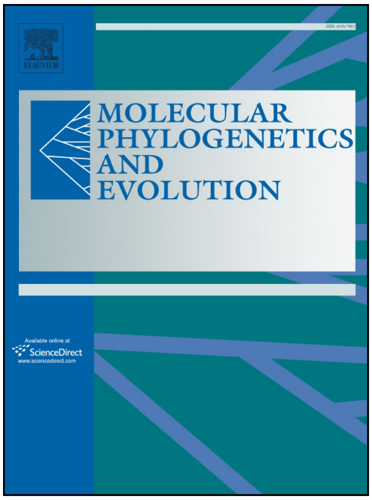

Out of the shadows: Multilocus systematics and biogeography of night monkeys suggest a Central Amazonian origin and a very recent widespread southeastward expansion in South America

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Abbreviations: My = Millions of years; Ma = Millions of years ago; Ky = Thousand years; ML = Maximum Likelihood; BI = Bayesian Inference; COI = Cytochrome Oxidase subunit I; COII = Cytochrome Oxidase subunit II; Cyt *b* = Cytochrome *b*; mtDNA = mitochondrial DNA; nuDNA = nuclear DNA; UTR = Untranslated Region; BS = bootstrap; pp = posteriori probability; HPD = Highest Posterior Density; AIC = Akaike Information Criterion; AICc = corrected Akaike Information Criterion; MCMC = Markov Chain Monte Carlo; EA = Eastern Amazon; RN = Rondonia; IN = Inambari; GH = Guiana Highlands; NW = Northwestern Amazon; IM = Imeri; NS = North of South America; CP = Cerrado plus Pantanal; BO = Bolivia.

ABSTRACT

Night monkeys (*Aotus*, Cebidae) are a widely distributed genus of Neotropical primates with a poorly understood taxonomy and biogeography. The number of species in the genus varies from one to nine, depending on the author, and there are at least 18 known karyotypes, varying from 2n = 46 to 2n = 58. Historically, night monkeys are divided into two species groups: red- and grey-necked groups from south and north of the Amazon-Solimões River, respectively. Here, we used 10 nuclear and 10 mitochondrial molecular markers from a wide taxonomic and geographic sample to infer phylogeny, divergence times, and biogeography of the genus. For phylogenetic reconstruction we used Maximum Likelihood (ML) and Bayesian Inferences (BI). Biogeographic models were generated using the 'BioGeoBEARS' software. We found support for nine taxa of *Aotus* and rejected the existence of monophyletic "red necked" and "grey necked" species groups. We suggest a taxonomic reclassification of the genus, which is better represented by two clades named northern group, which contains *Aotus miconax*, *A. nancymae*, *A. trivirgatus*, *A. vociferans*, *A. lemurinus*, *A. griseimembra*, *A. zonalis*, and *A. brumbacki*, and southern group, which contains *A. nigriceps*, *A. boliviensis*, *A. infulatus*, and *A. azarae*. The results suggest that the most recent common ancestor of all species of *Aotus* arose in the central Amazon basin in the Early Pliocene. The evolutionary history of night monkeys was guided by dispersal, vicariance and founder events. The end of the Andean uplift and the subsequent changes in the Amazon landscape, as well as the Amazon-Solimões and Tapajós rivers may have played an important role in the origin and diversification of *Aotus*, respectively. However, most of the Amazonian rivers seem not to have been geographical barriers to dispersal of night monkeys. The herein named southern group is fruit of a very recent diversification guided by dispersal, crossing the Tapajós, Xingú, Tocantins, and Guapore rivers and reaching the Cerrado in the last 1.6 My.

Key words: Andean uplift, *Aotus*, molecular systematics, Owl monkeys, phylogeography.

1. INTRODUCTION

Night monkeys, also known as owl monkeys (*Aotus*, Illiger 1811), are the only extant nocturnal anthropoids (Fernandez-Duque et al., 2010). The genus is also one of the most widely distributed among Neotropical primates, with species occurring from southern Panamá to northern Argentina, and occupying different biomes, such as the Amazonian rainforest, Cerrado savannas, Pantanal wetlands and the humid Chaco (Fig. 1).

There is no consensus as to how many species comprise *Aotus* (Plautz et al., 2009; Ruiz-García et al., 2016). In the first modern taxonomic appraisal of the genus, Hershkovitz (1949) recognized only one species of *Aotus*, *A. trivirgatus*, with two subspecies. In the 1970's, several karyological studies challenged the traditional taxonomy of the genus. Brumback et al. (1971) and Brumback (1973) proposed two species, *Aotus griseimembra* (2n = 52, 53 and 54) and *A. trivirgatus* (2n = 54). In the same period, Ma et al. (1976a, 1977, 1978, 1980) proposed the revalidation of *A. vociferans* (2n = 46) and additionally showed that Panamanian individuals had karyotypes of 2n = 55 and 56, suggesting additional species diversity in the genus.

Taking into account the karyotypic diversity, geographic distribution, susceptibility to malaria and pelage traits, Hershkovitz (1983) proposed nine species and four subspecies of *Aotus* classified in the red-necked and the grey-necked species groups. The red-necked species group comprising *A. miconax* Thomas, 1927, *A. nigriceps* Dollman, 1909, *A. infulatus* Kuhl, 1820, *A. azarae* (with *A. a. azarae* Humboldt, 1811, and *A. a. boliviensis* Elliot, 1907, as subspecies) and *A. nancymae* Hershkovitz, 1983, occurs south of the Amazon-Solimões River. The grey-necked species group, comprised of *A. lemurinus* I.

Geoffroy, 1943 (*A. l. lemurinus* and *A. l. griseimembra* Elliot, 1912, as subspecies), *A. vociferans* (Spix, 1923), *A. trivirgatus* (Humboldt, 1812) and *A. brumbacki* Hershkovitz, 1983, occurs north of the Amazon-Solimões River. Additionally, Ramírez-Cerqueira (1983) described *A. hershkovitzi*, as a species of the grey-necked group.

Based on morphological traits, Ford (1994) and Groves (2001), reaffirmed the two species groups proposed by Hershkovitz. Ford (1994) recognized seven species (two in the grey-necked group and five in the red-necked one), while Groves (2001) recognized eight (four in each group). The major taxonomic reviews of the genus are presented in the Table 1.

Based on lack of differences in the karyotype, Pieczarka and Nagamachi (1988) and Pieczarka et al. (1993) did not recognize *Aotus azarae boliviensis* as a species distinct from *A. infulatus*. Defler et al. (2001) questioned the validity of *A. hershkovitzi*, and Defler and Bueno (2007) described *A. jorgehernandezi* (2n = 50), a grey-necked species. To the best of our knowledge, there are at least 18 distinct karyotypes currently described for the genus varying in their diploid numbers from 2n = 46 to 2n = 58 (Defler and Bueno, 2007; Defler et al., 2001; Galbreath, 1983; Ma et al., 1976a,b, 1985; Menezes et al., 2010; Pieczarka et al., 1992; Ruiz-García et al., 2016; Torres et al., 1998).

Until now, only a few molecular phylogenetic hypotheses were proposed for the species of *Aotus* and most of them used only a single mitochondrial locus. Ashley and Vaughn (1995), based on DNA sequences of the Cytochrome Oxidase subunit II (*COII*) gene, questioned the monophyly of the red- and grey-necked groups of Hershkovitz (1983), showing that *Aotus azarae boliviensis*, a red-necked species, was sister to *A. griseimembra*, a grey-necked species, rather than to *A. nancymae*, a red-necked species. Subsequent phylogenetic studies of *Aotus* by Plautz et al. (2009), Menezes et al. (2010), Babb et al. (2011) and Ruiz-García et al. (2011, 2013, 2016) also found no support for the monophyly of Hershkovitz's groups, however, all but Menezes et al. (2010) were mostly based on the analyses of the *COII* data.

Menezes et al. (2010) used three mitochondrial genes and the nuclear gene SRY to infer the *Aotus* phylogeny. Despite the absence of phylogenetic signal in SRY, their concatenated mitochondrial dataset recovered *A. nancymae*, a red-necked species, as the sister species of all other *Aotus* (Menezes et al., 2010). Furthermore, Ruiz-García et al. (2016) found a small genetic differentiation between *A. nancymae* and *A. vociferans*, a grey-necked species, based on 12 nuclear DNA microsatellites. The phylogenetic relationships reported in all these molecular studies were, in general, weakly supported, and conflicting among studies.

This agrees quite well with the morphometric and dental study of Ford (1994), who could not discriminate among *A. lemurinus*, *A. griseimembra*, *A. brumbacki*, *A. zonalis*, and *A. vociferans*. Due to this, she concluded that all these taxa belong to a single species, *A. vociferans*. However, *A. vociferans* and *A. trivirgatus* (the other grey-necked species) were morphologically differentiated.

