Primates (2011) 52:77–87
DOI 10.1007/s10329-010-0226-2

ORIGINAL ARTICLE

Coalescent analysis of mtDNA indicates Pleistocene divergence among three species of howler monkey (*Alouatta* spp.) and population subdivision within the Atlantic Coastal Forest species, *A. guariba*

Felipe de Mello Martins · Cristiani Gifalli-Iughetti · Celia Priszkulnik Koiffman · Eugene E. Harris

Received: 5 May 2010 / Accepted: 26 October 2010 / Published online: 26 November 2010

Abstract We have used coalescent analysis of mtDNA cytochrome *b* (cyt *b*) sequences to estimate times of divergence of three species of *Alouatta*—*A. caraya*, *A. belzebul*, and *A. guariba*—which are in close geographic proximity. *A. caraya* is inferred to have diverged from the *A. guariba/A. belzebul* clade approximately 3.83 million years ago (MYA), with the later pair diverging approximately 1.55 MYA. These dates are much more recent than previous dates based on molecular-clock methods. In addition, analyses of new sequences from the Atlantic Coastal Forest species *A. guariba* indicate the presence of two distinct haplogroups corresponding to northern and southern populations with both haplogroups occurring in sympatry within Sao Paulo state. The time of divergence of these two haplogroups is estimated to be 1.2 MYA and so follows quite closely after the divergence of *A. guariba* and *A. belzebul*. These more recent dates point to the importance of Pleistocene environmental events as important factors in the diversification of *A. belzebul* and *A. guariba.* We discuss the diversification of the three *Alouatta* species in the context of recent models of climatic change and with regard to recent molecular phylogeographic analyses of other animal groups distributed in Brazil.

Keywords *Alouatta* · Coalescent estimates · Pleistocene · Cytochrome *b* · Phylogeography · Platyrrhini · Neotropical primates

Electronic supplementary material The online version of this article (doi:10.1007/s10329-010-0226-2) contains supplementary material, which is available to authorized users.

F. de Mello Martins (✉)
Departamento de Zoologia, Instituto de Biociências, Universidade de São Paulo, Rua do Matão, Travessa 14, número 101, São Paulo, SP CEP 05508-900, Brazil
e-mail: felipemartins1305@usp.br

C. Gifalli-Iughetti · C. P. Koiffman
Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade de São Paulo, São Paulo, Brazil

E. E. Harris
Department of Biological Sciences and Geology, Queensborough Community College, City University of New York, New York, USA

Introduction

Paleopalynological evidence suggests that Neotropical forested areas have been very dynamic. Although the Atlantic and the Amazon forests were connected in the past (Oliveira-Filho and Ratter 1995; Vivo 1997), they became separated as increasing aridity in the tertiary period triggered the development of a belt of xeromorphic formations between them (Bigarella et al. 1975). There seemed to be a predominance of arboreal vegetation during most of the Pleistocene, with Amazon and Atlantic forest tree species in areas that today lie in the semi-arid Caatinga formation that separates these two biomes (Ledru 1993; de Oliveira et al. 1999). These climatic fluctuations and associated vegetation changes are very likely to have been important in shaping the patterns of distribution and diversification of the forest-associated fauna.

The genus *Alouatta*, which comprises the Neotropical howler monkeys, is the most widely distributed of all New World monkeys. The genus extends from southern Mexico to northern Argentina, from sea level to over 2300 m, and in wet evergreen to highly seasonal semi-deciduous habitats (Crockett and Eisenberg 1987; Crockett 1998). According to Groves (2001) there are ten species of howler

monkey, with eight of these species having distributions within South America. Species occur in tropical and subtropical environments but are absent from the Andes cordillera and the dry diagonal of savannah-like and xeric formations of Brazil. Because most of the species occur in fragments of forest (Crockett 1998), these monkeys seem to have a high tolerance for habitat disturbance; thus, only drastic environmental disturbances are likely to cause howler monkeys to disappear from an area. As a result, allopatric speciation would presumably take place only across strong barriers to dispersal.

In Brazil there are three species of howler monkey separated by a savannah-like formation (the Cerrado): *Alouatta caraya* (the Black howler), *A. guariba* (the Brown howler) and *A. belzebul* (the Red-handed howler) (Fig. 1). *A. caraya* occurs west of the Paraná River in the wet areas of northeast Argentina into Bolivia, Paraguay, and the midwest of Brazil along the Paraguay River. *A. guariba* is distributed entirely within the Atlantic forest, from south of the state of Bahia to the south of Brazil, and is limited by the Paraná River to the west. *A. belzebul* has a disjunct distribution, occupying a large area of the Pará (between the Tapajós and Tocantins rivers) and Maranhão states in the west, but with an isolated or relict population in the Atlantic forest in Pernambuco state in the east. The western and eastern populations of *A. belzebul* are separated by over 1200 km of dry, open formations (Hill 1962).

Most molecular phylogenies are in agreement concerning phylogenetic relationships among the three species (*A. caraya, A. belzebul* and *A. guariba*). *A. belzebul* and *A. guariba* are hypothesized to be most closely related and *A. caraya* is believed to occupy a sister group position to this clade. These results are supported by analyses of mtDNA sequences (Bonvicino et al. 2001; Cortés-Ortiz et al. 2003; Villalobos et al. 2004) and analyses of sequences of two nuclear genes, γ^1-globin pseudogene (Meireles et al. 1999) and calmodulin (Cortés-Ortiz et al. 2003). In contrast, a phylogenetic analysis of chromosomal morphology indicated that *A. caraya* and *A. belzebul* shared synapomorphic traits to the exclusion of *A. guariba*, possibly pointing to a sister group relationship between *A. caraya* and *A. belzebul* (de Oliveira et al. 2002).

Despite the disjunct distribution of *A. belzebul*, the single phylogeographic study of this species based on cytochrome *b* (cyt *b*) sequences could not detect geographic structure (Nascimento et al. 2005). In *A. guariba*, which today has a continuous distribution within the Atlantic Forest, Harris et al. (2005) described two distinct

