no DNA mitocondrial e claro caracteres não genéticos aconselham contra elevar ao status de espécie ou subespécie. A análise de "Bayesian Skyride plot" mostrou que *A. guariba* sofreu uma expansão populacional há aproximadamente 50.000 anos atrás seguida por uma recente redução do tamanho populacional a 7.500 anos. Esta expansão foi somente observada no clado A. Esta flutuação no tamanho populacional pode ter ocorrido devido a mudanças climáticas ou competição com *A. caraya* devido a alta sobreposição de nicho destas espécies.

ABSTRACT

Phylogeography of the brown howler monkey, *Alouatta guariba* (Primates, Atelidae)

Here we examined the mitochondrial (control region and cytb gene) and microsatellites genetic diversity of the New World primate, endemic of Atlantic Forest, *Alouatta guariba*, in order to uncover its genetic structure and evolutionary history as well as it bearing on its taxonomic status. The mtDNA phylogeny shown a deep divergence between clade A (southern of the Santa Catarina state) and the northern part of the distribution, and the latter diverged in a more central clade B (Rio de Janeiro state) and a northernmost clade C (Espírito Santo state), although a population from São Paulo state present haplotypes from the three clades with 16 of 102 individuals in the clade A, 11 of 16 in the clade B and 14 of 32 in the clade C. The divergence time estimated between A and B/C clades was approximately 750 thousand years ago (kya) and between B and C clades was ~600 kya. Microsatellite data showed a clear isolation between the southern and the central+northern areas, in agreement with the mtDNA results. Therefore, our data consistently refute the hypothesis of a northern subspecies or species separated from a largely distributed central+southern one (*A. g. clamitans*). However, although the two isolated groups identified here certainly deserve appropriate conservation strategies, the absence of: complete concordance between the mtDNA and microsatellite data, reciprocal monophyly in the mtDNA, and clear cut non-genetic diagnostic characters advice against presently erecting then at subspecies or species status. The Bayesian Skyride plot showed that *A. guariba* underwent a sudden population expansion started ~50 kya followed by a recent reduction ~7 kya. This expansion was only observed in clade A. This fluctuation in population size may have occurred due to climate changes or to competition with *A. caraya* due to the high niche overlap of these species.

1. APRESENTAÇÃO

O presente trabalho teve por objetivo estudar a história evolutiva e a estruturação geográfica de *Alouatta guariba* ao longo da sua distribuição. *Alouatta guariba* (Cabrera & Yepes 1940) pertence à família Atelidae, a qual engloba os maiores primatas neotropicais (Oliveira *et al.* 2002). Esta espécie é endêmica da Mata Atlântica, sendo encontrada desde o sul da Bahia até o Rio Grande do Sul no Brasil, chegando à região de Misiones na Argentina (Crockett 1998; Gregorin 2006).

Nesta espécie, as populações são organizadas em grupos formados por 2 a 13 indivíduos, sendo que cada grupo possui de 1 a 2 machos adultos e um número maior de fêmeas do que de machos, além de machos e fêmeas subadultos e infantes (Miranda *et al.* 2004; Miranda & Passos 2005; Steinmetz 2005; Miranda *et al.* 2006). A reprodução ocorre durante todo o ano, sendo a taxa anual de natalidade inferior a um nascimento por fêmea adulta (Strier *et al.* 2001; Miranda & Passos 2005). Contudo, a maioria dos estudos a respeito dos aspectos biológicos desta espécie foram realizados apenas com a subespécie *A. guariba clamitans*.

Alouatta guariba encontra-se atualmente na lista de espécies ameaçadas de extinção da "International Union for Conservation of Nature" (IUCN) na categoria "pouco preocupante", pois apesar de encontrar-se amplamente distribuída, esta espécie habita a Mata Atlântica, um dos ambientes mais fragmentados pela ação antrópica no Brasil (Morellato & Haddad 2000; Mendes *et al.* 2008). A persistência desta espécie ao longo de toda sua distribuição provavelmente se deve à pequena área de vida necessária a qual varia de 4 a 41 ha; sendo mais comum os grupos habitarem pequenas áreas (4 a 8 ha) com uma área nuclear inferior a 2 ha (Aguiar *et al.* 2003; Steinmetz 2005; Cunha & Jalles-Filho 2007). Além disso, tem sido relatada uma alta capacidade dos indivíduos do gênero *Alouatta* de sobreviver

em ambientes altamente fragmentados quando não há pressão de caça (Crockett 1998; para revisão ver Bicca-Marques 2003).

Por outro lado, a fragmentação da paisagem pode modificar aspectos da biologia das espécies como, por exemplo, a migração. Oklander *et al*. (2010) demonstraram que a fragmentação pode modificar o padrão de migração de *Alouatta caraya*. Os autores mostraram que em áreas de ambiente contínuo, como esperado para este gênero, tanto machos quanto fêmeas migram. Entretanto, em áreas de ambiente fragmentado, a dispersão é diferenciada entre os sexos. Nestas áreas, os machos dispersam mais que as fêmeas. Além disso, os machos de áreas fragmentadas migram 50% menos se comparados com machos de ambientes contínuos (Oklander *et al*. 2010). É provável que esta modificação no padrão de migração em ambientes fragmentados também ocorra em *A. guariba*, pois Fortes & Bicca-Marques (2008) atribuem à limitação da migração e consequentemente ao endocruzamento a coloração anormal encontrada em algumas populações do Rio Grande do Sul que habitam uma região altamente fragmentada.

Um aspecto importante para a conservação e para a classificação da vulnerabilidade de uma espécie é a correta identificação das unidades evolutivas e táxons, pois a definição do *status* taxonômico do grupo se faz extremamente necessária para justificar os esforços de conservação de uma espécie. Em *A. guariba* ainda há uma indefinição quanto ao *status* taxonômico. Diversos autores têm sugerido diferentes classificações para este grupo. Cabrera (1958) e Hill (1962) sugerem a existência de três subespécies [*A. guariba beniensis* da Bolívia, possivelmente *A. seniculus* (Mittermeier *et al.* 1988), *A.g. clamitans* e *A.g. guariba*], enquanto Rylands *et al.* (1995) e Groves (2001) consideram a existência de apenas duas, mas utilizam nomenclaturas diferentes para as mesmas. Rylands *et al.* (1995) dividiram a espécie em *A. fusca clamitans* e *A. f. fusca,* ao passo que Groves (2001) dividiu em *A. guariba clamitans* e *A. g. guariba*. Finalmente, Gregorin (2006), em uma ampla revisão do gênero, sugeriu a elevação do nível taxonômico das atuais subespécies *A. g. guariba* e *A. g clamitans* para duas espécies distintas, sendo elas *Alouatta fusca* e *Alouatta clamitans*. A primeira seria restrita à parte central da costa leste brasileira, distribuindo-se pelos Estados da Bahia, Espírito Santo, Minas Gerais e Rio de Janeiro, enquanto que a segunda ocorreria desde a região de

Misiones na Argentina até os Estados do Rio de Janeiro e Minas Gerais no Brasil. Apesar da ampla revisão realizada por este autor, a elevação das subespécies para diferentes espécies não foi completamente aceita pela comunidade científica (Mendes *et al.* 2008). Assim, devido à complexidade e a incertezas taxonômicas deste grupo, neste trabalho usamos a nomenclatura *A. guariba* seguindo Rylands & Brandon-Jones (1998).