It was not until Perelman et al. (2011) and Kiesling et al. (2015) that nuclear multilocus approaches were used to investigate the phylogenetic relationships among all primates and the Platyrrhini, respectively. While Perelman et al. (2011) recovered the two species groups of Hershkovitz, Kiesling et al. (2015) did not. Perelman et al. (2011) used only two species of the grey-necked group (*A. griseimembra* and *A. trivirgatus*) and two species of the red-necked (*A. nancymae* and *A. azarae*). Kiesling et al. (2015) also used four, but different species and showed *A. lemurinus* as sister to the other species of *Aotus*, and *A. vociferans* as the sister species of the clade comprised by *A. nancymae* and *A. azarae*. Unfortunately, both studies used only one specimen of each species, and almost all of them were from medical research animal colonies.

Most studies have considered western Amazonia north of the Amazon River as the ancestral area of the night monkeys (Ford, 1994; Hershkovitz, 1983; Ma, 1981; Menezes et al., 2010). Ma (1981) suggested that Pleistocene climatic fluctuations generating Amazonian refugia was the main force for the diversification of *Aotus*, although subsequent studies disagree with this proposal due to the antiquity of the estimated species divergences (Ashley and Vaughn, 1995; Babb et al., 2011; Menezes et al., 2010; Plautz et al., 2009; Ruiz-García et al., 2011). Ford (1994) and Ma (1983) also proposed that *A. nancymae* is the ancestor of the south and easternmost lineages of night monkey, south of the Amazon River. Plautz et al. (2009) and Menezes et al. (2010) suggest that sea level rise of about 100 m during the past 5 million years, resulting in three refugia, was an important driving force in the diversification of the genus. In this context, *A. trivirgatus* would have arisen in the Guyana Shield refugium, *A. azarae* would have arisen in the Brazilian Shield refugium, and *A. nancymae* plus the other grey-necked species would have arisen in the refugium at the foothills of the Andes (Menezes et al., 2010; Plautz et al., 2009). Plautz et al. (2009) also pointed out that different rivers, such as the Amazon, Negro, Orinoco and Tapajós may have played a significant biogeographic role in the evolutionary history of the night monkeys.

To date, no studies has used statistical methods to test different biogeographical models to explain the historical evolution of night monkeys and to infer which paleogeoclimatic factors may have influenced their evolution in the Neotropics. Different features of land surface evolution have been used to explain the evolution and biogeography of the Neotropical biota, such as the uplift of the Andes and its consequences in changing climate, drainage and sedimentation patterns (Hoorn et al., 2010; Mora et al., 2010), as well as the establishment of the Amazon River network (Lynch Alfaro et al., 2015a,b; Ribas et al., 2012; Ruiz-García et al., 2018) and the Pleistocene climatic fluctuations (Haffer, 1969).

In the case of night monkeys, vicariance was the primary process invoked to explain their diversification. However, with the advent of event-based biogeography, it is possible to integrate other relevant processes, such as founder-event, dispersal, sympatry and extinction, using explicit models, permitting comparison of alternative evolutionary/biogeographical scenarios.

In this study, we used DNA sequences from multiple nuclear and mitochondrial loci to infer the phylogenetic relationships of night monkey lineages. Using a dated phylogeny, we infer the biogeographic history of the genus. This first multilocus phylogeny of night monkeys using mtDNA and nuDNA (coding and non-coding regions) resulted in new insights of evolutionary history of *Aotus*, such as (i) the monophyly of the red and grey-necked groups is not supported, (ii) the two species groups of *Aotus* are better named accordingly to the distribution of the species in the northern and southern groups, (iii) the end of uplift of the Andes in the Pliocene seems to have been important for the establishment of the night monkeys in the Amazon basin, (iv) the biogeographical inferences suggest that *Aotus* arose in the north and south of the central Amazon basin, (v) the analyses indicate that rivers of southeastern Amazon were not geographical barriers to the expansions of *Aotus* and (vi) the southeasternmost night monkey species are a very recent group, a result of a widespread southeastward expansion.

2. MATERIALS AND METHODS

2.1 Ethics statement

This research adhered to the principles for the ethical treatment of primates of the American Society of Primatologists.

2.2 Biological sample collection and laboratory procedures

Forty-five samples of *Aotus* representing eight taxa – *Aotus nancymae*, *A. vociferans*, *A. trivirgatus*, *A. infulatus*, *A. boliviensis*, *A. nigriceps*, *A. griseimembra* and *A. lemurinus* – were used (Table 2). Species-level identification of the specimens was based on external pelage traits and the geographical location where the specimens were collected.

Total DNA was obtained from liver, blood, skin and muscle using the Wizard Genomic/Promega DNA purification kit. Samples from outside Brazil complied with all the protocols approved by the Ethical Committee of the Pontificia Universidad Javeriana (No 45677) and the laws of the Ministerio de Ambiente, Vivienda y Desarrollo Territorial (R. 1252) and were processed in the laboratory of Molecular Population Genetics and Evolutionary Biology of the Pontificia Universidad Javeriana. In the other hand, samples from Brazil were processed in the Laboratory of Genetics and Evolution in the Institute for Coastal Studies and Center for Genomics and Systems Biology at Universidade Federal do Pará.

Twenty molecular markers were amplified using Polymerase Chain Reactions. Ten of which were nuclear markers previously used by Perelman et al. (2011) and Kiesling et al. (2015) – PNOC, FBN1, CHRNA1, ABCA1, LUC7L, mC19_03, mC13_04, m219, m64T3, and m022 – and the other 10 were mitochondrial ones: Cytochrome *b* gene (Cyt *b*), NADH dehydrogenase subunit 1 (ND1), ATP synthase F0 subunit 6 (ATP6), ATP synthase F0 subunit 8 (ATP8), 12S ribosomal RNA (12S), 16S ribosomal RNA (16S), Cytochrome Oxidase subunit I (COI), tRNA-Glutamine (Gln), tRNA-Isoleucine (Ile) and tRNA-Leucine (Leu) (Table S1).

The PCR conditions were as follows: a total volume of 15 µL with 8.6 µL of Milli-Q H_2O, 0.6 µL of $MgCl_2$ (25 mM), 1.5 µL of 10X buffer (200 mM Tris-HCl, 500 mM KCl), 2.4 µL of dNTPs (1.25 mM), 30 ng of DNA, 1 Unit of Invitrogen™ *taq* DNA polymerase and 0.2 µM of each forward and reverse primers (see Table S1). The PCR amplifications were carried out by an initial denaturation step at 96 ^{o}C for 5 min, followed by 30 cycles of denaturing at 95 ^{o}C for 45 sec, different temperatures of primer annealing (see Table S1) for 45 sec, and extension at 72 ^{o}C for 1 min, followed by a final extension of 5 min at 72 ^{o}C. Each amplicon that presented a single size fragment was subsequently purified and sequenced using the BigDye sequencing kit V 3.1 (Thermo Fisher, Calrsbad, CA, USA). The products were transferred to the Applied Biosystems 3500 XL automatic Genetic Analyzer (Thermo Fisher, Calrsbad, CA, USA).

2.3 Sequence alignment, evolutionary models and data partition selection

We downloaded GenBank sequences for each molecular marker from representative species of the genera *Saimiri*, *Sapajus*, *Ateles*, *Alouatta*, *Callithrix*, *Saguinus*, *Chiropotes* and *Callicebus* that were used as outgroup in the analyses (Table S2). For each individual marker, the sequences were aligned using the MUSCLE software (Edgar, 2004) plug-in in the software PhyDE® (Müller et al., 2010) and checked manually. The aligned markers were concatenated to produce three datasets (concatenated mtDNA/nuDNA, nuclear, and mitochondrial) as shown in Table S3. All three datasets were deposited in Mendeley Data (doi:10.17632/b246vvx3yg.1). The concatenated mtDNA/nuDNA dataset was used for phylogenetic analyses and divergence time estimates, whereas nuclear and mitochondrial datasets were used only for phylogenetic analyses (see below). The best-fit partitioning scheme and the evolutionary nucleotide substitution model for each dataset were estimated in the software PartitionFinder 2.1.1 (Lanfear et al., 2016). The coding regions were separated according to the codon positions. For the concatenated mtDNA/nuDNA dataset, all models implemented in BEAST and RAxML were tested using the greedy searching method, while for the nuclear and mitochondrial datasets, only the models implemented in the RAxML were tested. The best models were selected using AICc values. Complete information on the partitioning schemes, substitution models and other features of the schemes for each dataset are presented in Table S3.

2.4 Phylogenetic, species tree and divergence time analyses

We used the Maximum Likelihood (ML) algorithm for the phylogenetic reconstructions among night monkey lineages. For each dataset, a ML analysis was carried out in the RAxML 8.2.10 software (Stamatakis, 2014) using the selected partitioning schemes and substitution models (Table S3). Node support was estimated via bootstrap, using 1,000 pseudoreplicates. Only bootstrap values > 90% were considered satisfactory.