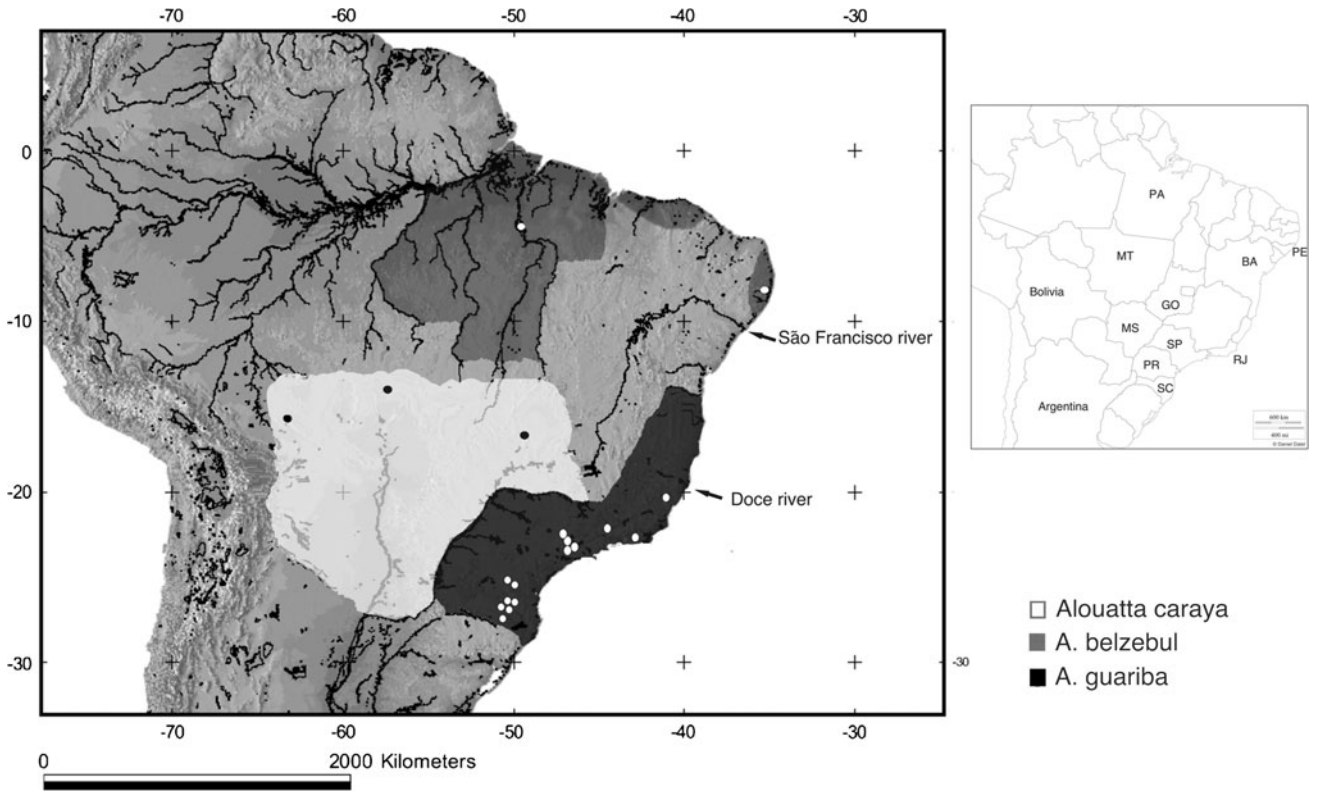

Fig. 1 Map showing the distribution of the three species studied and the specimen sampling locations (*filled circles*). In detail, political map of South America indicating the Brazilian states mentioned in the text: Espírito Santo (*ES*), Goiás (*GO*), Mato Grosso (*MT*), Pará (*PA*), Paraná (*PR*), Pernambuco (*PE*), Rio de Janeiro (*RJ*), São Paulo (*SP*), and Santa Catarina (*SC*)

mitochondrial lineages representing northern and southern ranges of this species with a contact zone in the state of São Paulo, near the Tropic of Capricorn. According to the author, this result could be related to contraction and expansion of the Atlantic Coastal Forest because of climate changes. A phylogeographic analysis of *A. caraya* with limited geographical sampling characterized the Brazilian specimens as a monophyletic group but also found evidence of a dynamic demographic history with possible population expansion within central Brazil (Nascimento et al. 2007a, b).

In recent years, coalescent approaches have become fundamental in phylogeographic and population genetic analysis (Avise 2009). A coalescent approach is a retrospective population genetic model developed within the last 20 years that is based on probability, and traces gene copies from different individuals within and between species back to their single ancestral gene copy (Nordborg 2001; Wakeley 2008). Coalescent methods enable estimation of multiple population parameters simultaneously with reduced variance in the estimates (Rannala and Yang 2003). These parameters enable the testing of hypotheses concerning the species' demographic history and can implement formal statistical tests with the use of simulations. Studies on Neotropical monkeys so far have used only classic phylogenetic, population genetics and divergence-based molecular-clock methods for historical inference.

Coalescent methods bring elements that are often ignored in the other analyses. Molecular-clock-based methods calculate time of divergence between the DNA sequences that form the clades, not the time of divergence between the biological entities (species/populations). The most recent common ancestor (MRCA) of the DNA sequences is likely to be present in the ancestral population that gave origin to the extant species and is therefore older than the species or populations (Edwards and Beerli 2000). This means there is a time lag between the speciation event and the MRCA of a particular gene. For deep-level phylogenies, this time lag is only a small fraction of the total divergence time and can therefore be ignored. For recent events, for example Pleistocene vicariant events, this time lag can represent a substantial fraction of the estimated time of divergence. It is necessary to discriminate between substitutions that have accumulated before the species divergence (ancestral polymorphism) and those after it (Takahata and Satta 1997). Coalescent methods account for this by implementing likelihood functions that discriminate between these two classes of substitutions based on the observed nucleotide variation within species/populations (Rannala and Yang 2003). Therefore, coalescent methods are recommended for estimating the timing of recent events of speciation/population differentiation.

The objective of this research was to use published and newly generated sequences of a mitochondrial marker (cyt *b*) and coalescent-based methodology to:

1. estimate times of divergence of *A. guariba*, *A. belzebul* and *A. caraya*;
2. estimate the time of divergence of the two lineages within the species *A. guariba*;
3. compare new coalescent-based estimates with previous estimates; and
4. use historical demography to investigate the processes responsible for the patterns observed.

Results are discussed in the context of recent findings concerning the dynamics of forest cover within the Atlantic and Amazonian regions where the three howler species are distributed, and in relation to new phylogeographic findings from other animal species with similar distributions.

Methods

Sampling, DNA extraction and sequencing

We used cyt *b* sequences of *A. guariba* generated for this study, and cyt *b* sequences previously published for the three species. The total sequences used and their respective GenBank accession numbers are given in the supplementary material. The 68 sequences from *A. belzebul* were collected by Nascimento et al. (2005, 2007a). These comprise 62 sequences from individuals from the left bank of the Tocantins River where today there is a lake, formed because of the construction of the Tucuruí hydroelectric dam. We also used seven sequences from the small Atlantic forest population of *A. belzebul* (Fig. 1). For *A. caraya*, we analyzed 42 sequences originally collected by Nascimento et al. (2005, 2007b) from three main localities: Chapada dos Guimarães, Mato Grosso state, Brazil and Serra da Mesa, Goiás state, Brazil and one locality in Bolivia (Fig. 1).

