



# Molecular phylogenetic inference of the howler monkey radiation (Primates: *Alouatta*)

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## Abstract

Howler monkeys (*Alouatta*), comprising between nine and 14 species and ranging from southern Mexico to northern Argentina, are the most widely distributed platyrrhines. Previous phylogenetic studies of howlers have used chromosomal and morphological characters and a limited number of molecular markers; however, branching patterns conflict between studies or remain unresolved. We performed a new phylogenetic analysis of *Alouatta* using both concatenated and coalescent-based species tree approaches based on 14 unlinked non-coding intergenic nuclear regions. Our taxon sampling included five of the seven South American species (*Alouatta caraya*, *Alouatta belzebul*, *Alouatta guariba*, *Alouatta seniculus*, *Alouatta sara*) and the two recognized species from Mesoamerica (*Alouatta pigra*, *Alouatta palliata*). Similarly to previous studies, our phylogenies supported a Mesoamerican clade and a South American clade. For the South American howlers, both methods recovered the Atlantic Forest endemic *A. guariba* as sister to all remaining South American species, albeit with moderate support. Moreover, we found no support for the previously proposed sister relationship between *A. guariba* and *A. belzebul*. For the first time, a clade composed of *A. sara* and *A. caraya* was identified. The relationships among the other South American howlers, however, were not fully supported. Our estimates for divergence times within *Alouatta* are generally older compared to estimates in earlier studies. However, they conform to recent studies proposing a Miocene age for the Isthmus of Panama and for the uplift of the northern Andes. Our results also point to an early genetic isolation of *A. guariba* in the Atlantic Forest, in agreement with the hypothesis of biotic exchange across South American rain forests in the Miocene. Collectively, these findings contribute to a better understanding of the diversification processes among howler monkey species; however, they also suggest that further comprehension of the evolutionary history of the *Alouatta* radiation will rely on broadened taxonomic, geographic, and genomic sampling.

**Keywords** *Alouatta* · Molecular phylogeny · Multi-locus · Systematics · Primates

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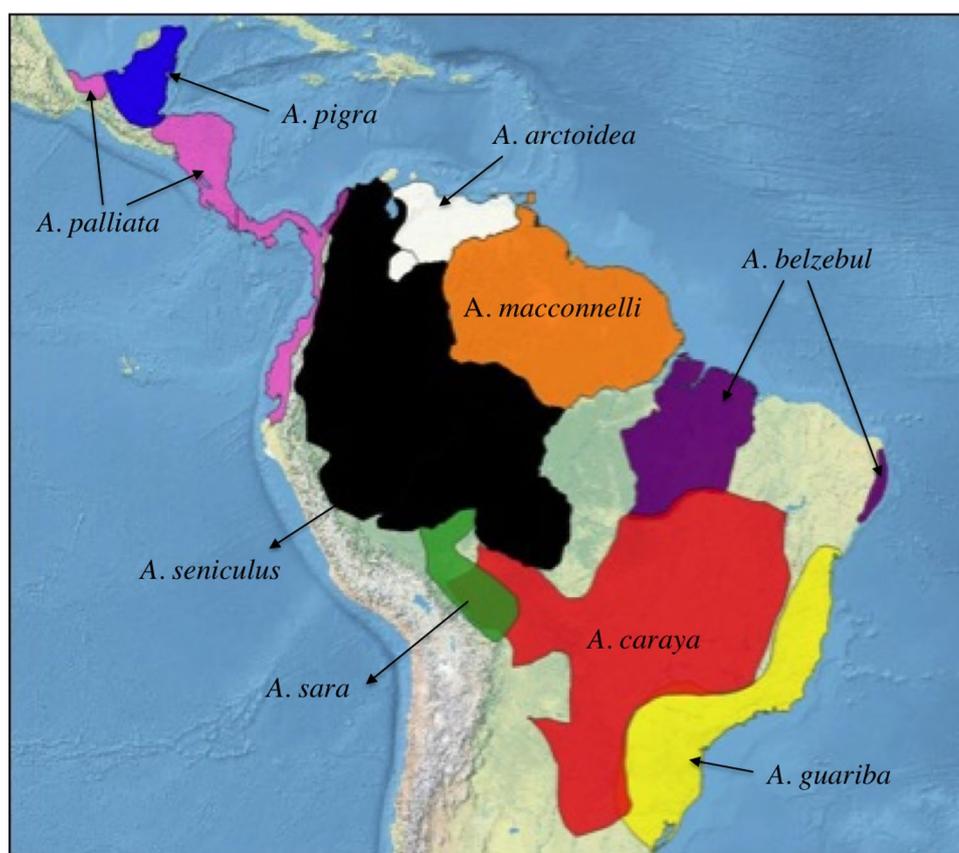
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## Introduction

Howler monkeys (order Primates, family Atelidae, genus *Alouatta*) are the most widely distributed group of platyrrhines, ranging from southern Mexico to northern Argentina. They inhabit a variety of forests—tropical rain forests, flooded forests, and gallery forests—as well as deciduous and semi-deciduous seasonal environments (Crockett and Eisenberg 1986; Zunino et al. 2001a, b). Their diet consists of leaves and fruits, with the proportions of these dependent on the productivity and size of the forest areas inhabited, as well as the density of their groups (see Dias and Rengel-Negrín 2015). Howler monkeys play important roles in seed dispersal by feeding on a wide variety of plant species, and are likely to be the only seed dispersers for certain plant species. Howlers help in forest regeneration and assist in the restoration of degraded habitats that, differently to other large-bodied frugivores, they are able to inhabit (de Moura and McConkey 2007; Arroyo-Rodríguez et al. 2015). Unfortunately, many *Alouatta* species are threatened by habitat loss and extreme habitat alteration (International Union for Conservation of Nature 2019).