A multilocus Bayesian species tree representing the main lineages estimated in previous analyses was generated in BEAST2 software (Bouckaert et al., 2014) through the STARBEAST2 analysis (Ogilvie et al., 2017) using the concatenated mtDNA/nuDNA dataset. A strict molecular clock with the Yule speciation tree prior was used for each subset. The tree priors were linked for the mitochondrial partitions with a linear and constant root prior, and the ploidy of the mitochondrial partitions was one quarter of the nuclear genes. Four independent runs were carried out with 1.5 x 10^9 generations sampled every 1000 generations.

The concatenated mtDNA/nuDNA dataset was used to conduct four independent MCMC runs of 10^8 generations sampled every 10,000 generations in BEAST 1.8.4 (Drummond et al., 2012) to estimate the divergence times between the lineages recovered in the species tree topology. We constrained the topology, to produce the same branching pattern found in the species tree. The tree priors and the clock models were linked for the mitochondrial partitions. The Birth-Death Branching Process (Gernhard, 2008) with an uncorrelated lognormal molecular clock was implemented. The topology was calibrated using four Platyrrhini fossils under a lognormal distribution: †*Stirtonia* for crown Atelidae (12.6 Ma) (Hershkovitz, 1970; Kay et al., 1987), †*Proteropithecia* for crown Pitheciidae (15.7 Ma) (Kay et al., 1998), †*Neosaimiri* for crown Cebinae (12.5 Ma) (Rosenberger et al., 1991; Takai, 1994), and †*Lagonimico* for crown Callitrichinae (13.4 Ma) (Kay, 1994).

The convergence of the chains was checked in Tracer 1.7 software (Rambaut et al., 2018). All .log and .trees files were summarized in the software LogCombiner and TreeAnnotator, respectively, with a burn-in of 20% for each independent run. The models employed and the species assignment of each sample are described in Table S3 and Table 2, respectively.

2.5 Biogeographical analysis

To infer the ancestral geographical area and the historical biogeography of night monkeys, we used the R package 'BioGeoBEARS' (Matzke, 2013, 2014). A consensus species-level phylogeny was built in R and used as input file for the analyses. Each single representative terminal branch was allocated to different biogeographical areas according to the known geographical distribution of the taxa (Table 2). Areas used were as follows: Eastern Amazon (EA) encompassing the Belém, Xingu and Tapajós centers of endemism and Marajó island, Rondonia (RN), Inambari (IN), Guiana Highlands (GH) defined by the typical distribution of *A. trivirgatus* east and north of Negro to Orinoco River and west of Trombetas River, the Northwestern Amazon (NW) encompassing the Napo center of endemism, Imeri center of endemism (IM), the North of South America regions (NS), the Cerrado + Pantanal (CP), and Bolivia (BO) (Fig. 2). These biogeographical zones were chosen taking into account three criteria: 1. They are considered centers of endemism in the Neotropics (Cracraft, 1985), 2. many of them are delimited by rivers and 3. some of them coincide with the Pleistocene refuges proposed by Haffer (1969). This allows us to analyze the influence of each of these geological features on the evolution of the taxa. As we are trying to understand the role of the different processes and patterns such as vicariance, sympatry, extinction, dispersal and founder-event in the evolution of *Aotus*, all six models implemented in 'BioGeoBEARS' were tested: DEC, DEC-J, DIVALIKE, DIVALIKE-J, BAYAREALIKE and BAYAREALIKE-J.

3. RESULTS

3.1 Phylogeny, divergence times and species tree

A total of 469,218 nucleotides base pairs representing 854 sequences of 20 nuclear and mitochondrial loci were produced for 45 samples of *Aotus*. All mitochondrial sequences showed to be true mtDNA instead of numts, since none of them presented irregular stop codons and showed high similarity with previously published sequences of night monkeys (comparisons carried out using the BLASTn software). Sequences were deposited in GenBank and the accession numbers are presented in Table S4.

The ML topologies derived from the three datasets and the species tree were virtually the same, although support values varied among the analyses (Figs. 3, S1, S2 and S3). Differences in topologies were restricted to two important points of discordance: the phylogenetic position of *Aotus nancymae* and of the sample from the Imeri center of endemism CTGAM759. Concatenated mtDNA/nuDNA, mitochondrial ML trees and the species tree recovered *A. nancymae* within the northern group, but without strong support (Figs. 3, S1 and S3), whereas the nuclear ML tree recovered this species as sister group to all the other southern species plus the samples CTGAM759, albeit also with low bootstrap support (Fig. S2). The CTGAM759 sample appeared within the southern group or sister to this group in all ML phylogenies (Figs. S1, S2 and S3). In the concatenated mtDNA/nuDNA and mitochondrial phylogenies, the sister taxon relationship of CTGAM759 and all southern taxa was strongly supported (Figs. S1 and S3), whereas in the nuclear phylogeny, its nested phylogenetic position was poorly supported (Fig. S2). However, in the species tree, the CTGAM759 sample was recovered as the first diverging taxon of the northern clade with low posterior probability support (Fig. 3). It is interesting to note that the nuclear tree presented the lowest bootstrap values for the branching pattern (Fig. S2), while the mitochondrial tree had better support values, generally higher than 90% (Fig. S3). The concatenated mtDNA/nuDNA phylogeny showed the best support values (Fig. S1).

The monophyly of the genus was recovered with maximum support in all analyses with a crown age of 4.11 Ma [95% Highest Posterior Density {HPD}: 3.27 – 5.02] in the Early Pliocene (Figs. 3, S1, S2 and S3). With the exception of the nuclear dataset that showed low support values, nine lineages were recovered within the genus, distributed in two main clades that almost entirely represent Hershkovitz's red and grey-necked groups (Figs. 3, S1 and S3). In the southern group are *Aotus infulatus* from the right bank of the Tocantins River, *A. infulatus* from the left bank of the Tocantins River, Marajó Island and the right bank of the Xingu River, *A. boliviensis* from the Jamari River, and *A. nigriceps* from the Purus River and from the vicinity of Sena Madureira from Brazil and from Madre de Dios River from Peru. In the northern group, we recovered *A. vociferans* from Iquitos in Peru, and Letícia and Puerto Nariño in Colombia, *A. lemurinus* and *A. griseimembra* (captive specimens from uncertain localities), and *A. nancymae* from Leticia in Colombia and Iquitos and Tapiche River in Peru. *Aotus trivirgatus* from left bank of the Japurá River in Brazil, was recovered either as sister to the southern group in the gene trees or sister to the northern group in the species tree. All these nine lineages had high statistical support, except for the nuclear dataset (Figs. 3, S1, S2 and S3).

In most cases, the species trees and gene trees were similar. However, given that the gene tree may not always match the species tree, the branching pattern described here is the one present in the species tree. For the nine lineages recovered, all diverged recently in the Pleistocene, except for *Aotus trivirgatus* (Fig. 3).

The first divergence in southern group occurred at 1.66 Ma [95% HPD: 1.22 – 2.22] in the Calabrian age of the Early Pleistocene originating *A. nigriceps* and the ancestor of the *A. infulatus*/*A. boliviensis* clade. In the Middle Pleistocene, the second divergence at 0.58 Ma [95% HPD: 0.41 – 0.78] separated *A. infulatus* from *A. boliviensis* (Fig. 3).

The northern group diversified earlier than the southern group. The first lineage leading to *A. trivirgatus* diverged at 3.67 Ma [95% HPD: 2.95 – 4.55] from the ancestor of the remaining northern group plus *A. nancymae*. This lineage subsequently diverged at 2.95 Ma [95% HPD: 2.37 – 3.37] in the Late Pliocene, giving rise to two clades: one comprised of *A. nancymae* and *A. vociferans* which diverged 2.56 Ma [95% HPD: 2.01 – 3.2]; and other comprised of *A. lemurinus* and *A. griseimembra* which diverged 1.63 Ma [95% HPD: 1.11 – 2.18].

3.2 Biogeographical analysis

The two 'BioGeoBEARS' models DIVALIKE and DIVALIKE-J were deemed the two best models to explain the data (Table 3). While the likelihoods of these two models were not significantly different and their AIC values were similar, the AICc value of the DIVALIKE model was lower than that estimated for the DIVALIKE-J. The DIVALIKE model indicates a biogeographical scenario in which the daughter lineages inherit the ancestral area considering dispersal and vicariance (parameters *d* and *v*, respectively) for subset speciation. The founder-event (+ J) implies the colonization of new areas distinct from those of the ancestors, which was observed, for instance, in *Aotus boliviensis* (Fig. 2). Both models, however, indicated that the most-probable area of origin of the genus *Aotus* were the Rondonia and Imeri centers of endemism (Figs. 2 and S4). The analysis also suggests that a vicariance event in the early diversification of the genus isolated southern-like and northern-like populations in the Rondonia and Imeri centers of endemism, respectively (Figs. 2 and S4).