For *A. guariba* we used a dataset of sequences from the cyt *b* gene from 38 individuals from three Brazilian states—Rio de Janeiro, São Paulo, and Santa Catarina—that occupy a north-to-south relationship along the Atlantic Coast. Half of the 38 sequences are new to this study and the geographic details of these sequences are given in the supplementary material. Methods for DNA extraction, amplification and sequencing for *A. guariba* samples are described by Harris et al. (2005). Our coalescent analyses also incorporated 15 sequences published previously by Harris et al. (2005) and geographic details of these samples are described in that paper. In addition, we incorporated four sequences of *A. guariba* published by Bonvicino et al. (2001) and Cortés-Ortiz et al. (2003) that are derived from

individuals from São Paulo and Rio de Janeiro states in Brazil. All samples of *A. guariba* we analyze in this paper are presumably attributable to the southern subspecies of *A. guariba* known as *A. g. clamitans* (with *A. g. guariba* being the northern subspecies). Kinzey (1982) and Rylands et al. (1988, 1996) have hypothesized locations of the geographic boundaries between subspecies within *A. guariba*.

Samples of *A. g. guariba* from São Paulo were collected under the auspices of the Brazilian govenmental organizations DEPAVE (Departamento de Parques e Áreas Verdes do Estado de São Paulo) and CEMAS (Centro de Estudo e Manejo de Animais Silvestres, Instituto Florestal, Fundação Florestal, São Paulo). All samples from Santa Catarina were supplied by Projeto Bugio, FURB (Universidade Regional de Blumenau) by its director, Dr Zelinda Hirano Braga. All extracted DNA samples are maintained in the laboratory of one of the authors (CPK) in the Departamento de Biologia, Universidade de São Paulo, Brazil. As outgroup sequences for analyses, from GenBank we downloaded cyt *b* sequences from the closely related genera, *Ateles* and *Brachyteles* (Steiper and Ruvolo 2003). Sequences were aligned by eye using Se-Al (Rambaut 2000).

Phylogenetic and coalescent analyses

We used the software ModelTest (Posada and Crandall 1998) to select the best-fitting model of molecular evolution. The software selected the HKY model (Hasegawa et al. 1985) with a gamma shape parameter of 0.1819 and a Ti/Tv ratio of 11.77. We used this model to implement maximum likelihood (ML) phylogenetic analysis using the software Garli (Zwickl 2006). Bootstrap values for clades (Felsenstein 1985) were estimated using 1000 replicates. Additionally, we used BEAST (Drummond and Rambaut 2007) for Bayesian inference (BI) of the phylogenetic relationships between species of *Alouatta*.

The first step in obtaining divergence times consisted of calculating a mutation rate for the group. We used the nucleotide divergence between the ancestor of *Ateles* and *Brachyteles* with *Alouatta* as the calibration point. The date for this split was set to 15 million years ago (MYA), a date in accordance with several published papers using different markers (Goodman et al. 1998; Schneider et al. 2001; Cortés-Ortiz et al. 2003; Nascimento et al. 2005). We used the HKY model (selected by ModelTest) to calculate nucleotide divergence between the sequences used in this study and cyt *b* sequences of *Ateles* downloaded from GenBank. The resulting mutation rate was 5.5×10^{-9}, slightly higher than that calculated by Nascimento et al. (2005) using uncorrected distances.

We divided coalescent-based analyses into two categories: interspecific and intraspecific. For interspecific analyses, we used the software MCMCcoal (Rannala and Yang 2003). This method is recommended for calculating divergence times between closely related species. This software implements a Bayesian Markov Chain Monte Carlo algorithm for calculating population sizes ($\theta = 4N_e\mu$ parameter) and divergence times (τ) between closely related species using a coalescent model that assumes no gene flow. The software implements a Jukes-Cantor model of sequence evolution (Jukes and Cantor 1969).

For calculating the time of separation between the two major Atlantic forest haplogroups found to exist within the species *A. guariba*, we used the software IM (Hey and Nielsen 2004). The population parameters estimated were: time of separation between populations (t), θ of each population (north and south) and the ancestral population, and two migration rates (m_1 and m_2). This analysis was implemented using a finite-sites model (HKY, identical with the model obtained by ModelTest). We used 10^5 cycles as burn-in. All runs were carried over with over 10^{10} chains after burn-in, to ensure proper smallest effective sample sizes (ESS) for the parameters and stable parameter trendlines. Multiple runs were carried out for each dataset using distinct random seeds to check for convergence of the results. We also compared the log-likelihoods of models that allow historical gene flow ($m \neq 0$) with models that do not allow gene flow ($m_1 = m_2 = 0$), following Hey and Nielsen (2007). As a complimentary analysis, we calculated times since divergence using a non-coalescent approach developed by Takahata and Nei (1985) based on the net-nucleotide divergence.

Additional analyses, including F_{st} calculations, the neutrality tests Tajima's D (Tajima 1989) and Fu's F (Fu 1997), and estimations of time since expansion (Rogers and Harpending 1992), were carried out using the software Arlequin (Schneider et al. 2000). The neutral mutation model makes several assumptions including a panmictic population, the absence of selection and constant population effective size (Kimura 1968). Neutrality tests are capable of determining if the observed dataset is significantly different from the expected pattern under a model of mutation–drift equilibrium. When the marker is neutral (i.e. has not experienced selection within the evolutionary time frame of interest), statistically significant values from neutrality tests indicate a violation of the neutral mutational model caused by demographic processes. Populations that experienced a recent demographic expansion from an ancestral population with small effective size are expected to show significant negative values for the tests implemented (Lessa et al. 2003). A significant departure from neutrality also affects estimation of divergence times, which is likely to bring the coalescent event closer to the bottleneck event and losing information on demographic events before the bottleneck (Wakeley 2008).

Results

The results from our phylogenetic analyses are presented in Fig. 2. The phylogenetic tree topologies resulting from maximum likelihood and Bayesian Inference (using BEAST) analyses were identical. Thirteen cyt *b* haplotypes were found for *A. caraya*, 10 for *A. guariba* and 24 for *A. belzebul*. The two species *A. guariba* and *A. belzebul* were found to form a monophyletic clade, with *A. caraya* occupying a sister-group position relative to this clade. The brown howler monkey, *A. guariba*, was found to have two very distinct mitochondrial lineages: one representing the northern end of the sampling distribution, and one representing the south (Fig. 2), a result consistent with the findings of Harris et al. (2005). These two lineages meet in a contact zone in the São Paulo state, where a presumably interbreeding population results in distinct northern and southern cyt *b* haplotypes. The average sequence divergence between these lineages is 1.3% (maximum is 1.95%), a value greater than the intraspecific divergence within either of the other two species of *Alouatta.* For instance, the average sequence divergence is 0.3% (maximum is 0.7%) in *A. caraya* and 0.6% (maximum is 1.2%) in *A. belzebul*.