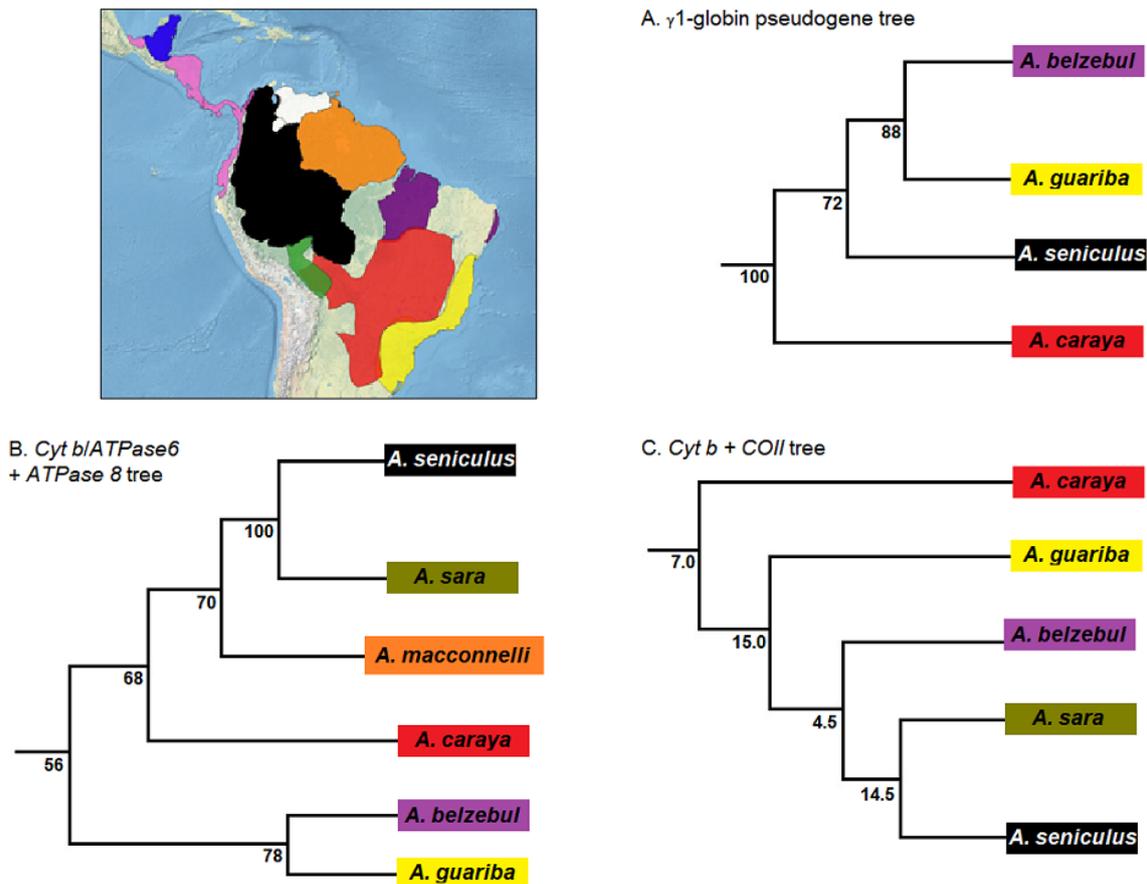
Although several studies used morphometric, molecular or cytogenetics to infer species boundaries within the howlers over the last few years, the number of species within *Alouatta* is still a matter of debate (Hershkovitz 1949; Meireles et al. 1999; de Oliveira et al. 2002; Cortéz-Ortiz et al. 2003). Hill (1962) estimated that the genus *Alouatta* was composed of six species. Decades later, Gregorin (2006) used a large morphological dataset (1286 specimens sampled for skin, fur, cranium and hyoid bone information) to separate the genus into ten species. In the most recent assessment, Cortés-Ortiz et al. (2015) recognized two Mesoamerican species (*Alouatta pigra* and *Alouatta palliata*) and nine South American species (*Alouatta pigra*, *Alouatta palliata*, *Alouatta seniculus*, *Alouatta macconnelli*, *Alouatta caraya*, *Alouatta belzebul*, *Alouatta arctoidea*, *Alouatta guariba*, and *Alouatta sara*; see Fig. 1 for their known distributions). Such taxonomic studies of *Alouatta* have focused largely on diagnosing the overall number of howler species, drawing on anatomical characters (Hershkovitz 1949) and aspects of pelage patterns and coloring (Gregorin 2006). Despite often incorporating genetic information, those studies aimed to resolve the instability of the group's alpha taxonomy, with limited focus on the phylogenetic relationships within *Alouatta*.

**Fig. 1** Geographic distributions of currently recognized species of howler monkeys, genus *Alouatta*: *Alouatta pigra* (dark blue), *Alouatta palliata* (pink), *Alouatta seniculus* (black), *Alouatta macconnelli* (orange), *Alouatta caraya* (red), *Alouatta belzebul* (purple), *Alouatta arctoidea* (white), *Alouatta guariba* (yellow), *Alouatta sara* (green) {distributions modified from the Red List of Threatened Species [International Union for Conservation of Nature (IUCN) 2016], <https://www.iucnredlist.org>, downloaded 24 December 2017}



Moreover, phylogenetic reconstructions of howler monkeys based on various DNA datasets disagree on the topology of the *Alouatta* species tree (Cortés-Ortiz et al. 2003; Villalobos et al. 2004). For instance, based on a single nuclear gene ( $\gamma 1$ -globin pseudogene), Meireles et al. (1999) found a moderately supported clade grouping *A. guariba* and *A. belzebul* as sister taxa, with *A. seniculus* and *A. caraya* as respective sister lineages to this clade (Fig. 2a). However, the number of species sampled in that study was limited; for instance, no Mesoamerican howler species were included. A study by Cortés-Ortiz et al. (2003) increased the number of South American howler taxa sampled, including *A. sara* and *A. macconnelli* (a total of six South American taxa) as well as two Mesoamerican howler species (*A. pigra* and *A. palliata*). Their neighbor-joining analysis of concatenated sequences of the *CYTB*, *ATPase 8* and *ATPase 6* mitochondrial genes yielded a hypothesis in which a Mesoamerican group was strongly supported as sister to a South American clade. Within the South American clade, two clades were supported: one including *A. seniculus*, *A. sara*, *A.*