Esta indefinição taxonômica se deve principalmente às características selecionadas para a distinção das espécies/subespécies. Apesar de alguns autores utilizarem caracteres como descrições do crânio e osso hióide, a principal característica utilizada é a coloração da pelagem (Gregorin 2006). *Alouatta guariba* apresenta dicromatismo sexual, sendo que há variação geográfica na coloração de machos e fêmeas (Gregorin 2006). Essa variação parece seguir um gradiente latitudinal, sendo que a coloração nos machos tende a escurecer gradativamente no sentido sul-norte enquanto o processo inverso, em menor intensidade, ocorre nas fêmeas. Além disso, há populações ao norte onde o dicromatismo sexual é ausente (Gregorin 2006; Fortes & Bicca-Marques 2008). Segundo Gregorin (2006), as populações que apresentam dicromatismo sexual pertencem a *A. clamitans*, enquanto que as que não o apresentam pertencem a *A. fusca*. O problema associado à utilização da variação da coloração como característica na distinção de categorias taxonômicas é que esta variação pode não refletir distinções reais nesta espécie, mas apenas a ocorrência de polimorfismo na coloração.

O principal motivo apresentado por Fortes & Bicca-Marques (2008) para adotarem apenas parcialmente a nomenclatura sugerida por Gregorin (2006) é o trabalho realizado por Harris *et al.* (2005). Neste trabalho, o único com enfoque genético populacional realizado com *A. guariba* até o momento, Harris *et al.* (2005) utilizaram o gene mitocondrial citocromo b de 19 indivíduos do Rio de Janeiro, São Paulo e Santa Catarina. Os autores encontraram fortes evidências de uma subdivisão entre as populações do Rio de Janeiro e de Santa Catarina, sendo que os espécimes de São Paulo estão representados em ambos os grupos. Segundo os autores, esta subdivisão pode ter surgido no final do Pleistoceno médio (400.000 anos atrás) seguindo a fragmentação das florestas e/ou a formação de ecoregiões distintas que se tornaram centros de endemismo. Para Fortes & Bicca-Marques (2008) esta subdivisão indicaria a

existência de duas espécies na atual *A. clamitans* sugerida por Gregorin (2006). Contudo, Harris *et al.* (2005) utilizaram em seu estudo uma baixa amostragem, o que impossibilita chegar a conclusões a respeito de aspectos evolutivos e taxonômicos da espécie.

Questões referentes à taxonomia das espécies podem ser mais bem elucidadas quando há compreensão dos diversos processos ocorridos no passado das espécies. A disciplina que procura compreende-los é a filogeografia. Esta área busca identificar através de evidências deixadas no DNA dos indivíduos de uma espécie quais processos históricos que podem ser responsáveis pela sua distribuição contemporânea (Avise 2001; Avise 2009). O DNA mitocondrial é o marcador genético mais utilizado para realizar inferências filogeográficas, pois possui herança materna, ausência de recombinação e taxa de mutação maior que o nuclear, além de possuir um quarto do tamanho populacional efetivo em relação a regiões nucleares. Estas características, principalmente as duas últimas, tornam possível estudar como eventos recentes, como as glaciações do Quaternário, influenciaram as espécies (Avise 2001; Charlesworth 2009).

Outra ferramenta molecular amplamente utilizada nos estudos filogeográficos são os microssatélites. Esses marcadores moleculares são constituídos de sequências de DNA de dois a seis pares de bases repetidos em *tandem*. Devido à sua alta taxa de mutação, os microssatélites são extremamente úteis para identificação individual, estudos populacionais e esclarecimento de questões biológicas relacionadas à taxonomia, reprodução e história evolutiva das populações (Chambers & MacAvoy 2000).

Assim, com a utilização do DNA mitocondrial e dos microssatélites, espera-se, através de um artigo científico a ser submetido à revista “Molecular Ecology” contribuir para um maior conhecimento de *A. guariba* e que os resultados obtidos possam auxiliar na resolução das questões pendentes nesta espécie. Além disso, pretende-se contribuir para um maior entendimento de como as mudanças climáticas recentes influenciaram as espécies neotropicais.

2. ARTIGO

Phylogeography of the brown howler monkey, *Alouatta guariba* (Primates, Atelidae)

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Running title: *A. guariba* phylogeography

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2.1 Abstract

Here we examined the mitochondrial (control region and cytb gene) and microsatellites genetic diversity of the New World primate, endemic of Atlantic Forest, *Alouatta guariba*, in order to uncover its genetic structure and evolutionary history as well as it bearing on its taxonomic status. The mtDNA phylogeny shown a deep divergence between clade A (southern of the Santa Catarina state) and the northern part of the distribution, and the latter diverged in a more central clade B (Rio de Janeiro state) and a northernmost clade C (Espírito Santo state), although a population from São Paulo state present haplotypes from the three clades with 16 of 102 individuals in the clade A, 11 of 16 in the clade B and 14 of 32 in the clade C. The divergence time estimated between A and B/C clades was approximately 750 thousand years ago (kya) and between B and C clades was ~600 kya. Microsatellite data showed a clear isolation between the southern and the central+northern areas, in agreement with the mtDNA results. Therefore, our data consistently refute the hypothesis of a northern subspecies or species separated from a largely distributed central+southern one (*A. g. clamitans*). However, although the two isolated groups identified here certainly deserve appropriate conservation strategies, the absence of: complete concordance between the mtDNA and microsatellite data, reciprocal monophyly in the mtDNA, and clear cut non-genetic diagnostic characters advice against presently erecting then at subspecies or species status. The Bayesian Skyride plot showed that *A. guariba* underwent a sudden population expansion started ~50 kya followed by a recent reduction ~7 kya. This expansion was only observed in clade A. This fluctuation in population size may have occurred due to climate changes or to competition with *A. caraya* due to the high niche overlap of these species.

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2.2 Introduction

Phylogeographic studies allow knowing the impact that paleogeoclimatic events had on species through the analyzes of the present distribution and demographic history imprinted in the genomes of the individuals. In this context, many studies have analyzed the influence of recent climatic changes on the evolutionary history of species (for review see Hewitt 2004; Lister 2004; Barnosky 2005; Rull 2006). The Quaternary period started about 2,588 million before present (BP) and was characterized by multiple climatic oscillations which involved changes of 7 to 15 ºC in few decades (Hewitt 2004; International Commission on Stratigraphy 2009). A well explored topic about this period is the occurrence of an elevation of the speciation rate that could explain the large biodiversity observed in some regions (Lister 2004; Barnosky 2005). With regard to mammals this is a controversial question. Lister (2004), reviewing the studies of Europe and northern Asia, concluded that there are evidence of the primary importance of the Quaternary in creating and molding most mammal species. On the other hand, Barnosky (2005) suggested that, in spite of the several climatic changes occurred in North America, the Quaternary promoted only genetic changes at population level for the majority of species because the climatic cycles were not long enough to permit reproductive isolation.

Unlike Eurasia and North America that were covering with ice sheet during glacial periods, the Neotropical land region did not present a significant portion under ice. However, the climate was considerably modified with cycles of dry and humid periods that caused large alteration in the distribution of the forests and consequently of the other organisms (Haffer 1969). The Atlantic Forest is an important biodiversity hotspot that has being highly threatened for anthropic actions (Myers *et al.* 2000). In the Quaternary interglacial periods, the Atlantic Forest distribution was similar to the present, however, in glacial periods the forest contracted and the region was dominated by grassland (Behling & Negrelle 2001; Behling *et al.* 2005; Carnaval & Moritz 2008; Pessenda *et al.* 2009; Woodburne 2010). These changes in Atlantic Forest should have high influence in herbivorous distribution, such as most species of primates (Lister 2004).