The most likely area for the origin of the southern group was within the Rondonia and Inambari regions in the Early Pleistocene (Fig. 2). The diversification of the southern group seems to be associated with a rapid colonization of eastern and southern Amazon, as well as Cerrado and Bolivian Llanos with several speciation event driven by dispersal and founder-event processes (Fig. 2).

The Imeri center of endemism was identified as the most probable ancestral area for the northern group (Fig. 2). From the Imeri region, northern populations appeared to reach the Guyana Highlands, northern South America, northwestern Amazon and, more recently, the Inambari regions, with speciation occurring via vicariance, founder-event and dispersal (Figs. 2 and S4).

The DIVALIKE model differs from the DIVALIKE-J in three points: (1) In the former, the evolution of the southern *Aotus* species seems to result from vicariance rather than founder event processes; (2) in the DIVALIKE model, the southern group reached the Cerrado and Pantanal 1 My earlier and the East Amazon 1 My later and; (3) the most-probable ancestral biogeographical area for northern populations was along the Imeri and Northern South American in the DIVALIKE, rather than just Imeri (see Figs. 2 and S4).

4. DISCUSSION

Here, we present the first dated phylogeny and phylogeographic analysis of night monkeys based on multiple loci sampled from mtDNA and different regions of nuDNA (coding – UTR, exon and introns – and non-coding loci). Our multilocus phylogeny based on the concatenated mtDNA/nuDNA dataset supported the monophyly of *Aotus* and the validity of all species-level taxa studied here, but did not support the monophyly of Hershkovitz's morphology-based species groups. We also showed that the most-probable ancestral area for the origin of the genus is the central-west Amazon basin, and that the end of the Andean uplift in the Pliocene seems to have been important for the origin of the genus. Our findings suggest that most of the Amazonian rivers were not geographical barrier for the dispersal of the night monkeys, supporting an old origin of the Amazon drainage system.

4.1 *Aotus* phylogeny

Curiously, one sample from the Jarupá River in the Imeri center of endemism (CTGAM759, latitude 1º39'22" S and longitude 69º12'10.8" W) was phylogenetically very distinct from the other *Aotus* included in our study (Figs. 3 S1, S2 and S3). Only *A. vociferans* supposedly occurs in the Imeri area of endemism, however all of our samples of *A. vociferans* are phylogenetically distinct from specimen CTGAM759. The pelage coloration pattern of this specimen matches that of *A. trivirgatus* which occurs on the Guyana Shield in the Negro/Trombetas interfluve. Unfortunately, we did not have any sample of wild-caught *A. trivirgatus* from its known occurrence area, and samples used in previous studies only had the COI and Cyt *b* genes sequenced. The specimen also shows minimal divergence (0.2 and 0.4%) in Cyt *b* from specimens of *A. trivirgatus* from known localities published by Menezes et al. (2010). Analyses of ddRAD and the Cyt *b* of CTGAM759 and specimens of *A. trivirgatus* from Roraima and north of the Negro River from the vicinity of Santa Isabel also show minimal divergences (IF and TH, data not included in this study). The limits of the distribution of *A. trivirgatus* are unclear, and it is supposedly limited to the left bank of the Negro River (Ford, 1994). Thus, our record of *A. trivirgatus* from the left bank of the Japurá River extends the distribution of the species to the Negro/Japurá interfluve.

Hershkovitz (1983) proposed the distribution of the red-necked species group to be from south of the Amazon-Solimões River and the grey-necked group from the north of the Amazon-Solimões River. In light of the molecular phylogeny presented here, this classification is untenable due to the phylogenetic position of *A. nancymae*, a red-necked species, nested within the grey-necked species in the concatenated mtDNA/nuDNA and mitochondrial ML trees (Figs. S1 and S3), as well as in the species tree (Fig. 3).

This phylogenetic position of *A. nancymae* indicates that pelage color on the side of the neck is not diagnostic for the two main clades of night monkeys. Previous studies using mitochondrial (Ashley and Vaughn, 1995; Babb et al., 2011; Menezes et al., 2010; Plautz et al., 2009; Ruiz-García et al., 2011, 2013, 2016) or nuclear markers (Kiesling et al., 2015) have also questioned the utility of this character for classification, but all of these studies either used few molecular markers or only few species of *Aotus*. From a morphological perspective, Ford (1994) demonstrated that *A. nancymae* exhibit several distinct pelage characteristics when compared to other red-necked night monkeys. Similarly, Galbreath (1983) highlighted various chromosomal rearrangements that are absent in the other red-necked species.

It is interesting to note, however, that the phylogenetic position of both *A. nancymae* and *A. trivirgatus* was poorly supported and disagreed among analyses. For example, in the nuclear tree *A. nancymae* is placed within the southern group with low support (BS = 65%, Fig. S2), while in the other analyses it was recovered within the northern group, also with low support values (Figs. 3, S1 and S3).

Similarly, all gene tree analyses recovered *A. trivirgatus* in the southern group although in the nuclear ML tree with low support (BS of concatML tree = 98%, Fig. S1; BS of nucML tree = 23%, Fig. S2; BS of mitML tree = 87%, Fig. S3), while in the species tree *A. trivirgatus* was recovered within the northern group, but also with low support (pp = 0.65, Fig. 3).

The position of *A. trivirgatus* as sister to the *A. nigriceps* + *A. boliviensis*/*A. infulatus* clade (Fig. S1 and S3) was already reported in previous studies (Menezes et al., 2010; Ruiz-García et al., 2011, 2016), but all of these studies used only mitochondrial markers, except for Ruiz-García et al. (2016) who grouped *A. trivirgatus* and these southern species using 12 microsatelite loci. The phylogenetic position of *A. trivirgatus* reported in Perelman et al. also (2011) agrees with that in our species tree showing *A. trivirgatus* to be nested within the northern group, as sister to *A. griseimembra*.

As gene trees do not necessarily represent species tree (Heled and Drummond, 2010), we consider here the branching pattern observed in the species tree as the most plausible and we suggest that the observed discrepancies among the different analyses can be explained by the occurrence of incomplete lineage sorting or historical hybridization (Choleva et al., 2014; Menezes et al., 2010; Peters et al., 2007; Plautz et al., 2009), as well as the kind of the molecular markers used in the analyses.

The disagreement in the position of *A. nancymae* observed between the nuclear and other phylogenies can be explained by the poor phylogenetic signal of the nuclear markers in contrast to the strong phylogenetic signal of the mitochondrial markers. The evolutionary history of night monkeys is marked by recent and successive diversifications that occur mainly in the Pleistocene (Fig. 3). This pattern of successive diversifications in a short frame time does not seem to have allowed nuclear markers, which present a low evolutionary rate, to accumulate significant differences to establish the phylogenetic relationships among *Aotus* species. This is reinforced by the low statistical support obtained throughout the nuclear tree as a whole. On the other hand, as the mitochondrial genome is associated with higher evolutionary rates, the divergence times between the different lineages seems to have been enough to recover the phylogenetic relationship with stronger supports. However, since the support value for the position of *A. nancymae* was not higher than 90% – although it was closer in the mitochondrial tree (BS = 86%, Fig. S3) – historical hybridization may have acted in the evolution of the genus.

The disagreement among datasets in the phylogenetic position of *A. nancymae* may be explained by past hybridization with mitochondrial introgression (Linder and Riesenberger, 2004; Sequeira et al., 2011). If historical hybridization occurred in *Aotus*, it may have occurred between ancestral populations from the north and south of the Amazon-Solimões River, which has already been proposed for other Neotropical monkeys (Lima et al., 2018; Martins-Junior et al., 2018).

The possibility of historical hybridization is strengthened by the observation that some species currently occur in sympatry, such as *A. vociferans* and *A. nigriceps* along the Purus River in Brazil (Hershkovitz, 1983) and *A. nancymae* and *A. vociferans* between Amazon/Napo and Tigre rivers (Aquino and Encarnación, 1988).

It is important to consider, however, that the current hybridization between *A. nancymae* and *A. vociferans* seems highly improbable because their karyotypes are very different (2n = 54 versus 2n = 46, 47, 48, respectively). In the areas where both species coexist in sympatry, no natural hybrids are reported (for example, Marañon or Tigre rivers), as well as when in captivity crosses between both species occurred, the hybrids were aborted or died few days after birth (Ruiz-García, unpublished observations). Further studies investigating the possible incomplete lineage sorting or/and historical introgression are necessary to better understand the evolutionary history of night monkeys.

Although we did not use *Aotus miconax* in our analyses, it is possible to make some remarks. Morphological data indicate *A. miconax* as a red-necked night monkey, similar to (Hershkovitz, 1983; Groves, 2001) or sister to *A. nancymae* (Ford, 1994). In the only molecular phylogenetic study using *A. miconax*, Ruiz-García et al. (2016) recovered it as sister species of *A. nancymae* and in the northern group. Taking this into account, we suggest that *A. miconax* should be considered a northern night monkey, sister to *A. nancymae*. Future molecular phylogenetic studies using more samples of *A. miconax* from multiple localities may be used to test this hypothesis.