*macconnelli* and *A. caraya*, and the other including *A. belzebul* and *A. guariba* (Fig. 2B). However, support for relationships within the South American clade was uniformly moderate to low; the highest level of support was that for a unique sister-species grouping of *A. belzebul* and *A. guariba* at only 78% bootstrap support. Cortés-Ortiz et al. (2003) also analyzed sequences from two nuclear loci: calmodulin 1 (*CALM1*; the intron between exons 3 and 4) and the recombination activating gene 1 (*RAG1*), although these analyses yielded trees with little to no resolution within and between all species of *Alouatta*, with no support for separate Mesoamerican and South American howler clades. Lastly, Villalobos et al. (2004) analyzed mitochondrial DNA (mtDNA) sequences (*CYTB* and *COII*) in a matrix combined with several morphological and karyotypic features for five South American *Alouatta* species: *A. seniculus*, *A. caraya*, *A. belzebul*, *A. guariba* and *A. sara*, as well as the Mesoamerican howler *A. palliata*. That study found yet a third topology: while *A. palliata* fell outside the South American clade, the South American howlers *A. sara*



**Fig. 2a–c** Color-coded map of the distributions of *Alouatta* species and the three gene trees that have been generated in previous studies to examine the phylogenetic relationships among the South American species. **a** Maximum parsimony tree based on the  $\gamma 1$ -globin pseudogene, including bootstrap values, adapted from Meireles et al. (1999).

**b** Neighbor-joining tree based on concatenated *CYTB*, *ATPase 8*, and *ATPase 6* sequences, including bootstrap values, adapted from Cortés-Ortiz et al. (2003). **c** Consensus mitochondrial (concatenated *CYTB* and *COII*) tree, including Bremer decay indexes, adapted from Villalobos et al. (2004)

and *A. seniculus* were inferred as sister taxa; an alternative arrangement was inferred for the relationships between *A. caraya*, *A. guariba*, and *A. belzebul* relative to the study of Cortés-Ortiz et al. (2003) (Fig. 2c). It is notable that Villalobos et al.'s (2004) results did not support a sister-species relationship between *A. guariba* and *A. belzebul*, which had been supported in studies by Cortés-Ortiz et al. (2003) and Meireles et al. (1999).

This persistent uncertainty in the phylogenetic relationships within *Alouatta* may result from limitations in the data types used for inference. For instance, chromosome structure has limited utility in phylogenetic inference because homologous chromosomal features are often difficult or impossible to determine; moreover, intraspecific diversity of chromosomal arrangements is high within *Alouatta* and closely related genera (de Oliveira et al. 1999, 2002). Other data that have been crucial to studying species relationships are DNA sequences of mitochondrial markers; however, they represent the evolutionary history of a single genomic region that is inherited in a linked and matrilineal mode, thus do not capture the influence of male population dynamics (see Moore 1995). In the face of these limitations, this study proposes a re-evaluation of evolutionary relationships within *Alouatta* on the basis of a combined analysis of several independent nuclear markers.

To help clarify the phylogenetic relationships among howler species, we generated DNA sequence data from 14 unlinked intergenic nuclear regions (Kiesling et al. 2015) for two Mesoamerican howler species and five South American howler species, making our nuclear DNA dataset the most comprehensive for the group to date. The nuclear regions sequenced have been found to be useful for resolving inter-generic phylogenetic relationships among South American monkeys (Kiesling et al. 2015). The selected loci are unlinked and thereby provide independent estimates of phylogenetic relationships that, if congruent, can shed light on the actual species relationships. Additionally, since these regions are intergenic and non-protein coding, the action of natural selection is expected to be less intense, with patterns of genetic variation being more likely to track species' relationships (Wildman et al. 2009; Kiesling et al. 2015). The data were first concatenated to generate a tree under Bayesian inference, and then analyzed under a coalescent framework to infer a species tree (Pamilo and Nei 1988; Edwards 2009). Using fossil calibrations, we also estimated divergence times for the howler genus *Alouatta* and its species clades.

## Materials and methods

Utilizing previously existing genomic extractions, we amplified and sequenced DNA from 41 individuals of seven species of *Alouatta*: *A. pigra* and *A. palliata* (Mesoamerican

howlers) and *A. guariba*, *A. caraya*, *A. belzebul*, *A. seniculus* and *A. sara* (South American howlers). DNA sequences were deposited in GenBank; the accession numbers are provided in the supplementary information (see online resource Table 1). Samples of two presumed species, *A. macconnelli* and *A. arctoidea* (Cortés-Ortiz et al. 2015), were not available to us. Our samples of *A. guariba* DNA were extracted from muscle and blood tissue samples as described in Harris et al. (2005); for *A. caraya*, *A. belzebul* and *A. seniculus*, DNA was extracted from blood samples using the protocol described by Schneider et al. (1996) and Sambrook et al. (1989). The sources of DNA samples used in our sequencing experiments are provided in the supplementary online resource Table 2. Although *A. belzebul* has a disjunct geographic distribution, with populations in both Amazonia and the coastal Brazilian Atlantic Forest (Fig. 1), we were only able to secure samples from the Amazonian population. As outgroups, orthologous DNA sequences from five species of closely related platyrrhines from the Atelidae family were downloaded from GenBank, including *Ateles paniscus*, *Ateles belzebuth*, *Ateles geoffroyi*, *Lagothrix lagotricha* and *Brachyteles arachnoides* (Kiesling et al. 2015; see online resource Table 3 for accession numbers). We designated these species as outgroups based on the results of recent phylogenetic studies by Wildman et al. (2009) and Kiesling et al. (2015), as well as previous phylogenies of platyrrhines (Villalobos et al. 2004; Schneider et al. 1996, 2001; Silvestro et al. 2017).