Alouatta guariba is a New World primate that inhabits exclusively the Atlantic Forest, distributing from south of Bahia to Rio Grande do Sul state in Brazil, and southwestern into Misiones region in Argentina (Crockett 1998; Gregorin 2006). *Alouatta guariba* has a generalist diet eating leaves, flowers and fruits according with their availability (Chiarello 1994; Aguiar *et al.* 2003; Miranda & Passos 2004). This feature, allied with their capacity to survive for a long time in small fragments, make the individuals of this species important seed dispersers with a crucial role in the regeneration process of Atlantic Forest (Martins 2006).

This species showed sexual dichromatism with the coloration changing according to a latitudinal gradient, although some populations do not present sexual dichromatism (Gregorin 2006). Males are lighter in southern regions and go darkening to the north while, although less intense, the inverse process happens in females. The taxonomy of *A. guariba* is still not well resolved, with different suggestions of taxonomic arrangement (Cabrera 1958; Hill 1962; Rylands *et al.* 1995; Groves 2001; Gregorin 2006). A recent revision recognized two distinct species, *A. fusca*, without sexual dichromatism and *A. clamitans,* with sexual dichromatism, the former being restricted to the central part of Brazilian east coastal, distributing through Bahia, Espírito Santo, Minas Gerais and Rio de Janeiro States (see fig. 9 in Gregorin 2006). Based in the genetic study carried out by Harris *et al.* (2005), Fortes & Bicca-Marques (2008) suggest that *A. clamitans* as proposed by Gregorin (2006) could be in fact two distinct species. Nevertheless, due to the very low sampling and genetic markers used by Harris *et al.* (2005), no conclusion with regard to taxonomic status could be proposed.

Despite the anthropic degradation and fragmentation of the Atlantic Forest, *A. guariba* is in the least concern category of risk according to International Union for Conservation of Nature (IUCN) (Mendes *et al.* 2008), given its wide distribution. However, the problematic taxonomy difficult the recognition of the real state of threat of this complex species.

In this study, we used segments of the control region and cytochrome b gene of the mitochondrial DNA (mtDNA), in combination with a set of microsatellites markers, to investigate the genetic diversity, genetic structure, and demographic history of *A. guariba* throughout its distribution.

2.3 Material and methods

2.3.1 Sample collection and laboratory techniques

The material for genetic analysis consisted of samples of 151 individuals of the *A. guariba* from 38 localities along the distribution of the species (Fig. 1; Table S1, supporting material), mostly from in situ samples. Total DNA was extracted from blood, tissue and feces using standard phenol-chloroform protocol (Sambrook *et al.* 1989) and QIAmp DNA Stool Mini Kit (QIAGEN).

Approximately 809 base pairs (bp) of the mtDNA cytochrome b gene (cytb) were amplified with the primers L14724 (Irwin *et al*. 1991) and (5'-TGGGTCGGTTAGAAGGTCAG-3') designed for us. A fragment of approximately 713 bp of control region (CR) was amplified with primers How-RA1 and How-S7 (Ascunce *et al.* 2003). For DNA extracted from feces, a nested PCR was performed with internal primers for CR RC_intF2 (5'-AAAATGTGGGCGGGTTGT-3'), and RC_intR1 (5'-CATAGCACATTCGTCCCGTA-3') designed for *A. guariba*. The PCR reactions contained around 10-100 ng of total DNA, 100-150 µM dNTPS, 1.5 mM $MgCl_2$, 0.15 µM each primer, 0.1 mg/ml BSA, 1x buffer and 0.4-1.25 units of Platinum Taq DNA Polymerase (Invitrogen). The first amplification started with initial denaturation at 94 ºC (3 min), followed for 8 cycles at 94 ºC (45 s), 57 ºC -1 ºC/cycle (1 min), 72 ºC (1 min 30 s) and 35 cycles at 94 ºC (45 s), 50ºC (1 min), 72 ºC (1min 30 s) and finished with final extension at 72 ºC (5 min). For amplifications with internal primers only 30 cycles at 50 ºC were used. Amplified fragments of mtDNA were sequenced using the DYEnamic ET Dye Terminator Cycle Sequencing Kit (Amersham Biosciences). For cytb analysis, three sequences of GenBank were incorporated (DQ679779, AY065898 and AY065899). As outgroup, we amplified two individuals of *Alouatta caraya* for cytb and CR and one individual for cytb of *Alouatta belzebul*. We included three more sequences of cytb deposited in GenBank of *A. belzebul* (AY374346, AY374356 and DQ387029) besides one individual of each species of *Alouatta* (Table S2, supporting material).

Eight microsatellites (AB17, AB7, AB10, AC17, 1118, 157, D17S804 and D8S165) previously characterized for *A. caraya* (Oklander *et al.* 2007) were

genotyped for 132 individuals. The PCR reactions contained approximately 10-100 ng of total DNA, 100 µM dNTPS, 1.8 mM $MgCl_2$, 0.02 µM of forward primer, 0.1 µM of reverse primer, 0.4 µM of fluorescence labeling (Schuelke 2000), 1 M betaine, 5 % trehalose, 1x buffer and 0.1 unit of Platinum Taq DNA Polymerase (Invitrogen). The amplification started with initial denaturation at 94 ºC (3 min), followed for 11-15 cycles at 94 ºC (45 s), 65 ºC -0,5 ºC/cycle (1 min), 72 ºC (1 min 30 s) and 26-30 cycles at 94 ºC (45 s), 58-60ºC (1 min), 72 ºC (1 min 30 s) and finished with a final extension at 72 ºC (30 min). For AB17 loci we used 6 cycles at 60 ºC (-1º/cycle) followed for 30 cycles at 50 ºC and for AC14 loci instead touchdown condition we used 30 cycles at 61 ºC. Both sequencing and microsatellites genotyping were performed in MegaBACE 1000 automated sequencer (Amersham Biosciences) according the manufacturer's protocols.

2.3.2 Analysis

2.3.2.1 Mitochondrial DNA

Sequences were aligned using ClustalW algorithm in Mega 4.0.2 (Tamura *et al.* 2007) and corrected by eye. The degree of saturation was investigated by plotting proportions of transitions and transversions against the pairwise divergence between the sequences in DAMBE 5.2.5 (Xia and Xie 2001). The number of variable sites (S) and haplotypes (h), haplotype (H_d) and nucleotide (π) diversity were calculated using DnaSP 5 (Librado & Rozas 2009).

2.3.2.2 Phylogenetic reconstruction

Bayesian Inference (BI) was used to infer the phylogenetic relationship using concatenated cytb and CR. The BI was performed in BEAST 1.6.1 (Drummond & Rambaut 2007) using SRD06 substitution model (Shapiro *et al.* 2006) for cytb and HKY+G with eight categories for CR. We used the strict molecular clock model with substitution rates estimated by us (see below). BEAST was run with 120 million iterations sampling each 12,000 chains and the first 10% iterations were discarded as burn-in. The runs were visually inspected

using TRACER 1.5, summarized in TreeAnnotator 1.6.1, and the inferred tree visualized in FigTree 1.3.1 (http://beast.bio.ed.ac.uk/Tracer).

The genealogical relationship between mtDNA haplotypes was estimated by the median-joining method (Bandelt *et al.* 1999) with Network 4.6 (http://www.fluxus-engineering.com). Pairwise F_{ST} estimated in Arlequin 3.5.1.3 (Excoffier *&* Lischer 2010) was used to analyze the differentiation between northern (ES, RJ, MG, SP) and southern (SC, RS, AR) groups of samples.