The single haplotype of *A. belzebul* described for the Atlantic Forest individuals (AbCRB2748; Fig. 2) is contained fully within the clades of *A. belzebul* haplotypes from the Amazon, i.e., no geographic structure is detected in the cyt *b* marker between Atlantic Forest and Amazonian individuals. Also, no identifiable geographic structure was detected for individuals of *A. caraya* sampled from Brazil by Nascimento et al. (2005, 2007a, b). As can be observed from Fig. 2, the individuals of *A. caraya* from Chapada dos Guimarães in Mato Grosso state, Brazil, form a

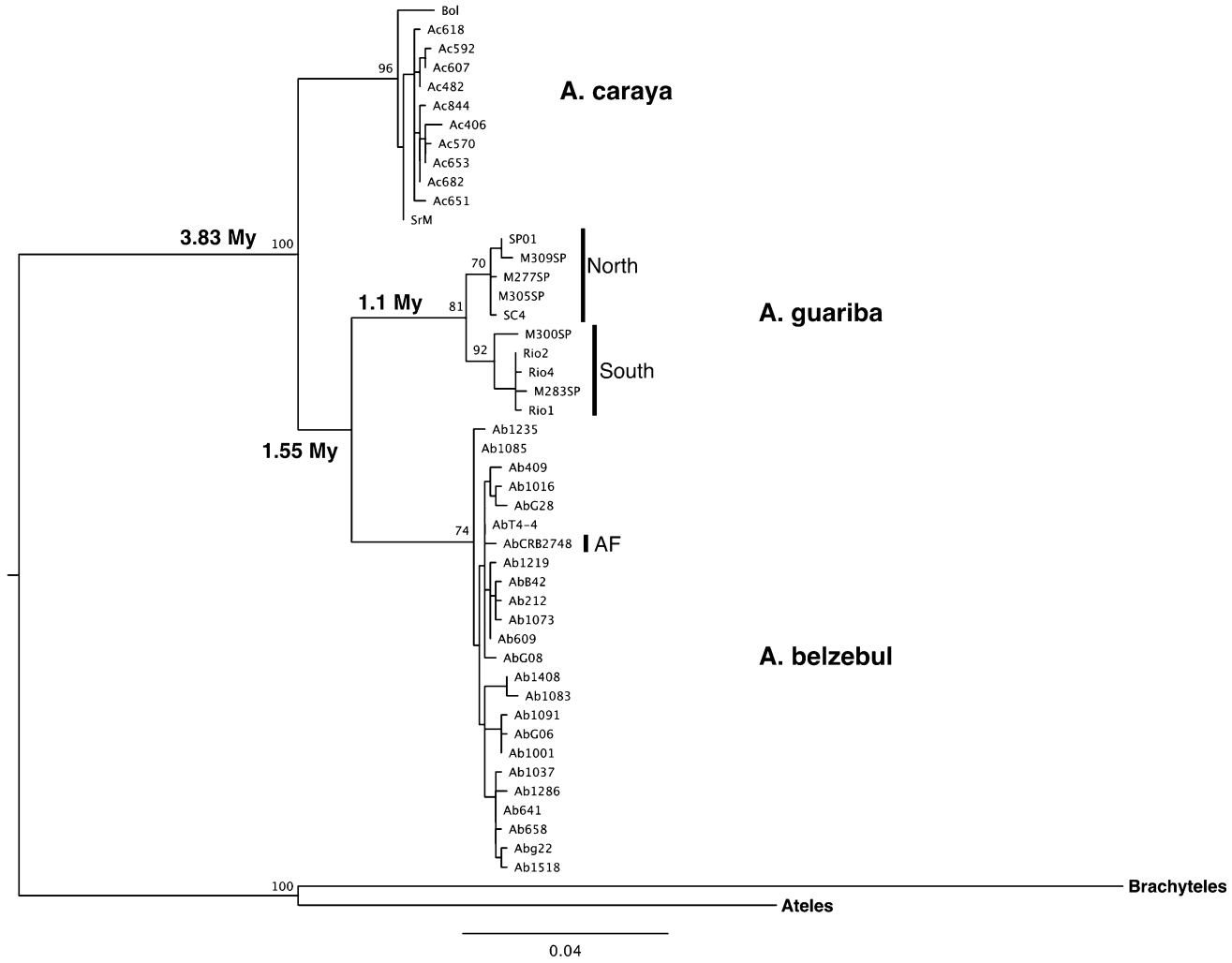

Fig. 2 ML phylogenetic tree of the haplotypes used in this study. *Vertical bars* highlight particular intraspecific lineages (north vs. south for *A. guariba* and the Atlantic forest haplotype of *A. belzebul*). *Values above nodes* indicate ML bootstrap support. *Numbers in bold* above the nodes indicate coalescent estimates of time of separation

Table 1 Summary of divergence time estimates

Pairwise comparison	Time estimate (MYA)	95% confidence interval (MYA)	Previous estimates (MYA)
A. belzebul × *A. guariba*	1.55	0.93–2.45	4
(*A. belzebul*, *A. guariba*) × *A. caraya*	3.83	2.9–4.73	5.1
A. guariba: N × S	1.1–1.2	0.38–2	0.4–0.5
A. belzebul: expansion	0.15	0.06–0.2	–

Table 2 Summary of population genetics analyses

The values of the neutrality tests, as well as their p values (when significant) are shown

	A. guariba		*A. belzebul*	*A. caraya*
	North (RJ)	South (SC)		
Tajima's D	n.s.	n.s.	$D = -1.37$ $p = 0.059$	n.s.
Fu's F	$F = -2.29$ $p = 0.007$	n.s.	$F = -13.22$ $p = 0.001$	$F = -10.77$ $p = 0.0000$

monophyletic clade with the haplotypes from Goiás state in Brazil. However, the haplotype carried by individuals of *A. caraya* from Bolivia is basal to the *A. caraya* haplotypes from Brazil.

The divergence time estimates between species using MCMCcoal are presented in Table 1. The divergence between *A. caraya* and the *A. guariba/belzebul* ancestor is estimated at 3.83 MYA whereas the split between *A. guariba* and *A. belzebul* is estimated at 1.55 MYA.

For the two haplogroups of *A. guariba*, the IM runs with migration rate estimates yielded a flat distribution of divergence times. When runs were carried out with a migration rate set to 0, a time of separation of 1.2 MYA was obtained. Comparison of log-likelihoods shows that the difference in likelihood between the two models is not statistically significant, i.e., it is an equal fit to assume 0 migration. Therefore, the estimate of 1.2 MYA for time of separation is the best coalescent estimate for the data. We have also estimated times of divergence between the *A. guariba* haplogroups using the molecular divergence methodology of Takahata and Nei (1985) and obtained a time of divergence of 1.1 MYA between north and south, a result similar to that obtained using the coalescent method.

Additional results of population genetic analyses are summarized in Tables 2 and 3. To estimate levels of population differentiation, we calculated F_{st} values between the two populations of *A. belzebul* and between the three sampled populations of *A. guariba*, specifically the northern *A. guariba* population (from Rio de Janeiro), the southern *A. guariba* population (from Santa Catarina), and the intermediate *A. guariba* population (from São Paulo), where the two clades overlap geographically (see Fig. 3 for details). For *A. belzebul*, comparisons of the Tucuruí population in the Amazon (Pará state) with the Atlantic Forest population yielded a statistically significant value of F_{st} ($F_{st} = 0.27$; $p = 0.000$). The F_{st} values for the *A. guariba* populations were exceptionally large, especially between

Table 3 Pairwise F_{st} values between *A. guariba* populations

	Northern	Intermediate	Southern
Northern	–		
Intermediate	0.41905 ($p = 0.000$)	–	
Southern	0.82953 ($p = 0.000$)	0.33943 ($p = 0.000$)	–

northern and southern clades, which are close to complete fixation ($F_{st} = 0.82953$). All F_{st} values between populations were statistically significant (Table 3).