Using protocols reported in Kiesling et al. (2015), we established an amplification panel for 16 unlinked intergenic regions using oligonucleotide primers developed by Wildman et al. (2009) (see online resource Table 4). Polymerase chain reaction (PCR) protocols were adapted from Meireles et al. (1997), including an initial denaturation of 3 min at 94 °C, 30 cycles of denaturation at 94 °C (30 s), annealing at 55 °C (45 s), extension at 72 °C (45 s), and a final extension of 10 min at 72 °C. For a given locus, annealing temperatures varied among species from 42 °C to 56 °C (for more details see online resource Table 5). PCR products were visualized by agarose gel electrophoresis (1%) and sequenced on an ABI 3730xl DNA Sequencer at MacroGen (New York, NY) and on an ABI 3500XL (School of Pharmaceutical Sciences, Universidade de São Paulo—Ribeirão Preto). To verify sequence accuracy we sequenced forward and reverse strands. Sequences were edited in Geneious version R6 [<https://www.geneious.com> (Kearse et al. 2012)], aligned using the global alignment method within Geneious, examined by eye, and then manually adjusted if needed. Limited numbers of samples per population might prevent confident estimation of the phase of heterozygous positions for coalescent-based phylogenetic inference (see below); therefore, we conservatively replaced all ambiguous positions that might correspond to heterozygous sites (K, M,

R, S, Y, W) by N (for the coalescent-based analysis only). We used Jmodeltest (Posada 2008) to determine models of nucleotide substitution for each locus and best-fit partition schemes, which were subsequently used in our phylogenetic analyses. To assess how informative the individual markers were for the purpose of phylogenetic inference, we estimated the number of variable sites across samples for each gene using the program Molecular Evolutionary Genetics Analysis (MEGA) version 4 (Tamura et al. 2007) (see Table 1).

We performed phylogenetic analyses using three approaches. First, we generated a tree under a concatenated approach using Bayesian inference in MrBayes 3.2.1 (Ronquist et al. 2012). For that purpose, we carried out three independent runs and four Markov chains of 20 million generations each, sampling every 1000 steps. Second, we used a coalescent-based analysis in \*BEAST (Heled and Drummond 2010) to generate a species tree. For that, we set up three independent runs of 100 million generations each, sampling every 10,000 steps. Lastly, we performed estimates of divergence times under a Bayesian framework using BEAST 1.10.4 (Drummond et al. 2012). For divergence time estimation, we implemented an uncorrelated lognormal relaxed clock (Drummond et al. 2006) with a uniform prior distribution (interval = 0–1) to the mean rate of the molecular clock (ucl.d.mean parameter). A birth–death tree prior was used, and the parameters that describe substitution rates and nucleotide frequencies were set to the defaults. We ran three independent chains of 100 million steps, sampling every 10,000 steps.

To calibrate the dated phylogeny, we used the ages of two primate fossils. These fossils were chosen after consulting Silvestro et al. (2017), who examined evolutionary rates

and trends among platyrrhines. The first fossil is *Stirtonia tatacoensis* (Stirton 1951), found in the late 1940s in Colombia, and widely recognized as closely related to the *Alouatta* group (e.g., Szalay and Delson 1979; Setoguchi et al. 1981; Delson and Rosenberger 1984; Rosenberger 1992; Hartwig and Meldrum 2002; but see Hershkovitz 1970). The specimen of *S. tatacoensis* consists of dental remains (Stirton 1951; Flynn et al. 1997). As per Silvestro et al. (2017), we used this fossil to calibrate the crown defined by all sampled *Alouatta* species by implementing a lognormal distribution with offset = 12.5 million years ago (MYA) ( $\mu = 0$ ,  $\sigma = 1$ ). We also calibrated the crown using *Solimoea acensis*, a taxon for which the description is based on a set of isolated dental elements from Brazil's Solimões Formation (Kay and Cozzuol 2006). Following Silvestro et al. (2017), we used this fossil to calibrate the crown clade defined by *Ateles*, *Brachyteles*, and *Lagothrix* by implementing a lognormal distribution with offset = 7.5 MYA ( $\mu = 0$ ,  $\sigma = 1$ ).

For all three approaches (MrBayes, BEAST, \*BEAST), we assessed stationary model parameters and convergence of model parameters in Tracer 1.7 (Rambaut and Drummond 2009), applied a 10% burn-in, and combined the three runs in LogCombiner 1.10.4. We then summarized a maximum clade credibility tree in TreeAnnotator 1.10.4 (Drummond et al. 2012). The final trees were visualized in FigTree 1.4. (<https://tree.bio.ed.ac.uk/software/figtree/>).