Our results disagree with the previous classifications of *Aotus lemurinus* and *A. griseimembra*, either as a synonym of *A. vociferans* (Ford, 1994) or as two subspecies of *A. lemurinus* (Hershkovitz, 1983; Groves, 2001). Clearly, *A. lemurinus* is the sister species of *A. griseimembra*, but *A. vociferans* is part of a distinct clade (Figs. 3, S1, S2 and S3). The sister group relationship of these two clades is only weakly supported. Our analysis does not include the other northern night monkey species, such as *A. zonalis* and *A. brumbacki*, however, the study of Ma et al. (1985) suggested that eastern Colombian population of *A. brumbacki* originated *A. vociferans* due to their shared acrocentric Y chromosomes. In the same way, Ford (1994) did not find substantial morphological differences between these two species, indicating that *A. brumbacki* is a synonym of *A. vociferans*. Ruiz-García et al. (2016) showed the existence of only very small DNA differences between *A. zonalis* and *A. lemurinus*. Similarly, Defler and Bueno (2007), based on karyotype, also suggested a close relationship between *A. zonalis* and *A. lemurinus*. All these lines of evidence point to *Aotus lemurinus* and *A. griseimembra* not being subspecies of *A. vociferans* or even very closely related to it, and, therefore, should be considered valid species.

Our phylogenetic results also are in agreement with previous morphological, karyological and mtDNA studies that recovered *Aotus nigriceps* as the sister species of the clade formed by *A. azarae*, *A. boliviensis* and *A. infulatus* (Babb et al., 2011; Ford, 1994; Menezes et al., 2010). It is concordant with the unique sexual chromosome system of $X_1X_1X_2X_2/X_1X_2Y$ and the reciprocal translocation between the Y chromosome and the short arm of an autosome shared by both species (Defler and Bueno, 2007).

One important debate in night monkey taxonomy regards the number of species in the *Aotus azarae* complex. Some morphological studies suggest *A. infulatus* as a full species and *A. azarae* as a polytypic taxon including *A. a. boliviensis* and *A. a. azarae* (Hershkovitz, 1983). Ford (1994) proposed *A. boliviensis* as a subspecies of *A. infulatus* and *A. azarae* as a monotypic taxon. Groves (2001) considered *boliviensis* and *infulatus* as subspecies of *A. azarae*. Conversely, karyological data indicates that *boliviensis* and *infulatus* are the same species (Pieckzarka and Nagamachi 1988; Pieckzarka et al. 1993).

Unfortunately, we did not analyze any sample of *Aotus azarae*. However, three distinct geographically-restricted lineages were recovered in our analysis: a clade of *A. infulatus* from right bank of Tocantins River, Brazil, and another from the left bank of Tocantins River and right bank of Xingu River, and a third clade of *A. boliviensis* from the area of the Samuel Hydroelectric Plant in Rondonia, Brazil (in the Madeira/Tapajós interfluve) (Figs. S1 and S3). Even if these lineages do not represent species, their divergence may be an indicative of an ongoing speciation. Schneider et al. (1989) suggested that the populations of *A. infulatus* from the right bank of Tocantins River may be undergoing rapid evolutionary change. Here, considering the molecular evidence framework produced in this study, we suggest that *Aotus infulatus* and *A. boliviensis* should be considered valid species. Future studies with samples from Paraguay, northern Argentina, and the Cerrado of Mato Grosso in Brazil, will help to elucidate the taxonomy of this group.

The taxonomy of *Aotus* is very controversial either based on morphology, karyology, or DNA sequences. Our molecular results provide significant insights into night monkeys taxonomy and in the light of the previous and the current evidences, we suggest a reclassification in the taxonomy of *Aotus*, considering two distinct groups: 1. the northern group, which is composed of *Aotus nancymae*, *A. miconax*, *A. vociferans*, *A. trivirgatus*, *A. griseimembra*, *A. lemurinus*, *A. zonalis*, *A. brumbacki*; and 2. the southern group, which is composed of *A. nigriceps*, *A. azarae*, *A. boliviensis*, and *A. infulatus* (Table 1).

4.2 Biogeographical history of night monkeys

The estimated time for the origin of the night monkeys was much more recent than those reported by Babb et al. (2011), Ruiz-García et al. (2011) and Kiesling et al. (2015), but similar to those of Ashley and Vaughn (1995), Plautz et al. (2009), and Menezes et al. (2010). The differences between these estimated ages may be related to methodological differences in divergence time estimation. The most obvious discrepancy is the use of the †*Aotus dindensis* fossil from the Miocene deposits of the Colombia La Venta formation as the crown *Aotus* for the calibration of divergence time estimates made by Babb et al. (2011) and Ruiz-García et al. (2011). The estimated age of this fossil is between 11.8 and 13.5 Ma. However, as showed by Kay et al. (2015) and Marivaux et al. (2016), this fossil is not a night monkey and is more probably related to the Callitrichinae lineage. Our estimated crown age of *Aotus* (4.11 Ma) also suggests that "†*Aotus dindensis*" is not a night monkey congeneric.

Due to few terminal tips in the phylogeny, it is expected that there will be low power to distinguish between the DIVALIKE and DIVALIKE-J models in BioGeoBEARS. We observed no significant differences between the likelihood values of these two models in the chi-squared test, as well as a very small difference between AIC values. However, the AICc value was much lower for the DIVALIKE model when compared to the DIVALIKE-J (Table 3). Both models recovered similar histories, but we chose to present the DIVALIKE-J in Fig. 2 and discuss the differences because of three main points: first, the way to reach the Andes and Northern South America – either via dispersal or founder events – does not change the discussion of the role of the Andes as a geographical barrier (see below); second, in the same way, both models corroborate the recent invasion of and expansion in the eastern Amazon albeit with different age estimates; and third, because there is no clear geological/climatic process to explain the vicariance between the Cerrado and Inambari groups within the estimated time frame (either following Hoorn et al. [2010] or Ribas et al. [2012]) as shown in the DIVALIKE model, since these areas are not contiguous. The results under the DIVALIKE model are available as supplementary material (Fig. S4).

Previous studies suggest two distinct ancestral areas for the night monkeys. One hypothesis states that the ancestral populations of night monkeys were distributed somewhere north of the Marañon/Amazon River in the northwestern Amazon basin and subsequently crossed the Amazon River to the south (Ford, 1994; Hershkovitz, 1983; Ma, 1981; Menezes et al., 2010). The other hypothesis states that the genus originated in the eastern Amazon with subsequent expansion westward over time (Babb et al., 2011).

Here, we tested different models and those selected by 'BioGeoBEARS' as the most likely to explain the biogeographical history of the night monkeys disagree with previous propositions. Both selected models (DIVALIKE-J and DIVALIKE) suggest the Central Amazon basin (both north and south of the Amazon River) as the area of origin of the night monkeys, with the ancestral populations of *Aotus* occurring throughout the Imeri and Rondonia regions (Figs. 2 and S4). This biogeographical scenario, both its spatial as well as temporal aspects, is very similar to that proposed for the diversification of the genus *Plecturocebus* (Byrne et al., 2018).

The estimated age of origin of *Aotus* (4.11 Ma [95% HPD: 2.93 – 4.27]) is chronologically close to the final uplift of the Central and Northern Andes (Burgos, 2006; Garver et al., 2005; Graham et al., 2001; Gregory-Wodzicki et al., 1998; Hooghiemstra et al., 2006; Mora et al., 2010). This tectonic event seems to have led to increased rainfall along the eastern slope of the Andes, the disappearance of the Acre system and subsequent establishment of lowland rainforests in central-west Amazon, very similar to the current Amazonian landscape (Hoorn et al., 2010).

This time is also concordant with the formation and stabilization of the modern Amazon basin river network, which was established in the Late Miocene and Early Pliocene (Figueiredo et al., 2009; Hoorn et al., 2010; Insel et al., 2010; Mora et al., 2010; Poulsen et al., 2010). The vicariance event separating the northern and southern ancestral night monkey populations seem to have been caused by the formation of the Amazon-Solimões River system.

Against this older origin of the Amazon River system, other geological data suggest a Plio-Pleistocene history for the transition of a western lacustrine system to the current drainage network (Campbell et al., 2006; Latrubesse et al., 2010; Nogueira et al., 2013). In this scenario, only recently the floodplains and lowland forests were formed in the western Amazon. Our data give more support to an "older" origin over a "younger" one for the establishment of the Amazon drainage system, given the older estimated age for the origin and vicariance events in *Aotus*, and its central-western Amazon ancestral area (Fig. 2).