2.3.2.3 Divergence time estimates

Time estimates using genetic data in Atelidae is difficult given the absence of reliable fossil record or accepted substitution rates for cytb and CR. As a first approximation, we estimated, using BEAST, the substitution rates for both fragments using as calibration points the dates estimated for Schrago (2007), since it is the best available analysis so far and one that used segments different from those used here. The cytb rate was estimated using the unique haplotypes of *A. guariba*, two sequences of *A. caraya* generated here and from GeneBank sequences representing of all families of Platyrrhini species (Table S2, supporting material). The BEAST analysis was as described above, modified by using a lognormal relaxed clock and 50 million iterations sampling each 5,000. Given the absence of control region sequences from most species of Platyrrhini, the substitution rate for this segment was estimated relative to the previously calculated rate for cytb (the latter used as a rate prior), in a partitioned analysis using only *A. guariba* that presented sequences for both fragments. The run was performed as before, using GMRF Bayesian skyride tree prior (Minin *et al.* 2008) and 120 million iterations.

2.3.2.4 Demographic history

DnaSP 5 and Arlequin 3.5.1.2 were used to calculate neutrality tests statistics such Tajima's D (Tajima 1983), Fu and Li's D and F and Fu's F_s (Fu 1997). GMRF Bayesian skyride plots (BSP) (Minin *et al.* 2008) using time-aware smoothing were used for estimate past population dynamics through time using the approach and parameters described above. Separate analysis were

performed for the whole species and for each clade found in the mitochondrial tree.

2.3.2.5 Microsatellites

The loci were tested for deviations from Hardy-Weinberg Equilibrium (HWE) (Guo & Thompson 1992) and linkage disequilibrium using Arlequin, corrected for simultaneous comparisons with the sequencial Bonferroni test (Rice 1989). Genetic diversity was measured as the number of alleles per locus (K), the mean number of alleles per locus (allelic diversity, A), observed heterozygosity (H_O) and expected heterozygosity (H_E) using Arlequin. We evaluated potential population subdivision in our samples using a Bayesian model-based clustering method implemented in Structure 2.3.3 (Pritchard *et al.* 2000). We conduct three independent runs for each K (number of cluster) between 1 and 5 using no prior information, the admixture model, and the correlated allele frequencies model. Burn-in length and length of simulations were set at 10,000 and 1,000,000 steps, respectively. The number of clusters was estimated by the ΔK statistic (Evanno *et al.* 2005). Pairwise Fst between the northern and southern areas was done as above for the mitochondrial data.

2.4. Results

2.4.1 Mitochondrial DNA

We obtained CR and cytb sequences for 121 and 135 individuals of *A. guariba* (Table 1), respectively. The 674 bp cytb alignment contained 21 variables sites, 20 being transitions and one transversion, comprising 16 haplotypes. The of 598 bp CR alignment presented 154 variables sites, 152 being transitions and 10 transversion, comprising 93 haplotypes. The CR sequences presented an insertion of 58 bp related to *A. caraya* with return no similarity with any sequence deposited in GenBank. This insertion is highly variable, presenting 12 variable sites. No evidence of substitution saturation were found in the datasets (data not shown). Genetic diversity indices are high, especially for control region, as expected, and for the whole species and each

clade. For the CR, haplotype diversity is very high and nucleotide diversity is around 5%.

2.4.2 Phylogenetic relationships

A Bayesian phylogenetic tree with both fragments combined revealed three strongly supported (posterior probability >0.9) and very divergent clades with a strong geographic structure (Fig. 2). The mean pairwise difference between the three clades was ~64 substitutions. Clade A contains samples from the southern part of the distribution, from São Paulo (SP) to Rio Grande do Sul (RS) and Argentina (AR). Clade B contains samples from the central area, from Rio de Janeiro (RJ), SP and one individual from Minas Gerais (MG). However, this sample is from a captive individual and its area of origin is not known with confidence. Clade C contains samples from the northernmost part of the distribution, from Espírito Santo (ES), MG and SP. Therefore, with the exception of the population from the São Paulo area, that present haplotypes from the three clades, all other regions (south: AR, RS, SC; central: RJ; and north, ES, MG) present haplotypes from a single major clade. Interestingly, even these samples from SP are not randomly distributed within clades A and C but are clearly associated in the haplotype tree (Fig. 2).

Only the haplotype network for clade A was shown (Fig. 3) since the other two are not very informative due to a low geographical coverage so far since we choose to analyze only individuals with sequences from both fragments, that are still lacking for some areas. Clade A network showed a complex relationship with the presence of several reticulations. Unlike the phylogenetic tree, the samples from SP do not group together, showing weak geographic structure. A F_{ST} of only 0.0128 was estimated between the northern and southern areas.

2.4.3 Divergence time estimates

The substitution rate for the Platyrrhini estimated with BEAST for cytb was 1.15×10^{-8} site/year (confidence interval 95%: 8.913×10^{-9} - 1.409×10^{-8}) and for CR was 2.815×10^{-7} (confidence interval 95% 1.459×10^{-7} - 4.399×10^{-7}). The

rate found for the cytb was similar to that reported for other mammals (e.g. $5.6x10^{-9}$ for *Tapirus terrestris*, Thoisy *et al.* 2010). The control region rate relatively higher than most mammals (e.g., $2.02x10^{-8}$ - $5.34x10^{-8}$ for *Cerdocyon thous*, Tchaicka *et al.* 2007) but within the range for humans and other primates (Santos *et al.* 2005; Endicott & Ho 2008). Using these rates in a combined mtDNA dataset, we estimated the divergence time between clades A and B/C around 700 thousand years ago (kya) (95% confidence interval ~505 - 1,000, kya) and between clade B and C ~600 kya (95% CI ~400 - 850 kya).

2.4.4 Demographic history

In general, most neutrality test statistics were not significant (Table 2), with the exception of F_s and especially for clade A, which seems the only clade with evidence for demographic changes. The significantly negative F_s suggest clade A underwent a historical population expansion. These suggestions are corroborated by the BSP for the whole species and for each clade (Fig. 4). The species BSP (Fig. 4A) shown a population expansion starting around 40-50 kya that was followed by a reduction of population size ~7 kya. The BSPs of the separate clades shown this pattern is only present in clade A history, since clades B and C are consistent with constant population size (Fig. 4b).

2.4.5 Microsatellites

All 8 loci were highly polymorphic with the number of alleles per locus ranging from 6 (AC17) to 23 (1118) with a mean of 12.625. The observed heterozygosity (H_o) ranged from 0.093 (AB10) to 0.604 (157) with a mean of 0.339 and the expected heterozygosity (H_E) ranged from 0.213 (AB10) to 0.825 (157) with a mean of 0.527 (Table 3). No significant deviation of HWE was seen at any loci. Pairwise comparisons of allele frequencies revealed no significant linkage disequilibrium after Bonferroni correction. The highest value of ΔK for K=2 in structure analysis suggests the existence of two major groups of populations in the species, one grouping individuals from the northern areas (SP, MG, RJ and ES) and the other individuals from the southern part of the distribution (SC and RS) (Fig. 5). Differently from the mtDNA tree, the

separation of these two groups based on microsatellite loci are very clear, including individuals from SP in the central+northern group. However, two individuals, one from SP and one from RS could not be unambiguously classified in any group (Fig. 5). A F_{ST} of 0.347 was estimated between the northern and southern groups of *A. guariba* populations.

2.5 Discussion

2.5.1 Taxonomic implications

The mtDNA phylogeny shown a deep divergence between the southern (AR, RS, SC) and the northern part of the distribution, and the latter later diverged in a central and a northern clade (Fig. 2). This geographic structure is not complete, however, since the populations from the central area of distribution, specifically from São Paulo State, present haplotypes from the three clades. Clades A and B were also found by Harris *et al*. (2005) previous analysis, but, given their limited sampling, they failed to find clade C, the northernmost clade. They also found in their SP sample haplotypes from both clades, supporting that this is not a technical error such as misidentification.