We used neutrality tests based on the frequency spectrum of mutations in the sampled sequences and detected no significant departure from neutrality in *A. guariba*, except for a statistically significant Fu's F value for the Rio de Janeiro (northern) population ($N = 5$). The Tucuruí population of *A. belzebul* has a marginally significant negative Tajima's D ($p = 0.059$) and a significant negative value of Fu's F ($p = 0.001$). The time since expansion (τ) was estimated at 148 KYA. A significant negative Fu's F value was also obtained for the species *A. caraya*.

Discussion

Phylogenetic results and divergence time estimates

The phylogenetic results presented here are consistent with previous phylogenetic findings that postulate that *A. guariba* and *A. belzebul* are each other's closest relatives with *A. caraya* as their sister clade (Meireles et al., 1999; Bonvicino et al. 2001; Cortés-Ortiz et al. 2003; Villalobos et al. 2004). When intraspecific lineages are considered, we have found very little or no geographic structure for cyt *b* within either *A. caraya* or *A. belzebul,* although it should be remarked that Bolivian samples of *A. caraya* do fall outside the clade of *A. caraya* from Goiás and Mato Grosso

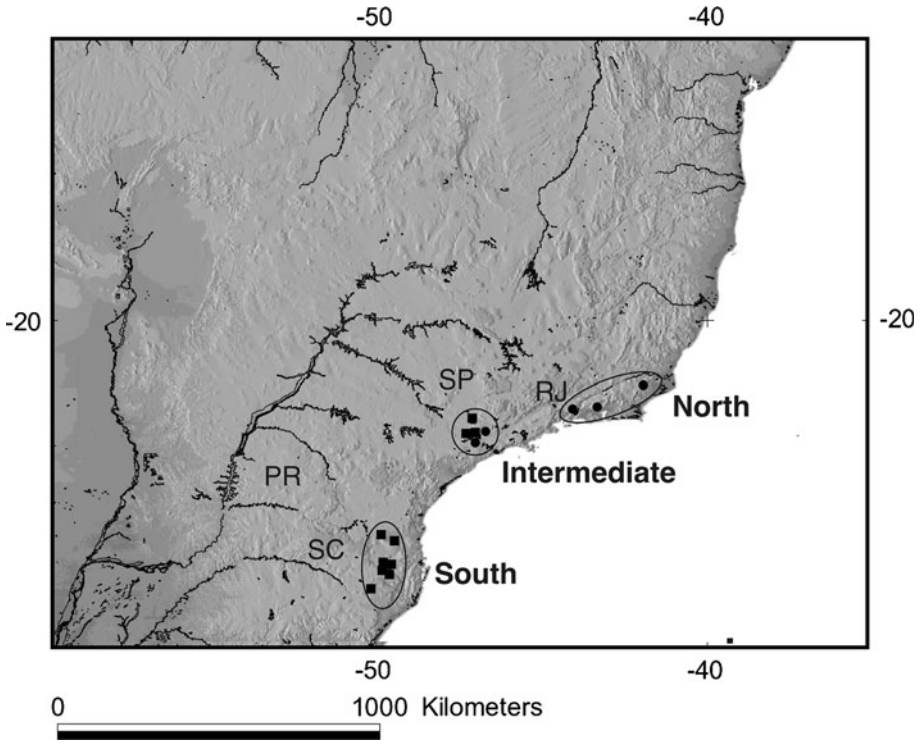

Fig. 3 Map showing the distribution of the north (*circles*) and south (*rectangles*) haplogroups in the coastal area in southeast Brazil. The two lineages can be found in sympatry in São Paulo state. *Circles* and *rectangles* represent sampled locations (for details, see supplementary material). Abbreviations are the same as for Fig. 1

states in Brazil. In contrast, *A. guariba* ha\s two markedly divergent mitochondrial haplogroups (or lineages) within the Atlantic Coastal Forest, with one haplogroup found in the northern state of Rio de Janeiro and the other in the state of Santa Catarina in the south. There seems to be a well-established and geographically intermediate contact zone between these two haplogroups in the state of São Paulo where divergent haplotypes are present within the same population.

The young (or recent) divergence dates estimated between *Alouatta* species in this study are important because they imply that Pleistocene (2.6–0.01 MYA) events affected the diversification of this group. All previous studies attributed the diversification of these species to Pliocene (5.3–2.6 MYA) events. It is well known that during the Pleistocene there were drastic climatic fluctuations that altered the distribution of all organisms, including the fragmentation of wet forested areas by dry, open formations (see references below). The debate surrounding the extent of these fluctuations on the Tropical landscape has become a controversial topic. Recent studies based on paleopalynology, paleovegetation, and paleoclimatic data clearly suggest that the Amazonian forest persisted in the central and western portions of the biome, with replacement by semi-deciduous dry forest or savanna happening only near ecotonal boundaries (Mayle et al. 2004; Bush et al. 2007). On the other hand, numerous paleopalynological and sediment studies suggest the Atlantic Forest region became fragmented during the Pleistocene, with the formation of dry open areas (Behling 1999, 2002; Behling and Lichte 1997; Lichte and Behling 1999) or deciduous temperate Brazilian pine forests during this time (Ledru et al. 1996).

Although our estimate of the time of divergence between *A. caraya* and the *A. belzebul/guariba* clade is still within the Pliocene (3.83 MYA), it is much more recent than previous estimates. On the other hand, our estimate of the divergence between *A. belzebul* and *A. guariba* is dated to the Pleistocene (1.55 MYA). The differentiation of these species may be causally related to increasing aridity in central Brazil where today there is found a dry diagonal occupied by open and non-forested formations. Similarly, Pleistocene climatic fluctuations are likely to have been responsible for the north–south population differentiation within *A. guariba* (i.e. the differentiation of the northern and southern haplogroups). Interestingly, the divergence between the mtDNA haplogroups within *A. guariba* is estimated at 1.2 MYA, a time that is only slightly more recent than the estimated divergence between *A. guariba* and *A. belzebul* (1.55 MYA). Thus, only shortly after the divergence between *A. guariba* and *A. belzebul*, it seems that environmental changes within the Atlantic Forest affected the divergence between the *A. guariba* populations. Pleistocene climatic fluctuations were also likely to be responsible for generating the disjunct distribution seen for *A. belzebul*. Given that the only haplotype described for the eastern population of *A. belzebul* (within the Atlantic forest) is fully nested within the clade containing all the

western haplotypes of this species (in the Amazon forest), separation of these regions of forest was probably caused by a much more recent climactic event.