The two main clades of *Aotus* diversified at markedly different times, with the northern group emerging in the Early Pliocene, and southern group more recently at Early to Mid-Pleistocene. Most species arose during the Pleistocene, a pattern also found in other Neotropical vertebrates (e.g. Boubli et al., 2018; Carneiro et al., 2018; Lima et al., 2017; Lynch Alfaro et al., 2015a; Martins-Junior et al., 2018; Ribas et al., 2012). This recent period of diversification of the genus (Plio-Pleistocene), after a long period that the *Aotus* lineage separated from other Cebidae at ~ 20 Ma (Kiesling et al., 2015; Perelman et al., 2011), may be associated to an intense landscape change in the Neotropics during this period (Hoorn et al., 2010; Insel et al., 2012; Zachos et al., 2001). This rapid diversification may also be related to the intense chromosomal rearrangements, as the group is one of the most diverse in terms of karyotypes among New World monkeys (Defler and Bueno, 2007).

The biogeographical model shown here weakens both sea rise level (Plautz et al., 2009) and the Pleistocene refuge (Ma, 1981) hypotheses to explain *Aotus* diversification. Despite the Pleistocene origin of the modern night monkey lineages, these hypotheses assume vicariance events caused by isolation of populations in separated refuges with subsequent accumulations of differences as the driving force for speciation (Haffer, 1969; Plautz et al., 2009). Vicariance events seem to be not an important force acting in the recent diversification of *Aotus*, given that all of them occurred prior to the Pleistocene and none of them led to the origin of the modern species (Fig. 2). Even in the DIVALIKE model, the recent vicariance event separating the populations of Bolivia and Cerrado/Pantanal at ~ 600 Ky (Fig. S4), may be associated with the drainage of the South American Chaco and the formation of the Paraguay River (Rosenberger et al., 2009).

Conversely, the major Amazonian rivers may have played an important role in subsequent diversification of the night monkeys, acting as geographic barriers for long periods, which were followed by occasional periods when river-crossing occurred (Figs. 2 and S4). These punctual migrations may have been influenced by the Quaternary geological events and climatic fluctuations, but not necessarily related to the forest refuges (Baker and Fritz, 2015; Hoorn et al., 2010).

In the early diversification of the northern group (at ~ 3.7 Ma), the selected models suggest that northern ancestral populations crossed the Negro River eastward expanding its distribution and reaching the Guyana Highlands, where *Aotus trivirgatus* originated (Figs. 2 and S4). This pattern of origin in the

Amazonian lowlands with subsequent dispersal to the highlands was also recovered in studies with other vertebrate taxa (Carneiro et al., 2018). However, this scenario should be interpreted with caution, due to the low statistical support for the phylogenetic position of *A. trivirgatus*.

Considering the DIVALIKE-J model (Fig. 2), some individuals from the ancestral population of Imeri likely crossed the Andes, colonizing the western slopes and lowlands, followed by a recolonization of the eastern slopes of the Andes in Ecuador, and subsequent expansion within the northwestern Amazon at ~ 3 Ma (Fig. 2). The DIVALIKE model, on the other hand, suggests a slightly older age for the crossing of the Andes and colonization of the trans-Andean areas at ~ 3.7 Ma (Fig. S4). In both cases, the estimated ages (between 3.7 – 3.0 Ma) are consistent with the timing of the final uplift of the Northern Andes (Hoorn et al., 2010). At this time, the elevation of the Northern Andes was already over 2000 meters (Gregory-Wodzicki, 2000; Hoorn et al., 2010; Mora et al., 2010) indicating that the Northern Andes probably were not a geographical barrier for dispersal of the night monkeys.

Another vicariance event at ~ 3 Ma divided the northwestern Amazon populations from northern South American ones (Figs. 2 and S4) where *Aotus lemurinus* and *A. griseimembra* arose in the last 1.6 My (Fig. 3). The species *A. zonalis* was not used in this study, but from a biogeographical perspective, it is plausible that this group is phylogenetically closely related to the *A. lemurinus*/*A. griseimembra* clade (Defler and Bueno, 2007; Ruiz-García et al., 2016) and may have arisen after its arrival in Central America, following the final stages of the formation of the Panama Isthmus (Hoorn and Flantua, 2015).

From the northwestern Amazon, two opposing dispersals, one towards the north and one towards the south, appear to have occurred (Figs. 2 and S4). In the northwestern Amazon, the Solimões-Marañon River seems to have acted as a southern geographical barrier until the ancestor of *Aotus nancymae* dispersed to Inambari in recent times. Similarly, the Japurá River acted as a northern geographical barrier in the northwestern Amazon until the recent dispersal into the Imeri region and the origin of *A. vociferans* (Figs. 2 and S4), occupying the same biogeographical area of *A. trivirgatus* in the Japurá/Negro interfluvium. As the distribution of *A. vociferans* is limited to the east by the Negro River, this river has acted as a geographical barrier for this species. Interestingly, the occurrence of *A. trivirgatus* in the Negro/Japurá interfluvium appears to be a result of an ancient colonization of this region instead of a recent one.

In the southern group, ancestral populations seem to have been restricted to the original area of Rondonia and Inambari centers of endemism (Figs. 2 and S4) until very recently. Based on the DIVALIKE-J model, some individuals from these ancestral populations crossed the Tapajós River eastward to eastern Amazon some 1.7 Ma. At approximately 600 Ky, this lineage diverged into *A. infulatus* (Fig. 2). It is interesting to note that in less than 600 Ky the *A. infulatus* crossed the Xingu and Tocantins rivers eastward and the mouth of the Amazon River on to the Marajó island, and expanded into the Cerrado and Pantanal biomes (Fig. 2). Its sister lineages, *A. boliviensis*, expanded from Bolivia biogeographical zone to Rondonia center of endemism to occupy this entire area. Even in the DIVALIKE model (Fig. S4), ancestral populations reached the eastern Amazon from the Cerrado/Pantanal in the last 600 Ky, crossing these rivers. At this time, the Xingu and Tocantins rivers were barriers for other groups of primates (Lynch Alfaro et al., 2015a; Ribas et al., 2012), but evidently not for the night monkeys. On the other hand, the Tapajós River seems to be an important geographical barrier for the night monkeys.

In the last 600 Ky, populations from the Pantanal seem to have crossed the Paraguay River towards Bolivia and expanded its distribution returning to the Rondonia endemism center, giving rise to *A. boliviensis* (Fig. 2). Until very recently, it has been assumed that night monkeys were limited to the east by the Cerrado biome in Brazil. Night monkeys became established in the Cerrado only at about 600 Ka (Fig. 2) or at ~ 1.6 Ma (Fig. S4). The occurrence of night monkeys in Cerrado is an indication that this region was not a strong geographical barrier for this genus and the absence of night monkeys in the eastern and southern Cerrado may be due to the very recent colonization of this biome. However, it seems that the genus continues to expand its distribution to the east reaching the Caatinga biome in Brazil (Pinto and Roberto, 2016). This pattern of dispersal out of the Amazon southeastward into the Brazilian plateau was already proposed for other Neotropical primates (Byrne et al., 2016).

The recent colonization of these biomes seems to be accompanied by physiological and ecological changes, given that night monkeys from higher latitudes exhibit diurnal activities (Fernandez-Duque and Erkert, 2006; Fernandez-Duque et al., 2010). Also, high trees, which are normally used by these monkeys for daily activities (Savagian and Fernandez-Duque, 2017; Wright, 1978), are scarce in the Cerrado and Caatinga, and animals in the Cerrado and Caatinga are generally found in shrubs and low trees. Further studies focusing on populations of eastern Amazon and adjacent areas of southern and eastern Brazil, Argentina and Paraguay, will clarify the palaeogeological and climatic factors that may be correlated with this range expansion.

5. CONCLUSIONS

The evolution of *Aotus* seems to be much more complex than previously thought, with intense chromosomal rearrangements within a very short timescale. We found nine lineages of night monkeys distributed in two main clades and, as suggested in previous studies, the clades are not concordant with Hershkovitz's species groups, and thus we propose a reclassification of the genus. The best way to refer to the two main groups is considering their geographical distribution. We proposed the northern group which contains species (*Aotus nancymae*, *A. miconax*, *A. griseimembra*, *A. lemurinus*, *A. vociferans*, *A. zonalis*, and *A. brumbacki*) that mainly occurs north of the Amazon-Solimões River, and the southern group composed of species (*A. nigriceps*, *A. boliviensis*, *A. infulatus*, and *A. azarae*) that occur only south of the Amazon-Solimões River.

We showed that the end of Andean uplift and the consequent alterations in the Amazonian landscape at the Miocene/Pliocene boundary and Pleistocene played a key role in the evolution of *Aotus*. The results do not corroborate the Pleistocene refuge hypothesis for night monkey speciation.

A number of major rivers impacted the diversification of some lineages of night monkeys by acting as geographical barriers for long periods, however, most of the Amazonian rivers did not. The northern Andes was not a geographical barrier for the dispersal of the genus, but some geological and climatic changes in this region may have influenced the speciation process. More studies adding more sample from the north of the South America and Central America will help to better understand the evolution in these regions.