The population structure found by the microsatellites data largely agrees with the mitochondrial results, showing the higher hierarchical structure distinguishing the same southern (RS, SC) and northern (all other) groups (Fig. 5). The two individuals not clearly assigned to any group may indicate incomplete differentiation or some ongoing gene flow between these areas.

Southern vs central+northern clades divergence date is relatively ancient, around 700 kya and between B and C clades was around 600 kya. The former date is older than that previously estimated by Harris *et al.* (2005) (~400 or 500 kya for different calibration points), but within our confidence interval.

How these findings compare with the previous non-genetic taxonomic suggestions? The traditional suggestions that *A. guariba* is divided in two subspecies (or even species for some, such as Gregorin 2006), one (*A. g. guariba*) restricted to the northernmost area of distribution, from ES to the north (Gregorin 2006) or northern of the Doce river (Kinzey 1982) is not corroborated here. However, if this subspecies is restricted to the area north of the Rio

Jequitinhonha (as suggested by Rylands *et al.* 1988), then we could not tests its status since we do not have samples from that area. Our microsatellite data do not suggest any important present day subdivision in our northern group, since the structure analysis considering 3 groups present a subdivision only within the southern group. However, all samples from the northern area (ES and MG) present our mtDNA clade C, suggesting some kind of ancient divergence that may not be valid any more, at least for the autosomal genome (see below).

In summary, our data consistently refute the traditional suggestion of a northern (north of the RJ or ES) subspecies or species separated from a largely distributed central+southern one (*A. g. clamitans*). On the other hand, we partially corroborate and largely extend the previous genetic evidence (karyological, eg. Oliveira *et al.* 1998 and Oliveira *et al.* 2002 and mitochondrial, Harris *et al.* 2005) on the existence of a large divergence between the southernmost and the central+northern areas of distribution of *A. guariba*.

What are the implications of these findings for the taxonomy of the species? Although it may be tempting to suggests (following Oliveira *et al.* 2002 and Harris *et al.* 2005) that the southern and the central+northern populations are two subspecies or even different species, the lack of complete concordance between the mtDNA and microsatellite data, the lack of reciprocal monophyly in the mtDNA and the absence of clear cut non-genetic diagnostic characters advise against such decision presently. However, the clear pattern of differentiation depicted in the structure analysis suggests these two groups have been isolated for some time (at least a few thousand years) and that they certainly deserve appropriate conservation strategies.

Phylogeographic patterns

The interpretation of the patterns found in Atlantic Forest is difficult because, despite an increase in research efforts in the past few years, the knowledge about the evolutionary history of the Atlantic Forest is still limited (Ribeiro *et al.* 2011). The most well-known hypothesis about the origin of diversity in Neotropical region is the refuge theory. According to this theory, proposed for Amazonian basin and expanded for Atlantic Forest, during the glacial ages the rainforests were reduced to refuges isolated by opens areas,

and organisms isolated in these refuges could have diverged and originated new lineages. Then, in the next interglacial period, the forest expanded and the new clades could be in contact. Thus, this hypothesis requires the expansion and contraction of open areas and the formation of forest refuges (Haffer 1969; Carnaval & Moritz 2008; Carnaval *et al.* 2009). One possible explanation for our mtDNA results is that these three clades could represent a differentiation of the populations due to vicariant events during the forest fragmentation following by expansions in favorable periods that induced secondary contacts (represented here by the SP populations).

Actually, the demographic history showed that *A. guariba* went by a sudden population expansion started approximately 50 kya followed by a recent reduction approximately 7 kya. However, this expansion is only observed in clade A. An expansion of clade A to the north could have introgressed this clade to the central population (SP), suggesting a secondary contact of previously geographically separated clades. However, the clear divergence between southern and central+northern areas found by the microsatellites results suggest that these populations are effectively isolated for several thousand years.

Contrarily to what was expected by the refuge theory depicted above, the expansion of *A. guariba* southern clade during the glacial period and even intensified during and soon after the Last Glacial Maximum (26-19 kya) when forests contractions occurred (Behling 2002; Behling *et al.* 2004). A possible explication for this expansion in a likely unfavorable period is the humidity variation during the Pleistocene that formed ecoregions favorable for this species. Palynological studies had showed that southern and southeastern Brazilian regions had predominance of grassland during the Late Pleistocene, mainly during the Last Maximum Glacial when tropical trees were almost absent (Behling 2002; Behling *et al.* 2004). However, the pollen data suggest that small populations of the *Araucaria* and the Atlantic rainforest trees may have been present on the wetter coastal slopes and deep and protected river valley, which may have served as a refuge (Behling *et al.* 2004). Besides, periods of increased humidity favored forests of conifers, which appear to have expanded during the glaciations when the climate was cool and moist enough for their development, forming mosaics with grassland (Ledru *et al.* 1996; Ledru *et al.*

2007; Pessenda *et al.* 2009). Thus, the generalist diet of the *A. guariba* that is composed for many vegetal species, including conifer species as *Araucaria angustifolia*, could allowed its survival in the expanded conifer forest and their associated vegetation (Miranda & Passos 2004; Martins 2006; Martins 2008).

The period of *A. guariba* expansion coincide with that found by Cabanne *et al.* (2007). The authors showed that the subspecies *Xiphorhynchus fuscus tenuirostris* that inhabit the northeastern of Minas Gerais and southern Bahia remains demographically stable whereas *Xiphorhynchus fuscus fuscus* that have a subdivision of the northern and southern presented a demographic expansion that started approximately 57 and 19 kya, respectively.

Also unexpectedly was the recent (since ~7 kya) decrease in population size detected in *A. guariba* southern clade, a period usually characterized by the expansion of the Atlantic Forest (Behling & Negrelle 2001). However, this period was marked by oscillations on the humidity and temperature. For example, Garcia *et al.* (2004) and Behling *et al.* (2005) showed that in SP and RS states between ~10 and ~4 kya the climate was much drier than today. These oscillations in humidity and temperature could have affected *A. guariba* demography. One alternative explanation to this reduction in population size in *A. guariba* is its partial coincidence with the expansion of *A. caraya* that would have occurred between 15 and 6 kya (Ascunce *et al.* 2007). These species present high degree of niche overlap, competing for resources and negatively affecting each other's population growth. This niche overlap is thought to have an important role in maintaining the allopatric distribution of these species (Agostini *et al.* 2010).

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2.8 Legends for figures

Figure 1. Geographical distribution of the *A. guariba* with sampling localities. Grey line showed the approximate limit of *A. guariba* distribution according Mendes *et al.* (2008). Dashed line represents the barrier between the two microsatellites groups. The circles yellow, red and green represent the mtDNA clades A, B and C, respectively. Abbreviations are for Brazilian states and Argentina.

Figure 2. Bayesian tree based on CR and cytb fragments combined. Node numbers indicate the posterior probability for main clades. Color vertical bars represent main clades. Red lines are samples from São Paulo state. Bottom rule is divergence time in years.

Figure 3. Median joining network of CR+cytb complete haplotypes for clade A. Each circle represents a different haplotype with size proportional to its relative frequency. White circles represent median vectors. In yellow RS, blue SC, red SP. Numbers represent the number of mutations in each branch.

Figure 4. Bayesian Skyride plot of CR+cytb sequences showing the effective population size fluctuation throughout time (in years). a) all individuals; b) for clades A-C. The solid line depicts median estimate, grey region represent 95% highest posterior density intervals.

Figure 5. Estimated population structure of 132 individuals using 8 microsatellite loci as performed by Structure. Thin bars represent individuals where each is probabilistically assigned to *K=2* populations (colored segments).