The fact that the divergence time estimates presented here between *Alouatta* species are much more recent than the estimates determined in previous studies deserves further discussion. Cortés-Ortiz et al. (2003) estimated the split between *A. guariba* and *A. belzebul* at 4.0 MYA, and the split between this clade and *A. caraya* at 5.1 MYA. Similarly, Nascimento et al. (2005) estimated the divergence between *A. belzebul* and *A. caraya* at 5.3 MYA, and the MRCA of *A. belzebul* at 4.14 MYA. Both studies implemented relatively simple molecular-clock-based analyses. A similar difference between coalescent and molecular-clock results (i.e. with estimates from coalescence analyses being more recent) was also observed when calculating time of divergence between hominoid lineages using the same coalescent approach used in this study (Rannala and Yang 2003; Burgess and Yang 2008). According to Rannala and Yang (2003), traditional molecular divergence methods tend to inflate estimates of times of separation and effective population sizes for datasets with an excess of low-frequency variants. All species sampled here show a frequency spectrum of polymorphisms (as measured by Fu's *F* test) that is inconsistent with the neutral-equilibrium model but is skewed towards low frequency variants (Table 2). Besides being more robust to violations of the neutral model, the coalescent method used here gives much smaller variances of the estimates of divergence compared with other methods (Rannala and Yang 2003). Another important feature is that coalescent methods estimate the divergence time between the species or populations while clock-based methods estimate the time of divergence between the sequences. Because the divergence between sequences will always predate the species divergence, clock-based methods can lead to substantial overestimates of species divergence times.

It is important to stress that the results presented here, and the previous estimates discussed above, were made using a single locus. Genealogies based on one single gene may be problematic because each genealogical reconstruction is just one point in the space of all possible genealogies. Lineage sorting may produce trees different from the real history of the population; this means that even if the tree and the demographic parameters were inferred correctly, they do not necessarily correspond to the actual species tree (Pamilo and Nei 1988). Mitochondrial markers are maternally inherited, which could also cause a bias if there is sex biased-dispersal; this, however, does not seem to be true for *Alouatta* (Kinzey 1997). In this context, the results shown here must be taken with some caution. New studies that analyze multiple loci and increase the geographic sampling of the species will help furnish a more complete picture of howler monkey evolution and diversification.

Population structure within *A. guariba* in the Atlantic Forest

The historical latitudinal division of the Atlantic Coastal Forest, with north and south components, has long been recognized in analyses of endemicity in amphibians (Lynch 1979), reptiles (Vanzolini 1988), birds (Bates et al. 1998) and harvestmen (Pinto-da-Rocha et al. 2005). More recently, phylogeographic studies using mtDNA sequences have described a similar north/south population structure in such diverse animals as birds (Cabanne et al. 2007), pit vipers (Grazziotin et al. 2006), non-volant small mammals (Costa 2003), the crab-eating fox (Tchaicka et al. 2007), and bats (Ditchfield 2000; Martins et al. 2007, 2009). Even without sampling areas further north within the Atlantic forest, the pattern described for *A. guariba* is remarkably similar to the patterns described above and demonstrates an intriguing consilience across species. Additionally, all of the above analyses that have estimated times for the onset of population structure for various Atlantic Forest species have yielded Pleistocene divergence times.

The exact geographic demarcation of the northern and southern clades in the Atlantic forest for the various animal groups varies to some degree among the different taxa. It is interesting, however, that the geographical pattern described in this study for *A. guariba* is almost identical with the pattern described previously for the lesser woodcreeper bird, *Xiphorhynchus fuscus* (Cabanne et al. 2007), with two divergent haplogroups meeting and possibly interbreeding in São Paulo state. As a possible explanation of the pattern detected in *A. guariba*, Harris et al. (2005) hypothesized that the different cyt *b* clades detected in Rio de Janeiro and Santa Catarina populations may stem from fragmentation of the Atlantic forest as a result of increasing aridity during the Pleistocene. This fragmentation could have led to distinct forest refuges in which *A. guariba* populations became isolated. The fact that we detect distinct cyt *b* haplotypes in São Paulo today—haplotypes that are otherwise found either in Santa Catarina or Rio de Janeiro—may result from recent expansion of the Atlantic forests that enabled individuals bearing these distinct cyt *b* haplotypes to come into contact.

Interestingly, analyses of chromosomal diversity in *A. guariba* have also found a north/south structuring of chromosomal variation. For instance, individuals from the states of Rio de Janeiro and São Paulo have a diploid number (2 N) of 49 (♂♂) or 50 (♀♀), whereas individuals from the more southern states of Paraná and Santa Catarina have a diploid number of 45 (♂♂) or 46 (♀♀) (de Oliveira

et al. 1995, 2002; de Oliveira 2001). In addition, the north/south chromosomal differentiation extends to several Robertsonian rearrangements, pericentric inversions, and chromosomal translocations (de Oliveira et al. 1995, 2002; de Oliveira 2001). These differences may indicate that the two groups are reproductively isolated from each other and that the southern subspecies of *A. guariba* (*A. g. guariba*) in fact constitutes two different subspecies or potentially different species (Harris et al. 2005). To help elucidate these questions analyses of genetic variation at multiple autosomal genetic markers is required. The interpretation of these analyses will be aided by field or laboratory studies that examine possible interbreeding between the populations.

Population structure within *A. belzebul*

The existence of a single mitochondrial haplotype in the eastern population of *A. belzebul* (in the Atlantic Coastal Forest population) is probably because of the very small effective population size, which, in turn, could be the result of small census sizes, very restricted range, and high endogamy. Given the fact that only a single mitochondrial lineage was found in this location, it is not surprising that the F_{st} value between the Atlantic Forest and Amazonian populations of *A. belzebul* was relatively high and significant (0.27). What is surprising is the result of the neutrality tests, pointing towards a recent population expansion for the Amazon population during the Pleistocene, *circa* 148 KYA. This is an unexpected result, because this pattern is expected for organisms susceptible to refugia-type historical fragmentation (Lessa et al. 2003; Moritz et al. 2009). Maybe the fact that *A. belzebul* occurs near the Amazon's eastern and southern ends made them susceptible to possible regression in the forest cover during the Pleistocene, especially in the south, because the eastern boundary is the Tocantins River. This regression could have caused the population to decrease substantially, resulting in the demographic expansion we detected.

Demographic parameters estimated give mixed results supporting refugia vicariance in the Atlantic Forest. There is no significant departure from neutrality in the *A. guariba* clades, as would be expected under this model. Therefore, there are still significant areas for improvement in the analyses of *Alouatta* diversification, including more extensive geographic sampling of the species, and use of *multiloci* data that will enable better resolution of historical demography and divergence time estimates.