The very recent and widespread southeastward expansion of the clade formed by *A. azarae*, *A. infulatus* and *A. boliviensis*, may be an indication that the Pleistocene climate changes did not produce geographic barriers for the night monkeys. More studies using more samples from Cerrado, Pantanal, Bolivia, Paraguay and Argentina are necessary to understand the factors that may have acted in the evolution of this young and widespread group.

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Appendix A. Supplementary material

Supplementary Table S1. List of the molecular markers sequenced, their respective primers, category and annealing temperature.

Supplementary Table S2. GenBank codes of the sequences for each marker used as outgroup.

Supplementary Table S3. Partitioning schemes, their respective length, and model for BEAST and RAxML analyses, estimated by PartitionFinder 2.1.1 (Lanfear et al., 2016).

Supplementary Table S4. List of the GenBank accession numbers for all sequences produced in the current study.

Supplementary Fig. S1. Maximum Likelihood topology estimated by the concatenated mtDNA/nuDNA dataset. Numbers above and below of the branches, or ahead the nodes indicate the bootstrap values. Grey and red bars represent the Hershkovitz's grey- and red-necked species.

Supplementary Fig. S2. Maximum Likelihood topology estimated by the nuclear dataset. Numbers above and below of the branches, or ahead the nodes indicate the bootstrap values. Grey and red bars represent the Hershkovitz's grey- and red-necked species.

Supplementary Fig. S3. Maximum Likelihood topology estimated by the mitochondrial dataset. Numbers above and below of the branches, or ahead the nodes indicate the bootstrap values. Grey and red bars represent the Hershkovitz's grey- and red-necked species.

Supplementary Fig. S4. Estimated ancestral area of *Aotus* and its phylogeographic evolutionary pattern showed by DIVALIKE model. NS = North of the South America, GH = Guyana Highlands, IM = Imeri, NW = Northwest Amazon, IN = Inambari, RN = Rondonia, EA = Eastern Amazon, CP = Cerrado and Pantanal, and BO = Bolivia.

Competing interests

The authors declare that they have no conflict of interest.

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Tables.

Table 1. Major taxonomic reviews of the night monkeys (*Aotus*).

Hershkovitz (1983)	**Ford (1994)**	**Groves (2001)**	**Present Study**
Red-necked group	**Red-necked group**	**Red-necked group**	**Southern group**
A. miconax	*A. miconax*	*A. miconax*	–
A. nigriceps	*A. nigriceps*	*A. nigriceps*	*A. nigriceps*
A. infulatus	*A. infulatus*	*A. azarae infulatus*	*A. infulatus*
A. azarae azarae	*A. azarae*	*A. azarae azarae*	*A. azarae*
A. a. boliviensis	–	*A. a. boliviensis*	*A. boliviensis*
A. nancymae	*A. nancymae*	*A. nancymae*	–
Grey-necked group	**Grey-necked group**	**Grey-necked group**	**Northern group**
A. lemurinus lemurinus	–	*A. lemurinus lemurinus*	*A. lemurinus*
A. l. griseimembra	–	*A. l. griseimembra*	*A. griseimembra*
A. brumbacki	–	*A. l. brumbacki*	*A. brumbacki*
–	–	*A. l. zonalis*	*A. zonalis*
A. vociferans	*A. vociferans*	*A. vociferans*	*A. vociferans*
A. trivirgatus	*A. trivirgatus*	*A. trivirgatus*	*A. trivirgatus*
–	–	*A. hershkovitzi*	–
			A. nancymae
			A. miconax

– Taxa not proposed or not included in the group of species.

Table 2. List of species used, sample information such as code name, tissue used for DNA extraction, locality origin and the biogeographical region assigned for the 'BioGeoBEARS' analysis.

Taxon	Code	Tissue	Locality	'BioGeoBEARS' areas
Aotus nigriceps	Ab1	Skin	Madre de Dios River – PER	Rondonia (RN) and Inambari (IN)
	TER12	Blood	Teresina Zoo – PI, BRA	
	Aot789	Muscle	Sena Madureira River – AC, BRA	
	CTGAM187	Muscle	LB Purus River – AM, BRA	
A. infulatus	Ai1801	Blood	RB Tocantins River – PA, BRA	Eastern Amazon (EA) and Cerrado/Pantanal (CP)
	Ai1673	Blood	RB Tocantins River – PA, BRA	
	MP01	Muscle	47 km NE of Tailândia – PA, BRA	
	Ai1686	Blood	LB Tocantins River – PA, BRA	
	AI-IM1	Blood	Marajó Island – PA, BRA	
	AI-IM2	Blood	Marajó Island – PA, BRA	
	At352	Blood	LB Tocantins River – PA, BRA	
	At279	Blood	LB Tocantins River – PA, BRA	
	MCB56	Liver	Xingu River – PA, BRA	
	MCB57	Liver	Xingu River – PA, BRA	
	MCB58	Liver	Xingu River – PA, BRA	
A. boliviensis	At2510	Blood	UHE Samuel, Jamari River, RN, BRA	Rondonia (RN) and Bolivia (BO)
	Aa8177	Blood	UHE Samuel, Jamari River, RN, BRA	
	Aaz14066	Blood	UHE Samuel, Jamari River, RN, BRA	
	Aaz4537	Blood	UHE Samuel, Jamari River, RN, BRA	
	An2476	Blood	UHE Samuel, Jamari River, RN, BRA	
	Aaz13341	Blood	UHE Samuel, Jamari River, RN, BRA	
	Aot2508	Blood	UHE Samuel, Jamari River, RN, BRA	
A. nancymae	An2025	Blood	Iquitos – PER	Inambari (IN) and Northwest Amazon (NW)
	Any3081	Blood	Vicinity of Letícia, SAR – COL	
	Any3141	Blood	Vicinity of Letícia, SAR – COL	
	An1994	Blood	Iquitos – PER	
	An1959	Blood	Iquitos – PER	
	An2031	Blood	Iquitos – PER	
	An1	Blood	Tapiche River – PER	
A. trivirgatus	CTGAM759	Muscle	LB Japurá River – AM, BRA	Negro (NE) and Guyana Highlands
A. vociferans	Av3051	Blood	Letícia – COL	Northwest Amazon (NW) and Negro (NE)
	Av2047	Blood	Iquitos – PER	
	Av1	Blood	Puerto Nariño, Loretoyaku River – COL	
	Av3	Blood	Puerto Nariño, Loretoyaku River – COL	
	Av4	Blood	Puerto Nariño, Loretoyaku River – COL	
	Av5	Blood	Puerto Nariño, Loretoyaku River – COL	
A. griseimembra	ALGC21	Blood	Captivity	North of South America (NS)
	ALG2N52	Blood	Captivity	
	ALGM71	Blood	Captivity	
	ALGM60	Blood	Captivity	

	ALGM20	Blood	Captivity	
	ALGM45	Blood	Captivity	
	ALGD13	Blood	Captivity	
	ALGD67	Blood	Captivity	
A. lemurinus	ALLUSA	Blood	Captivity	North of South America (NS)

Abbreviations: BRA = Brazil, COL = Colombia, PER = Peru, AM = Amazonas, AC = Acre, PA = Pará, PI = Piauí, RN = Rondonia, DEPT = Department, SAR = South of the Amazon River, LB = Left Bank, RB = Right Bank.

Table 3. Comparison among 'BioGeoBEARS' models. Log-Likelihood values (LnL), Dispersal (d), Extinction (e), Founder (j), Akaike Information Criterion (AIC), ΔAIC value, corrected AIC (AICc), and ΔAICc value.

Model	LnL	n. params	d	e	j	AIC	ΔAIC	AICc	ΔAICc
DIVALIKE+J	-26.54	3	0.081	1.0e – 12	0.084	59.08	0	65.08	3.4
DEC+J	-27.52	3	0.072	1.0e – 12	0.23	61.03	1.95	67.03	5.35
BAYAREALIKE+J	-28.05	3	0.35	0.21	0.17	62.1	3.02	68.10	6.42
DIVALIKE	-27.64	2	0.12	3.3e – 08	0	59.28	0.20	61.68	0.00
DEC	-28.80	2	0.49	0.23	0	61.61	2.52	64.01	2.33
BAYAREALIKE	-30.50	2	0.57	0.53	0	64.99	5.91	67.39	5.71

Figure 1. Distribution of night monkey genus *Aotus* species in South and Central America. The map is based on Hershkovitz (1983), Pieczarka et al. (1993), Ford (1994) and IUCN (2018).

Figure 2. Estimated ancestral area of *Aotus* and its phylogeographic evolutionary pattern. Biogeographic regions tested in 'BioGeoBEARS' analysis and the major rivers in *Aotus* distribution (left). Consensus chronogram tree showing the biogeographical history of night monkeys through the DIVALIKE-J model (right). NS = Northern South America, GH = Guyana Highlands, IM = Imeri, NW = Northwest Amazon, IN = Inambari, RN = Rondonia, EA = Eastern Amazon, CP = Cerrado and Pantanal, and BO = Bolivia.