## Results

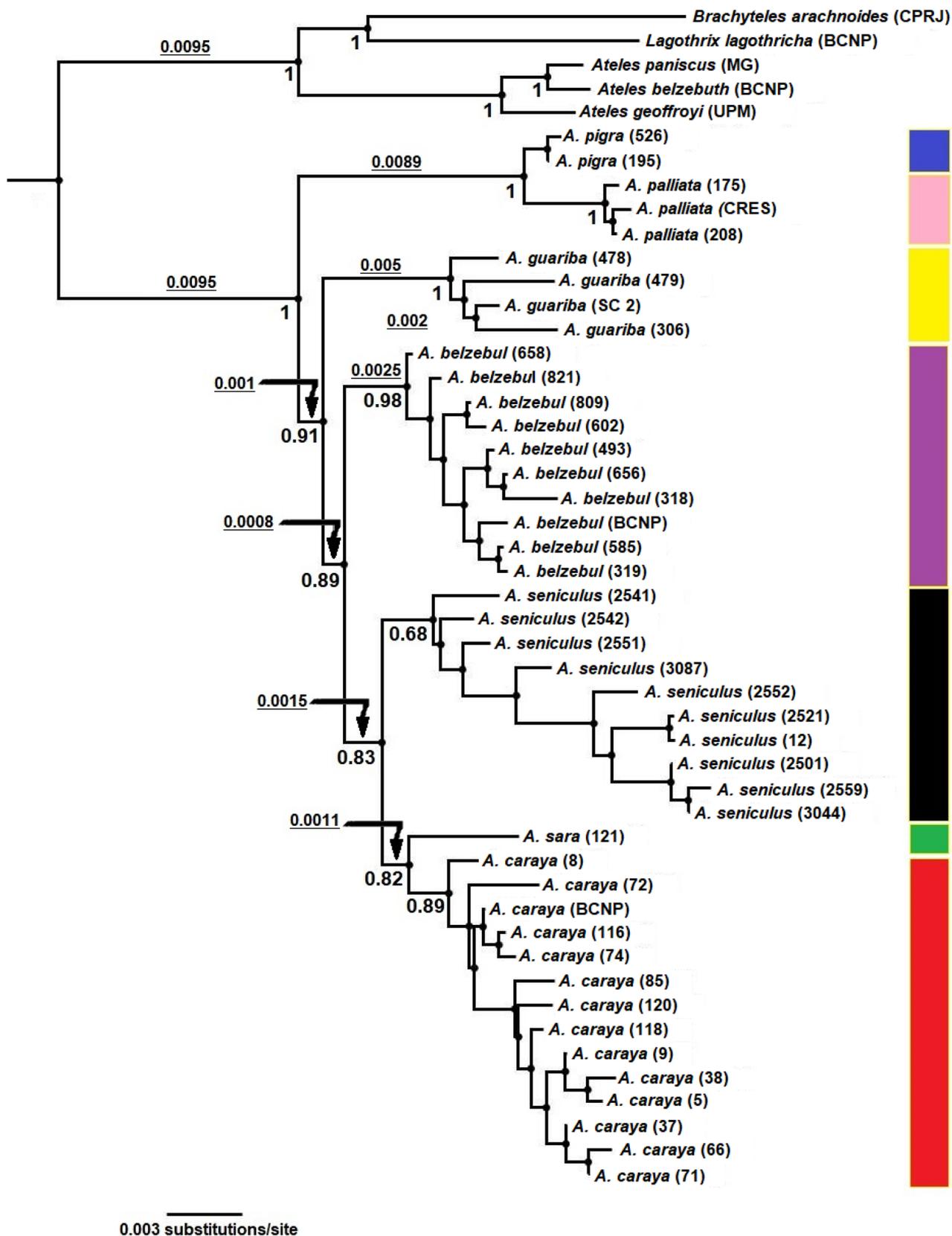
### Phylogenetic analyses

Of the 16 pairs of primers screened, we were able to successfully amplify and obtain high-quality DNA sequences from 14 loci (see online resource Table 6 for amplification success per species and per locus). Two loci (M113T7 and M1803) were not included in the subsequent analyses given the poor quality of most of their sequences. For the set of 14 loci that we successfully amplified, the size of sequenced fragments ranged from 332 to 904 base pairs (see Table 1). The mean number of variable sites across the 14 loci was 26 (ranging from 12 to 46), and the mean number of phylogenetically informative sites was 12 (ranging from 6–36; Table 1).

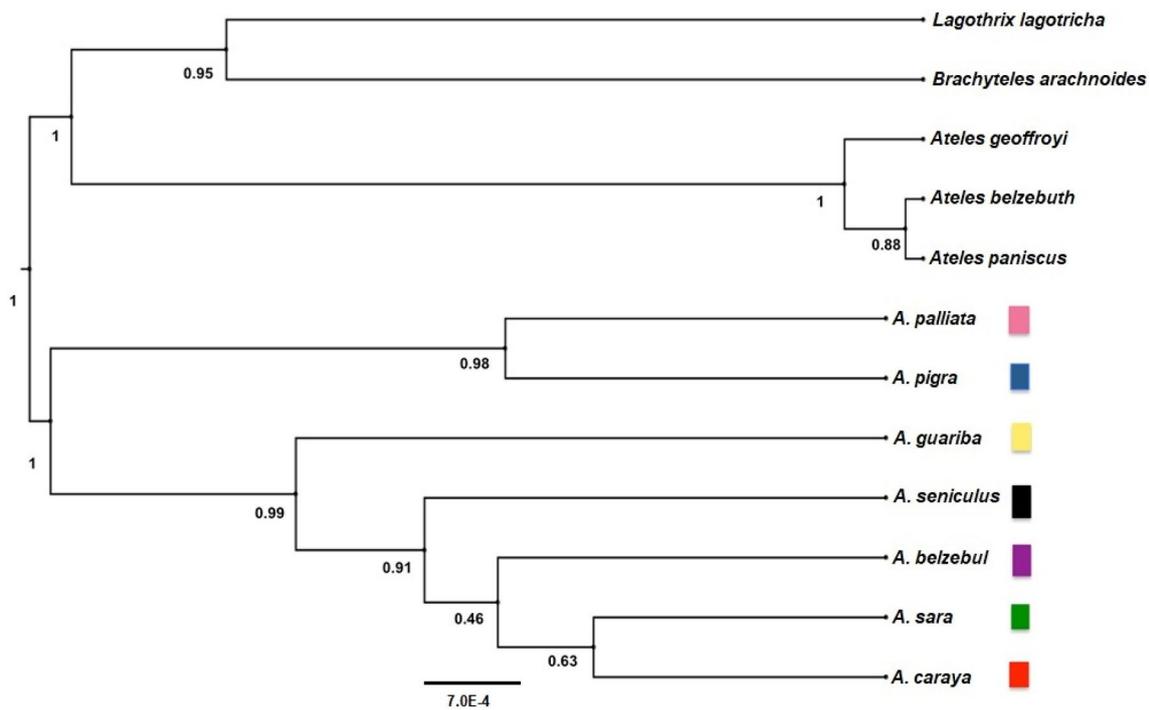
Both the concatenated and species tree approaches inferred high support [posterior probability (PP) of 1.0 and 0.98, respectively] for a Mesoamerican howler clade containing *A. pigra* and *A. palliata*. The South American howler clade comprising *A. guariba*, *A. belzebul*, *A. seniculus*, *A. caraya*, and *A. sara* also received high support [MrBayes tree PP = 0.91 (Fig. 3); \*BEAST PP = 0.99 (Fig. 4)]. All species for which multiple individuals were sequenced formed monophyletic clades with very high support, except for *A. seniculus* (PP = 0.68; see Fig. 3).