2.9 Figures and tables

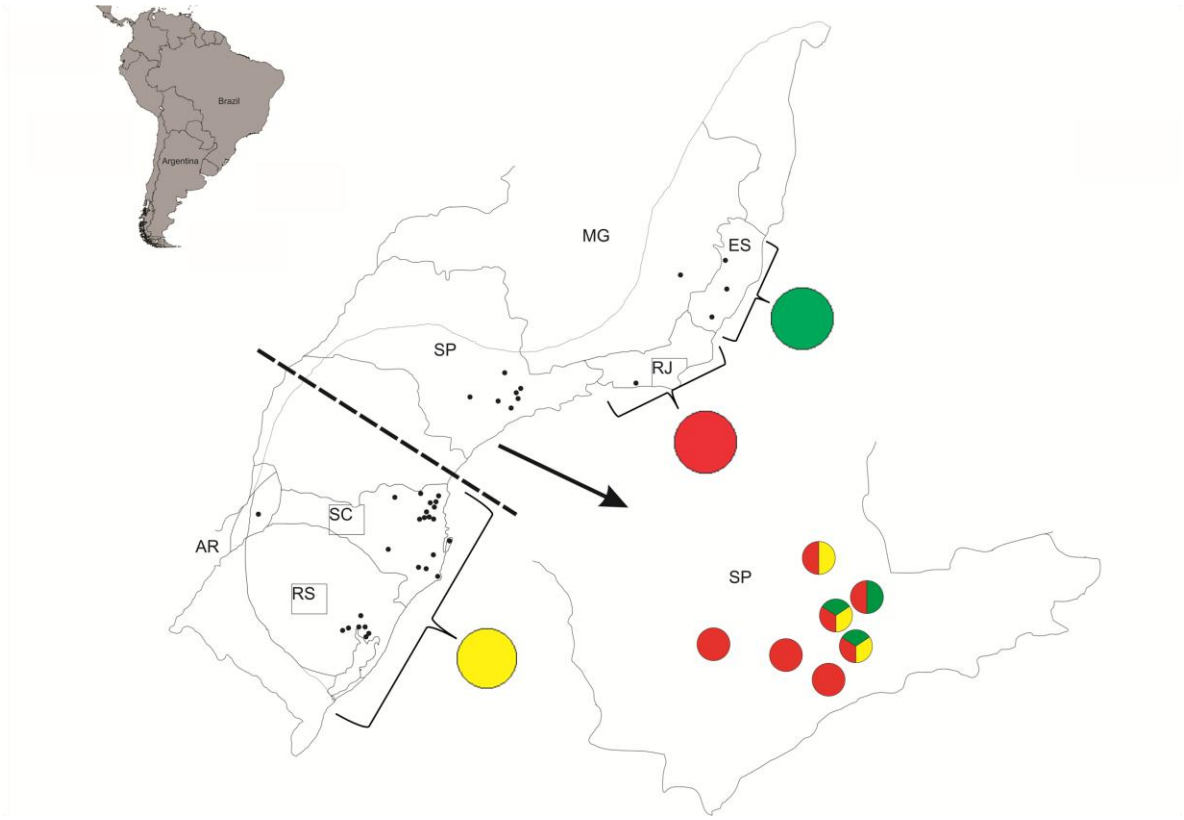

Figure 1

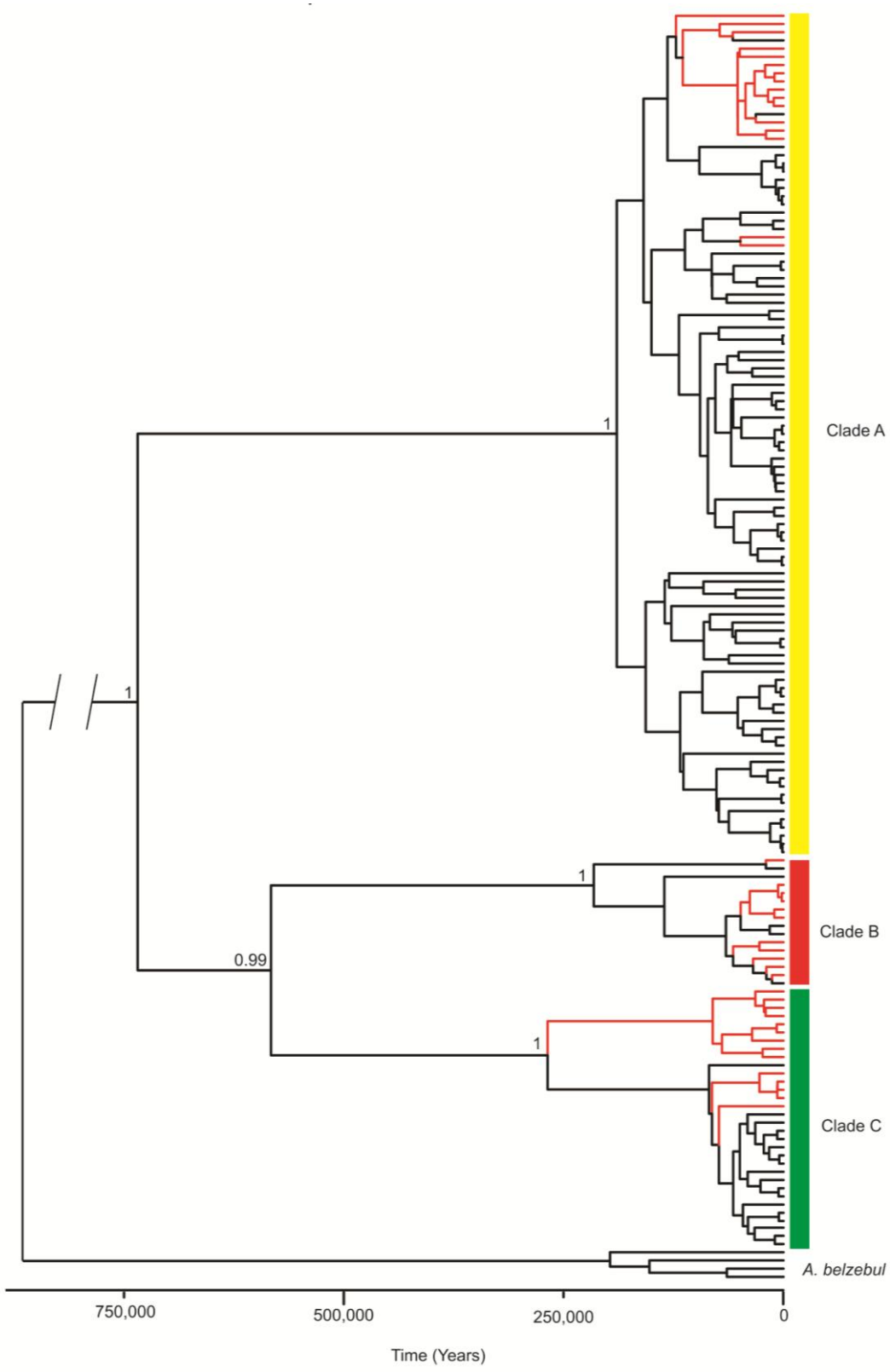
1
1
1
0.99
1
Clade A
Clade B
Clade C
A. belzebul
750,000
500,000
250,000
0
Time (Years)