Acknowledgments The authors would like to thank Rodrigo S. dos Santos for critical reviews. Thanks to Marcus V. Domingues, Leon Franciatto, Paulo Noffs, and Jose Grau for the figures. The authors wish to thank Drs Zelinda Hirano Braga and Julio César de Souza Júnior of FURB (Universidade Regional de Blumenau) for samples of Alouatta. We also would like to thank two anonymous reviewers and an anonymous associate editor for detailed and helpful comments. Part of this work was carried out by using the resources of the Computational Biology Service Unit from Cornell University which is partially funded by Microsoft Corporation. This work became possible through financial aid from CAPES PRODOC grant number 0072/087 (to FMM), from CEPED-FAPESP (to CPK), and CUNY RF Awards 67468-00-36 and 68625-0037 (to EEH). Sampling from live animals was carried out in accordance with Brazilian animal care regulations and laws, and also complied with ethical standards for the treatment of animals in the guidelines of the Primate Society of Japan.

References

Avise JC (2009) Phylogeography: retrospect and prospect. J Biogeogr 36:3–15

Bates JM, Hackett SJ, Cracraft J (1998) Area-relationships in the Neotropical lowlands: a hypotheses based on raw distribution of passerine birds. J Biogeogr 25:783–793

Behling H (1999) Late quaternary vegetational and climatic changes in Brazil. Rev Paleobot Palynol 99:143–156

Behling H (2002) South and southeast Brazilian grasslands during Late Quaternary times: a synthesis. Palaeogeogr Palaeoclimatol Palaeoecol 177:19–27

Behling H, Lichte M (1997) Evidence of dry and cold climatic conditions at glacial times in tropical southeastern Brazil. Quat Res 48:348–358

Bigarella JJ, Andrade-Lima D, Riehs PJ (1975) Consideracões a respeito das mudanças paleoambientais na distribuição de algumas espécies vegetais e animais no Brasil. An Acad Bras Cienc 47:411–464

Bonvicino CR, Lemos B, Seuánez HN (2001) Molecular phylogenetics of howler monkeys (Alouatta, Platyrrhini). A comparison with karyotypic data. Chromosoma 110:241–246

Burgess R, Yang Z (2008) Estimation of hominoid ancestral population sizes under bayesian coalescent models incorporating mutation rate variation and sequencing errors. Mol Biol Evol 25:1979–1994

Bush MB, Gosling WD, Colinvaux PA (2007) Climate change in the lowlands of the Amazon basin. In: Bush MB, Flenley JR (eds) Tropical rainforest responses to climatic change. Springer-Praxis, Berlin, pp 55–76

Cabanne GS, Santos FR, Miyaki CY (2007) Phylogeography of *Xiphorhynchus fuscus* (Passeriformes, Dendrocolaptidae): vicariance and recent demographic expansion in southern Atlantic forest. Biol J Linn Soc 91:73–84

Cortés-Ortiz L, Bermingham E, Rico C, Rodríguez-Luna E, Sampaio I, García-Ruiz M (2003) Molecular systematics and biogeography of the neotropical monkey genus, *Alouatta*. Mol Phylogenet Evol 26:64–81

Costa LP (2003) The historical bridge between the Amazon and the Atlantic Forest of Brazil: a study of molecular phylogeography with small mammals. J Biogeog 30:71–86

Crockett CM (1998) Conservation biology of the genus *Alouatta*. Int J Primatol 19:549–578

Crockett CM, Eisenberg JF (1987) Howlers: variations in group size and demography. In: Smuts BB, Cheney DL, Seyfarth RM, Wrangham RW, Struhsaker TT (eds) Primate societies. University of Chicago Press, Chicago, pp 54–68

de Oliveira EH (2001) Filogenia da subfamília Atelinae (Primates: Platyrrhini): analises comparativas por pintura cromossômica multicor. Doctoral thesis, Setor de Ciencias Biológicas, Universidad Federal do Paraná, Curitiba

de Oliveira EH, de Lima MMC, Sbalqueiro IJ (1995) Chromosomal variation in *Alouatta fusca*. Neotrop Primates 3:181–182

de Oliveira PE, Franca-Barreto AM, Suguio K (1999) Late Pleistocene/Holocene climatic and vegetational history of the Brazilian Caatinga: the fossil dunes of the middle Sao Francisco River. Palaeogeogr Palaeoclimatol Palaeoecol 152:319–337

de Oliveira EH, Neusser M, Figueiredo WB, Nagamachi C, Pieczarka JC, Sbalqueiro IJ, Wienberg J, Muller S (2002) The phylogeny of howler monkeys (*Alouatta*, Platyrrhini): reconstruction by multicolor cross-species chromosome painting. Chrom Res 10:669–683

Ditchfield AD (2000) The comparative phylogeography of Neotropical mammals: patterns of intraespecific mitochondrial DNA variation among bats contrasted to nonvolant small mammals. Mol Ecol 9:1307–1318

Drummond AJ, Rambaut A (2007) BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol Biol 7:214–221

Edwards SV, Beerli P (2000) Perspective: gene divergence, population divergence and the variance in coalescence time in phylogeographic studies. Evolution 54:1839–1854

Felsenstein J (1985) Confidence limits on phylogeny: using the bootstrap. Evolution 39:783–791

Fu Y-X (1997) Statistical methods, tests of neutrality against population growth, hitchhiking and background selection. Genetics 147:915–925

Goodman M, Porter CA, Czelusniak J, Page SL, Schneider H, Shoshani J, Groves CP (1998) Toward a phylogenetic classification of primates based on DNA evidence complemented by fossil evidence. Mol Phylogenet Evol 9:585–598

Grazziotin FG, Monzel M, Echeverrigarauy S, Bonatto S (2006) Phylogeography of the *Bothrops jararaca* complex (Serpentes: Viperidae): past fragmentation and island colonization in the Brazilian Atlantic Forest. Mol Ecol 15:3969–3982

Groves CP (2001) Primate taxonomy. Smithsonian Institution Press, Washington, DC

Harris EE, Gifalli-Iughetti C, Braga ZH, Koiffman CP (2005) Cytochrome *b* sequences show subdivision between populations of the brown howler monkey (*Alouatta guariba*) from Rio de Janeiro and Santa Catarina, Brazil. Neotrop Primates 13:16–21

Hasegawa M, Kishino H, Iano T (1985) Dating of the human–ape splitting by a molecular clock of mitochondrial DNA. J Mol Evol 21:160–174

Hey J, Nielsen R (2004) Multilocus methods for estimating populations sizes, migration rates and divergence times, with application to the divergence of *Drosophila pseudoobscura* and *D. persimilis*. Genetics 167:747–760

Hey J, Nielsen R (2007) Integration within the Felsenstein equation for improved Markov chain Monte Carlo methods in population genetics. PNAS 104:2785–2790

Hill WCO (1962) Primates comparative anatomy and taxonomy. V. Cebidae. Part. B. Edinburgh University Press, Edinburgh

Jukes TH, Cantor CR (1969) Evolution of protein molecules. In: Munro HM (ed) Mammalian protein metabolism. Academic Press, New York, pp 21–132

Kimura M (1968) Evolutionary rate at the molecular level. Nature 217:624–626

Kinzey WG (1982) Distribution of primates and forest refuges. In: Prance GT (ed) Biological diversification in the tropics. Columbia University Press, New York, pp 455–482