**Table 1** Number of polymorphic sites, number of phylogenetically informative sites, and models of nucleotide substitution per marker

Marker	Variable sites	Phylogenetically informative	Length (base pairs)	Model selected
M002	14	6	332	HKY + I
M003	12	11	480	HKY + I
M085	19	10	367	HKY
M093	14	6	450	HKY
M190	28	7	620	HKY + G
M194	18	10	472	HKY + G
M201	10	6	738	HKY + G
M252	46	36	763	HKY
M254	29	10	460	HKY
M258	27	8	726	HKY + G
M265	24	9	843	GTR + G
M271	46	19	703	GTR
M1701	29	11	600	K80
M4344	43	15	904	HKY



**Fig. 3** Bayesian inference tree built with Mr. Bayes, based on concatenated sequences. Posterior probability (PP) values are next to the *nodes*. Branch lengths are *underlined*



**Fig. 4** Species tree generated in \*BEAST, following a coalescence-based approach. PP values are next to the *nodes*

Within the South American howler clade, both MrBayes and \*BEAST found moderate support for a sister taxon relationship between *A. caraya* and *A. sara* (MrBayes PP = 0.82, \*BEAST PP = 0.63). The remaining South American species of *Alouatta* were placed as successive sister taxa to this clade, although occupying alternative positions for each of the two methods. The Atlantic Forest species *A. guariba* was positioned by both methods as the sister to all the other South American howlers, with moderate support (MrBayes PP = 0.89, \*BEAST PP = 0.91).