Figure 2

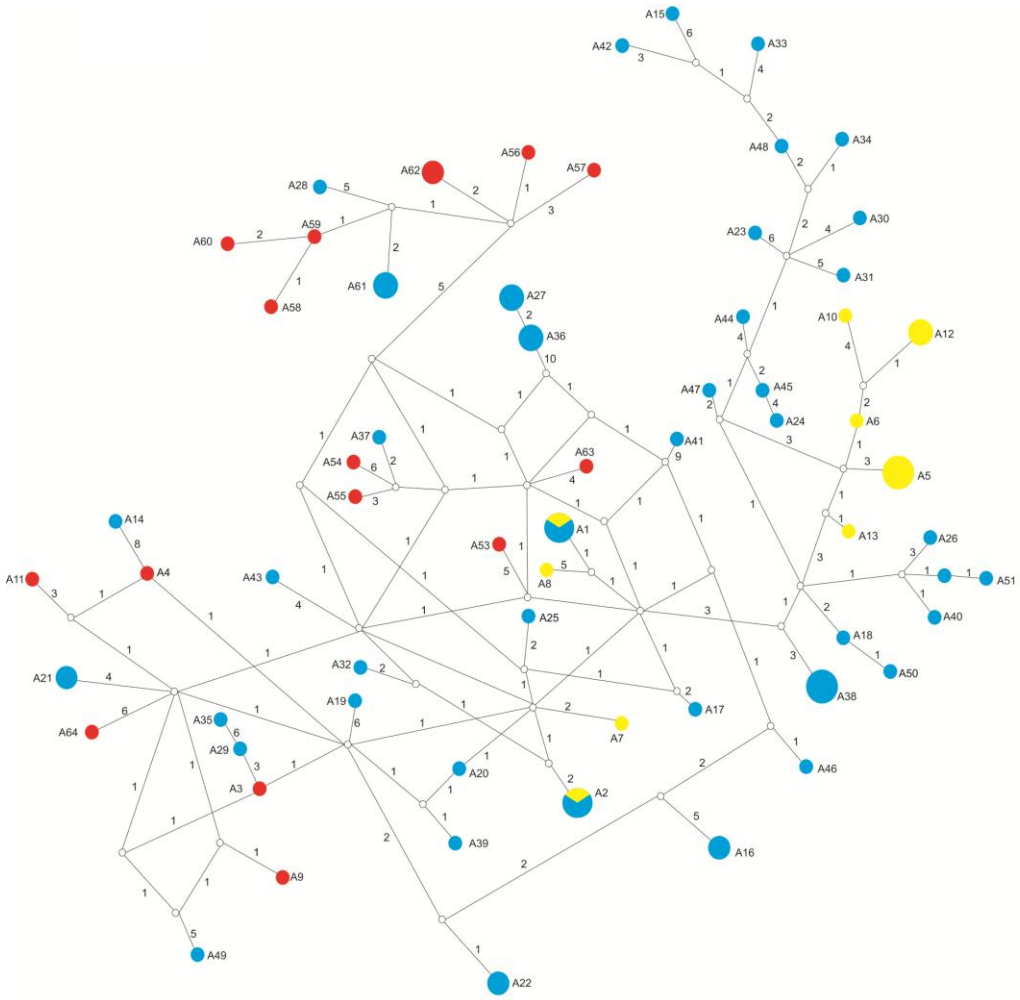
A15
A42
A33
A48
A34
A23
A30
A31
A44
A10
A12
A47
A45
A24
A6
A5
A41
A63
A37
A54
A55
A1
A53
A8
A13
A26
A51
A40
A18
A50
A38
A14
A4
A11
A43
A25
A21
A32
A19
A64
A35
A29
A3
A17
A7
A46
A20
A2
A39
A16
A9
A49
A22
A62
A56
A57
A28
A59
A60
A61
A58
A27
A36

Figure 3

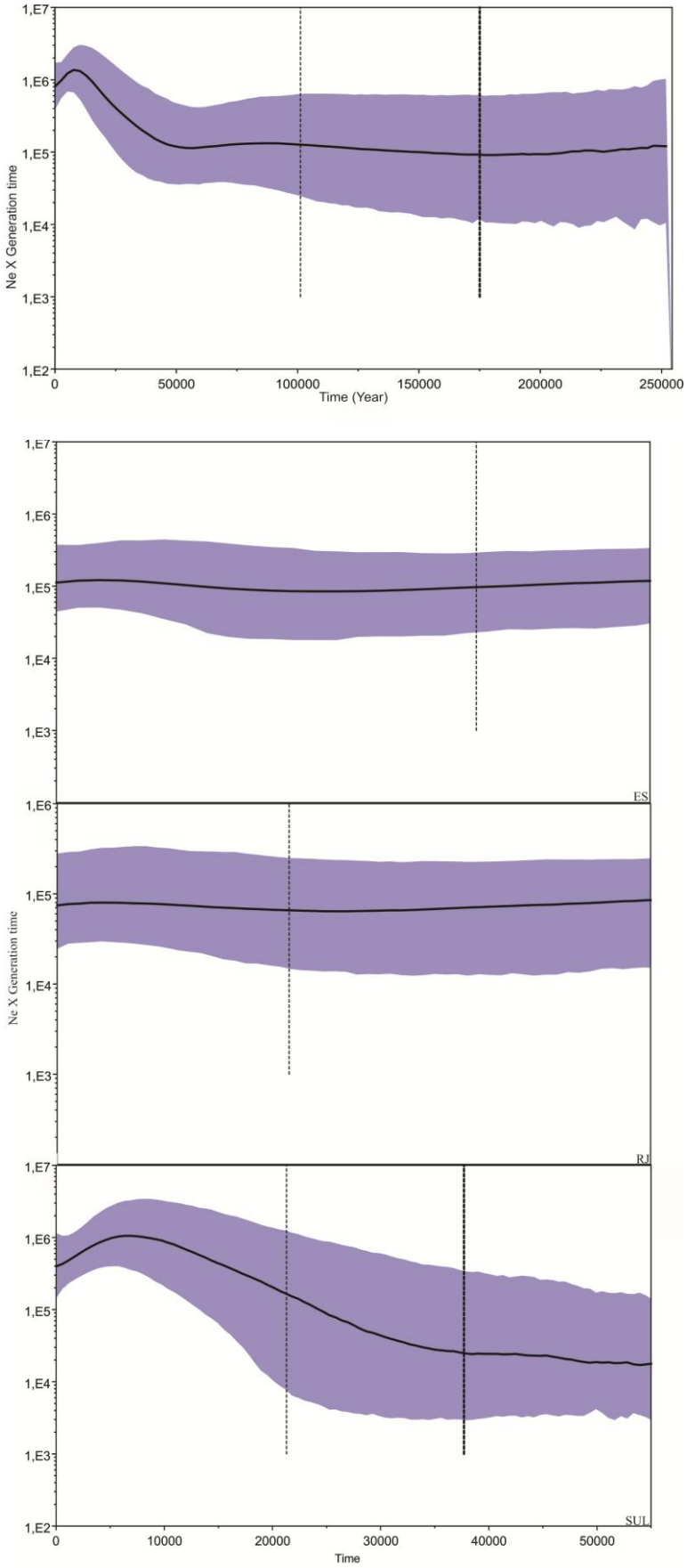
1,E7
1,E6
1,E5
1,E4
1,E3
1,E2
Ne X Generation time
0
50000
100000
150000
200000
250000
Time (Year)
ES
RJ
SUL
10000
20000
30000
40000
50000
Time

Figure 4

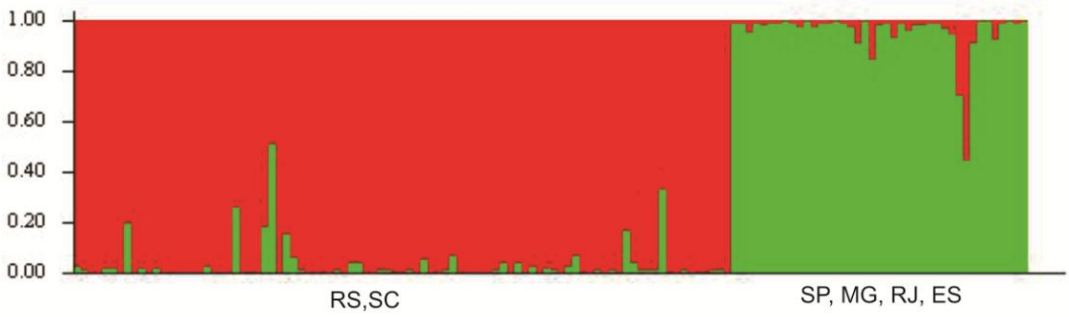

Figure 5

Table 1. Basic sequence statistics for each gene fragment in all samples and for each clade found in the phylogenetic analysis.