Kinzey WG (1997) New world primates: ecology. evolution, and behavior (Foundations of Human Behavior Series). Aldine de Gruyter, New York

Ledru M-P (1993) Late quaternary environmental and climatic changes in central Brazil. Quat Res 39:90–98

Ledru M-P, Braga PIS, Soubiés F, Fournier M, Martin L, Suguio K, Turcq B (1996) The last 50,000 years in the Neotropics (Southern Brazil): evolution of vegetation and climate. Palaeogeogr Palaeoclimatol Palaeoecol 123:239–257

Lessa EP, Cook JA, Patton JL (2003) Genetic footprints of demographic expansion in North America, but not Amazonia, during the late quaternary. PNAS 100:10331–10334

Lichte M, Behling H (1999) Dry and cold climatic conditions in the formation of the present landscape in Southeastern Brazil: an interdisciplinary approach to a controversial topic. Zeitschrift fuer Geomorphologie 43:341–358

Lynch JD (1979) The amphibians of the lowland tropical forests. In: Duellman WE (ed) The South American Herpetofauna: its origin, evolution and dispersal. University of Kansas Museum of Natural History, Lawrence, pp 189–216

Martins FM, Meyer D, Ditchfield AD, Morgante JM (2007) Mitochondrial DNA phylogeography reveals marked population structure in the common vampire bat, *Desmodus rotundus* (Phyllostomidae). J Zool Syst Evol Res 45:372–378

Martins FM, Templeton AR, Pavan ACO, Kohlbach BC, Morgante JM (2009) Phylogeography of the common vampire bat (*Desmodus rotundus*): marked population structure, Neotropical Pleistocene vicariance and incongruence between nuclear and mtDNA markers. BMC Evol Biol 9:294

Mayle FE, Beerling DJ, Gosling WD, Bush MB (2004) Responses of Amazonian ecosystems to climatic and atmospheric carbon dioxide changes since the last glacial maximum. Phil Trans R Soc B 359:499–514

Meireles CM, Czelusniak J, Schneider MP, Muniz JAPC, Brigido MC, Ferreira HS, Goodman M (1999) Phylogenetic relationships among Brazilian howler monkeys, genus *Alouatta* (Platyrrhini, Atelidae), based on γ^1 -globin pseudogene sequences. Gen Mol Biol 22:337–344

Moritz C, Hoskin CJ, Mackenzie JB, Phillips BL, Tonione M, Silva N, Vanderwal J, Williams SE, Graham CH (2009) Identification and dynamics of a cryptic suture zone in tropical rainforest. Proc R Soc B 276:1235–1244

Nascimento FF, Bonvicino CR, da Silva FCD, Schneider MPC, Seuánez HN (2005) Cytochrome *b* polymorphisms and population structure of two species of *Alouatta* (Primates). Cytogenet Genome Res 108:106–111

Nascimento FF, Bonvicino CR, de Oliveira MM, Schneider MP, Seuánez HN (2007a) Population genetic studies of *Alouatta belzebul* from the Amazonian and Atlantic Forests. Am J Primatol 70:423–431

Nascimento FF, Bonvicino CR, Seuánez HN (2007b) Population genetic studies of *Alouatta caraya* (Alouattinae, Primates): inferences on geographic distribution and ecology. Am J Primatol 69:1093–1104

Nordborg M (2001) Coalescent theory. In: Balding D, Cannings C, Bishop M (eds) Handbook of statistical genetics. Wiley, Sussex, pp 843–877

Oliveira-Filho AT, Ratter JA (1995) A study of the origin of central Brazilian forests by the analysis of plant species distribution patterns. Edinb J Bot 52:141–194

Pamilo P, Nei M (1988) The relationships between gene trees and species trees. Mol Biol Evol 5:568–583

Pinto-da-Rocha R, Bragagnolo C, da Silva MB (2005) Faunistic similarity and historical biogeography of the harvestmen of southern and southeastern Atlantic rainforest of Brazil. J Arach 33:290–299

Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. Bioinformatics 19:817–818

Rambaut A (2000) Se Al. http://evolve.zoo.ox.ac.uk/software/Se-Al/

Rannala B, Yang Z (2003) Bayes estimation of species divergence times and ancestral population sizes using DNA sequences from multiple loci. Genetics 164:1645–1656

Rogers A, Harpending HC (1992) Population growth makes waves in the distribution of pairwise genetic differences. Mol Biol Evol 9:522–569

Rylands AB, Spironelo WR, Tornisielo VL, Sa RL, de Kierulff MCM, Santos IB (1988) Primates of the Rio Jequitinhonha Valley, Minas Gerais, Brazil. Primate Conserv 9:100–109

Rylands AB, Fonseca GAB, da Leite YLR, Mittermeier RAM (1996) Primates of the Atlantic forest: origin, distributions, endemism, and communities. In: Norconk M, Rosenberger A, Garber PA (eds) Adaptive radiations of Neotropical primates. Plenum Press, New York, pp 21–51

Schneider S, Roessli D, Excoffier L (2000) Arlequin ver. 2000: a software for population genetics data analysis. Switzerland: Genetics and Biometry Laboratory, University of Geneva

Schneider H, Canavez FC, Sampaio I, Moreira MAM, Tagliaro CH, Seuánez HN (2001) Can molecular data place each Neotropical monkey in its own branch? Chromosoma 109:515–523

Steiper ME, Ruvolo M (2003) New World monkey phylogeny based on X-linked G6PD DNA sequences. Mol Phylogenet Evol 27:121–130

Tajima F (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123:585–595

Takahata N, Nei M (1985) Gene genealogy and variance of interpopulational nucleotide sequences. Genetics 110:325–344

Takahata N, Satta Y (1997) Evolution of the primate lineage leading to modern humans: phylogenetic and demographic inferences from DNA sequences. PNAS 94:4811–4815

Tchaicka L, Eizirik E, Oliveira TG, Candido JF Jr, Freitas TRO (2007) Phylogeography and population history of the crab-eating fox (*Cerdocyon thous*). Mol Ecol 16:819–838

Vanzolini PE (1988) Distributional patterns of South American lizards. In: Vanzolini PE, Hayer W (eds) Proceedings of a workshop on Neotropical distribution patterns: 12–16 January 1987. Academia Brasileira de Ciências, Rio de Janeiro, pp 317–342

Villalobos F, Valerio AA, Retana AP (2004) A phylogeny of howler monkeys (Cebidae: Alouatta) based on mitochondrial, chromosomal, and morphological data. Rev Biol Trop 52:665–677

Vivo M (1997) Mammalian evidence of historical ecological change in the Caatinga semiarid vegetation of northeastern Brazil. J Comp Biol 2:65–73

Wakeley J (2008) Coalescent theory: an introduction. Roberts and Publishers, Greenwood Village

Zwickl DJ (2006) Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion (dissertation). The University of Texas, Austin. Available from: http://www.molecularevolution.org/si/software/garli/