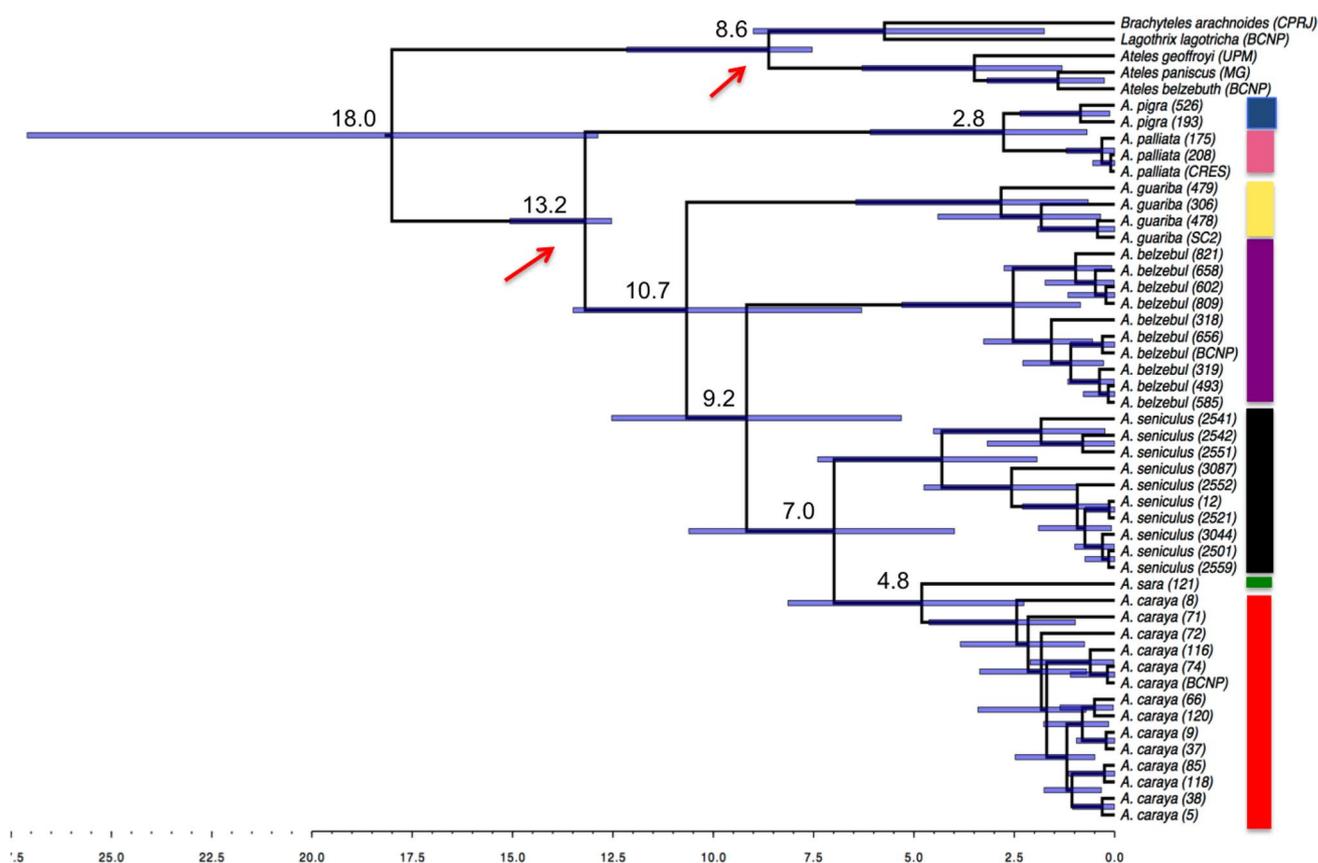
### Estimates of divergence times

Estimates of divergence times using BEAST and based on fossil calibrations placed the age of the genus *Alouatta* at ca. 13.2 MYA [95% highest posterior density (HPD) = 12.5–15.0 MYA; Fig. 5]. The Mesoamerican howler clade containing *A. pigra* and *A. palliata* was dated to ca. 2.8 MYA (HPD 0.68–6.0 MYA), while the node at the base of all South American howlers was dated to ca. 10.7 MYA (HPD 6.3–13.5 MYA). The clade that contains *A. sara* and *A. caraya* was dated to ca. 4.8 MYA (HPD 2.3–8.1 MYA) [see the figure in the supplementary online resources for the PP values for clades in the divergence tree].

### Discussion

All three phylogenetic trees generated from the 14 markers analyzed here support previous studies in recovering distinct Mesoamerican and South American howler clades, which are sister to each other (Villalobos et al. 2004; Figueiredo 1998; Cortés-Ortiz et al. 2003). We found maximum support for this relationship.

In disagreement with the trees of Meireles et al. (1999) and Cortés-Ortiz et al. (2003), we found no support for a sister relationship between *A. belzebul* and *A. guariba*. Instead, all trees in this study inferred the endemic Atlantic Forest howler species *A. guariba* to be the sister lineage to all other South American howlers, with moderate levels of support (see Figs. 3–5). Despite having distinct underlying models, both the concatenated and multispecies coalescent approaches recovered the same position for *A. guariba*. This differs from any topology previously published for this group. If this relationship is supported as more data are collected, it would suggest a biogeographical history for the genus in which the easternmost Atlantic Forest species split early from the ancestor of all the species that today occupy the Cerrado (dry savanna) and Amazonia (tropical rain forest). In breaking up the close sister-species relationship previously hypothesized for *A. guariba* and *A. belzebul*, the results here resemble



**Fig. 5** Dated Bayesian analysis based on concatenated sequences calibrated using two fossils, *Stirtonia* and *Solimoea*, placed as per arrows. Bars represent the 95% highest posterior densities (HPD),

above the bars are values of divergence in ages (million years) on nodes discussed in the text

those of Villalobos et al. (2004). As such, a close relationship between these two species can no longer be assumed and should be further analyzed on the basis of additional genomic evidence.

The relationships among the remaining South American species, however, are less clear, particularly regarding the placement of *A. seniculus* and *A. belzebul*. The concatenated MrBayes gene tree (Fig. 3) and the BEAST dated tree (Fig. 5) placed *A. belzebul* as sister to a clade that includes *A. seniculus*, *A. sara*, and *A. caraya*, with moderate support. In placing *A. belzebul* as external to a clade that contains *A. seniculus*, the tree recovered by MrBayes resembles that of Villalobos et al. (2004) and Cortés-Ortiz et al. (2003). By contrast, the species tree placed *A. seniculus* as the sister species to a clade that includes *A. sara*, *A. caraya*, and *A. belzebul*, but support for this arrangement was low. In addition, although *A. caraya* and *A. sara* were recovered as sister species in all analyses performed here, this relationship consistently had moderate support. This clade arrangement is a novel result relative to both Cortés-Ortiz et al. (2003) and Villalobos et al. (2004), which recovered *A. sara* as more closely related to *A. seniculus*, with *A. caraya* (see Fig. 6



**Fig. 6** *Alouatta caraya* (male)

black howler monkey) being placed more externally on the tree.

The low level of resolution among the Amazonian and Cerrado howlers (*A. belzebul*, *A. seniculus*, *A. sara*, and *A.*

*caraya*) may be related to the relatively recent time frame over which speciation transpired. Alleles along the lineage separating *A. guariba* from the remaining South American howlers seem to have experienced greater allele sorting, whereas the more recently derived species may still show high levels of incomplete lineage sorting. It is well known that incomplete lineage sorting of ancestral alleles imposes difficulties for phylogenetic reconstruction, even when coalescent-based methods are used (Pamilo and Nei 1988; Maddison and Knowles 2006; Edwards et al. 2016; Degnan and Rosenberg 2009). In other words, rapid speciation with relatively short internal branches separating species lineages or clades, as we see here, may make it problematic to resolve evolutionary relationships because random sorting of ancestral alleles obscures phylogenetic signals. In this study, while the length of internal branches separating South American howler species ranged from 0.0008 to 0.0015 nucleotide substitutions per site (Fig. 3), with the lowest values corresponding to weakly supported clades, the branch separating Mesoamerican and South American howlers were as much as 11 times longer (0.0089), and the PP support for this relationship was at the maximum value.

It may be challenging to find nuclear regions that possess levels of nucleotide divergence sufficient to resolve short internal divergences among species that have diverged over a recent time frame. Short branches may be inordinately difficult to resolve even with large multi-locus datasets (Wiens et al. 2008). Although the lengths (in base pairs) of the regions sequenced for each loci in this study were comparable to those in other multi-locus studies of primates (Pozzi et al. 2014; Wiens et al. 2008; Weisrock et al. 2012), nucleotide divergence was low; phylogenetically informative sites ranged from six to 36 (Table 1), with a mean of 12 informative sites over the 14 loci. Still, it may be possible to improve phylogenetic inference by including greater numbers of nuclear loci for *Alouatta* species, particularly through the generation of genome-scale datasets.