		N	S	H	H_d (SD)	π% (SD)
Control region	All	135	154	93	0.993 (0.002)	5.3 (0.32)
	A	96	105	68	0.990 (0.003)	2.0 (0.08)
	B	10	31	8	0.933 (0.077)	1.6 (0.31)
	C	29	52	17	0.956 (0.019)	2.3 (0.36)
Cytochrome b	All	121	21	16	0.664 (0.046)	0.4 (0.05)
	A	91	10	9	0.428 (0.062)	0.1 (0.02)
	B	16	3	4	0.525 (0.137)	0.1 (0.03)
	C	14	4	3	0.582 (0.092)	0.3 (0.04)
CR and cytb concatenated	All	105	171	79	0.993 (0.002)	2.4 (0.22)
	A	84	113	64	0.992 (0.003)	1.0 (0.04)
	B	10	34	8	0.933 (0.077)	0.8 (0.01)
	C	11	42	7	0.909 (0.066)	1.5 (0.15)

Number of sequences (N), number of variable sites (S), number of haplotypes (h), haplotype diversity (H_d,), nucleotide diversity (π), standard error (SD).

Table 2. Neutrality tests for each gene fragment in all samples and for each clade found in phylogenetic analysis.

		D	F'	D'	F_s
Control region	All	0.234	0.707	0.884	-23.854**
	A	-1.352	-1.198	-0.740	-24.292**
	B	-0.654	-0.721	-0.633	-0.429
	C	0.071	0.103	0.095	-0.424
Cytochrome b	All	-0.694	-0.337	-0.025	-2.339
	A	-1.611	-2.814*	-2.776*	-4.572**
	B	-1.002	-0.336	-0.039	-1.415
	C	1.224	0.621	0.305	2.163
CR and cytb concatenated	All	-0.335	0.350	0.778	-23.894**
	A	-1.388	-1.427	-1.050	-24.253**
	B	-0.784	-0.897	-0.799	3.273
	C	1.592	1.224	0.901	-0.293

Tajima's D (D), Fu and Li's F(F'), Fu and Li's D (D'), Fu's F_s (F_s). *P<0.05 and **P<0.02.

Table 3. Summary statistics for 8 microsatellite loci genotyped for brown howler.

Locus	N	K	H_o	H_E
AB17	212	17	0.454	0.587
AB7	131	11	0.397	0.631
AB10	118	9	0.093	0.213
AC17	111	6	0.261	0.462
1118	123	23	0.163	0.346
157	111	13	0.604	0.825
D17S804	116	11	0.431	0.589
D8S165	116	11	0.310	0.562
Average		12,625	0.339	0.527

Number individuals analyzed (N); alleles per locus (K); observed heterozygosity (H_o); H_E (expected heterozygosity).

2.10 Supporting material

Table S1. Sample collection.

State	Localization	N	Mit	H
MG	Caratinga	06	C	-
	Unknown	02	B, C	B8
SP	Campinas	02	A, B	A53
	Embu-Guaçu	01	A	A64
	Ibiúna	01	A	A63
	Itapetininga	01	A	A60
	Mairiporã	05	B, C	B2, C1, C2, C5
	São Paulo	13	A, B, C	A59, A61, A62, B3, B5, C5, C6, C7
	Serra da Cantareira	18	A, B, C	A54, A55, A56, A57, A58, B2, B6, B7, C1, C2, C3, C4
ES	Cachoeiro de Itapemirim	06	C	-
	Pancas	02	C	-
	Santa Maria de Jetibá	03	C	-
RJ	Rio de Janeiro	04	B	B1, B4
SC	Ascurra	02	A	A17, A28
	Blumenau	11	A	A19, A21, A27, A36, A37, A38, A43, 44
	Braço do Norte	01	A	A34
	Brusque	01	A	A29
	Florianópolis	02	A	A40, A48
	Gaspar	01	A	A41
	Guaramirim	01	A	-
	Indaial	12	A	A1, A2, A16, A18,A27, A47, A49, A52
	Jaraguá do Sul	01	A	-
	Joinville	01	A	A25
	Lages	02	A	A31
	Laguna	03	A	A26, A30, A46
	Massaranduba	01	A	A22
	Papanduva	01	A	A32
	Pomerode	08	A	A23, A24, A27, A35, A38, A39, A42,A51
	São Bento do Sul	03	A	A14, A15, A33
	São Bonifácio	01	A	A21
	Unknown	08	A	A16, A20, A22, A36, A38, A45, A50
RS	Águas Claras	01	A	-
	Arroio do Ratos	01	A	-
	Butiá	01	A	A4
	Itapuã	04	A	A1, A5
	Porto Alegre	06	A	A8, A9

	São Leopoldo	01	A	A6
	Viamão	01	A	A7
	Unknown	10	A	A2, A3, A5, A10, A11, A12, A13
Argentina	Misiones	02	A	-

Number of individuals and mitochondrial group found in phylogenetic analysis (N); mitochondrial group of BI (Mit); haplotypes of CR and cytb concatenated network (H). Brazilian states: Rio Grande do Sul (RS), Santa Catarina (SC) and São Paulo (SP); Argentina (AR).

Table S2. Sequences used for estimate cytb rate in Platyrrhini superfamily.

Family	Genus	Species	GenBank
Pitheciidae	*Callicebus*	*C. hoffmannsi*	AF524885.1
		C. lugens	AF524888.2
		C. moloch	AF524887.1
		C. torquatus	AF524890.2
	Pithecia	*P. irrorata*	AY226183.1
		P. monachus	FJ531668.1
	Chiropotes	*C. chiropotes*	FJ531667.1
		C. israelita	AY226187.1
		C. utahicki	AY226186.1
	Cacajao	*C. calvus*	FJ531664.1
		C. melanocephalus	FJ531646.1
Cebidae	*Cebus*	*C. albifrons*	FJ529108.1
		C. apella	FJ529103.1
		C. capucinus	AY065907.1
		C. cay	FJ529088.1
		C. olivaceus	FJ529107.1
	Saimiri	*S. boliviensis*	AJ315388.1
		S. oerstedii	EU232702.1
		S. sciureus	AJ489747.1
		S. ustus	EU232707.1
	Aotus	*A. azarai*	DQ098865.1
		A. lemurinus	DQ098871.1
		A. nancymaae	AJ489746.1
		A. trivirgatus	DQ098874.1
	Saguinus	*S. midas*	AJ489760.1
	Callithrix	*C. jacchus*	AF295586.1
Atelidae	*Alouatta*	*A. belzebul*	DQ387015.1
		A. macconnelli	AY065888.1
		A. palliata	AY065878.1
		A. pigra	AY065884.1
		A. sara	AY065887.1
		A. seniculus	AJ489759.1
	Ateles	*A. belzebuth*	FJ785422.1
		A. geoffroyi	AY065903.1
	Brachyteles	*B. arachnoides*	AY065905.1
	Lagothrix	*L. lagotricha*	AY671799.1

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