Our estimates of divergence dates differed from those reported in previous studies. The divergence dates estimated by Cortés-Ortiz et al. (2003) using mitochondrial DNA sequences recovered a split between the Mesoamerican and the South American howler clades at 6.8 MYA, while our analysis estimated this split at 13.2 MYA (HPD = 12.5–15.0 MYA). Cortés-Ortiz et al. (2003) placed *A. guariba* as the sister species of *A. belzebul* diverging at 4.0 MYA, whereas our analysis recovered *A. guariba* as sister to all other South American howlers with a divergence date of 10.7 MYA (HPD = 6.3–13.4 MYA). However, the divergence between the two Mesoamerican species, *A. pigra* and *A. palliata*, was estimated as 2.8 MYA (HPD 0.68–6.0 MYA), similar to Cortés-Ortiz et al. (2003), who estimated it at 3.0 MYA. The differences in estimated divergence dates between this and previous studies likely stem

from distinct dating strategies. Unlike Cortés-Ortiz et al. (2003), we used multiple nuclear genomic regions as well as lognormally distributed fossil-based priors (*Stirtonia* and *Solimoea*) for node calibration. The use of prior distributions aims to accommodate the possibility that the age of each calibrated node predates the minimum age defined by a fossil (Ho and Phillips 2009). By contrast, Cortés-Ortiz et al. (2003) employed Sanderson's (1997) nonparametric rate smoothing approach, and notably calibrated their tree based on a non-fossil estimate of the chimpanzee-human divergence. Such secondary calibration strategies, based on previous molecular estimates of divergence, can lead to underestimation of node divergence dates (Graur and Martin 2004; Sauquet et al. 2012).

Extinct species such as *S. tatacoensis* (Stirton 1951) from the Middle Miocene of Colombia are morphologically similar to *Alouatta* (Fleagle et al. 1997; Rosenberger et al. 2015), raising the possibility that the latter may have originated in that general area. From there, howlers may have expanded both northwards into Mesoamerica and southwards into the Amazon and Atlantic forests. Movement northwards would have depended on the formation of a land bridge connection, the Isthmus of Panama, whose date is still debated. Recent studies have suggested that the closing of the isthmus might have occurred around 10–6 MYA (Bacon et al. 2015) or 15–13 MYA (Montes et al. 2015), earlier than initial estimates at 3 MYA. In agreement with those studies, our analyses inferred that the split between Mesoamerican and South American howlers dates to around 13 MYA. Similarly, there is debate regarding the date of the formation of the Andes, which may have played a role in the early separation between Mesoamerican and South American howlers. Recent analyses have suggested that the Andes attained their present-day height as early as ~14 MYA (Evenstar et al. 2015), considerably earlier than hypothesized by previous studies [~6 MYA (Garziona et al. 2008; Molnar and Garziona 2007)]. This earlier date conforms to our estimates for the separation between Mesoamerican and South American howlers.

In addition, our analyses placed the time frame of species diversification within South American howlers considerably earlier (between 10.7 and 4.8 MYA) relative to the divergence between the two Mesoamerican howler species (at 2.8 MYA). We are unable to identify the factors contributing to this early South American diversification and to the relative lack of diversification among Mesoamerican forms. Importantly, our dates for the diversification of the South American species during the Late Miocene do not conform to a model of diversification driven by the climate effects of glaciation cycles as predicted by the Pleistocene refugia hypothesis (Haffer 1969).

One of our most striking results is the inference of the brown howler (*A. guariba*), endemic to the Atlantic Forest, as sister to all other South American howlers, with a

divergence date of 10.7 MYA. Such a date for the divergence may point to an early isolation of the Atlantic Forest. Rain forests in eastern South America are thought to have been continuous in the past, separating with increasing dry conditions through the Tertiary (Bigarella 1975), and perhaps becoming distinct towards the end of the Pliocene around 2.5 MYA. A dry and open diagonal formed between the Amazon and Atlantic forests in a northeast to southwest axis, encompassing three biomes: the Caatinga in northeastern Brazil, the Cerrado in central Brazil, and the Chaco in Argentina and Paraguay (Costa 2003; Bigarella 1975). Climatic changes throughout the Pleistocene are thought to have caused contractions and expansions of both the Amazon and Atlantic forests, which would have allowed intermittent connections and biotic exchange (Dal Vechio et al. 2018, 2020; Prates et al. 2016, 2018). Nevertheless, studies on many animal groups support the hypothesis that biotic connections between Amazonian and Atlantic forests have also happened much earlier, during the Miocene (Dal Vechio et al. 2018; Prates et al. 2017, 2020), in remarkable agreement with our estimates for the divergence between *A. guariba* and howler species from western South America.

### Concluding remarks

Our analyses of 14 intergenic loci provide strong support for separate Mesoamerican and South American howler clades and for the monophyly for each howler species. Regarding the interrelationships among species within the South American howler group, our results provide mixed support for the phylogenetic hypotheses published thus far. Our results provide no support for a sister relationship between *A. guariba* and *A. belzebul*, which was found in recent mtDNA studies (see Cortés-Ortiz et al. 2003). We also found moderate support for the early divergence of the Atlantic Forest-based *A. guariba* relative to the remaining South American howlers. Lastly, our estimates of divergence times are often older than estimates previously proposed for the group, which may reflect our conservative dating strategy based on prior distributions for node calibration. Two non-mutually exclusive reasons may explain the moderate to low support offered by the nuclear loci we sampled: low rates of nucleotide substitution in nuclear genes (even in intergenic regions, presumably less constrained by selection), and incomplete lineage sorting on short internodes between clades. Our study contributes to a better understanding of the diversification processes that led to howler monkey species; however, they also suggest that further comprehension of the evolutionary history of the *Alouatta* radiation will rely on broadened taxonomic, geographic, and genomic sampling.

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