Figure 3. Bayesian phylogenetic reconstructions in BEAST 1.8.4 and BEAST 2.4.8. Dated gene tree (left) and branching pattern of the species tree (right). In the gene tree, numbers above or below the branches indicate the divergence times, while in the species tree, numbers ahead of nodes indicate the posterior probability supports. Grey and red bars represent the Hershkovitz's grey- and red-necked species. Illustrations copyright 2013 Stephen D. Nash / IUCN SSC Primate Specialist Group. Used with permission.

Figure S1. Maximum Likelihood topology estimated by the concatenated mtDNA/nuDNA dataset. Numbers above and below of the branches, or ahead the nodes indicate the bootstrap values. Grey and red bars represent the Hershkovitz's grey- and red-necked species.

Figure S2. Maximum Likelihood topology estimated by the nuclear dataset. Numbers above and below of the branches, or ahead the nodes indicate the bootstrap values. Grey and red bars represent the Hershkovitz's grey- and red-necked species.

Figure S3. Maximum Likelihood topology estimated by the mitochondrial dataset. Numbers above and below of the branches, or ahead the nodes indicate the bootstrap values. Grey and red bars represent the Hershkovitz's grey- and red-necked species.

Figure S4. Estimated ancestral area of *Aotus* and its phylogeographic evolutionary pattern showed by DIVALIKE model. NS = North of the South America, GH = Guyana Highlands, IM = Imeri, NW = Northwest Amazon, IN = Inambari, RN = Rondonia, EA = Eastern Amazon, CP = Cerrado and Pantanal, and BO = Bolivia.

Figure 1

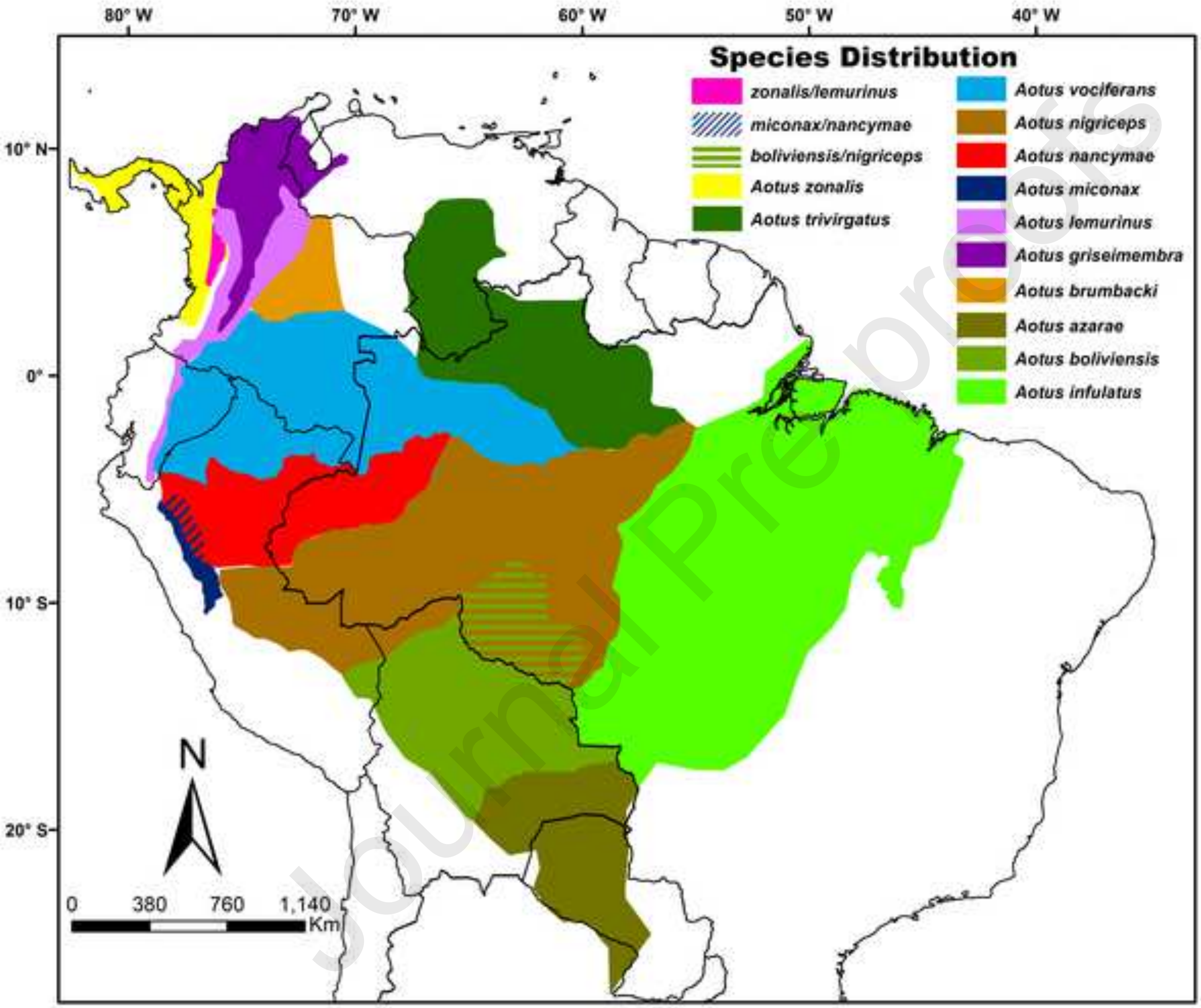
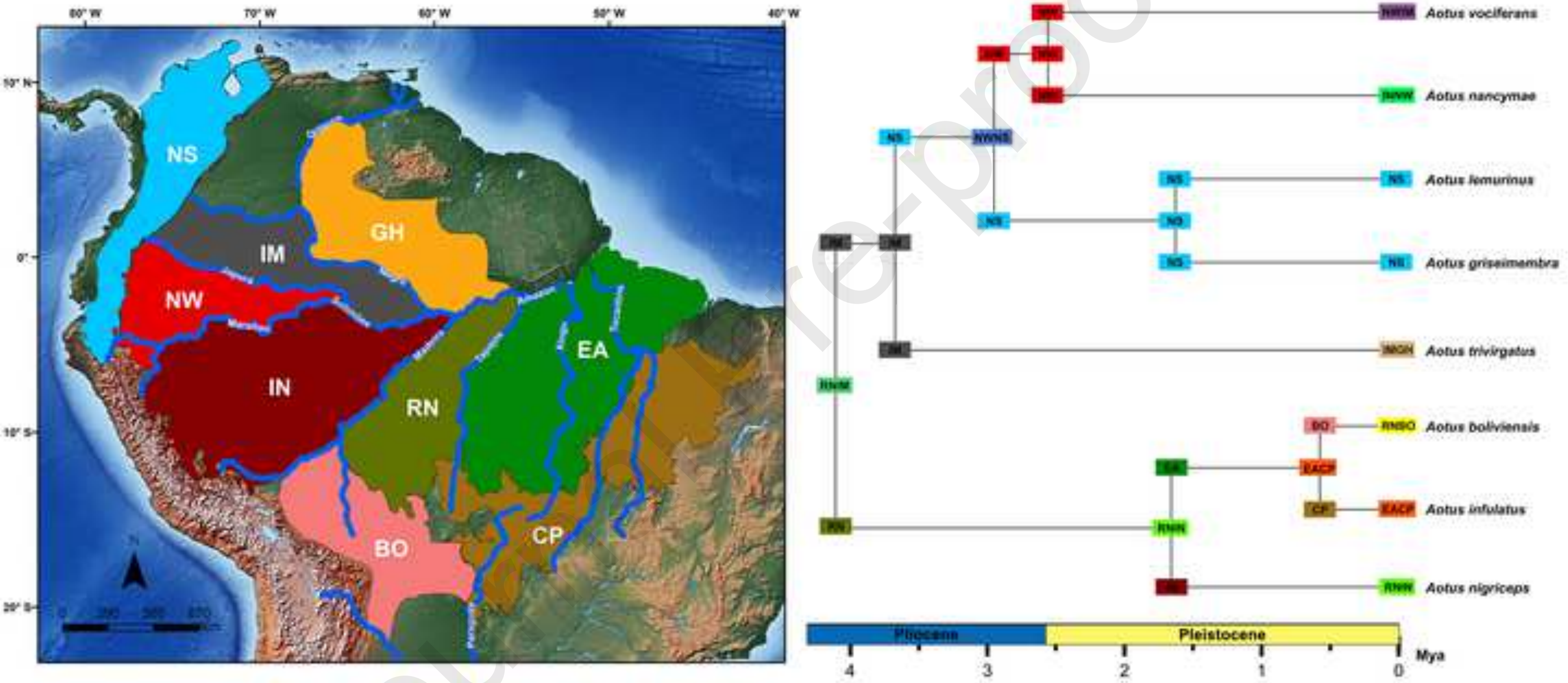
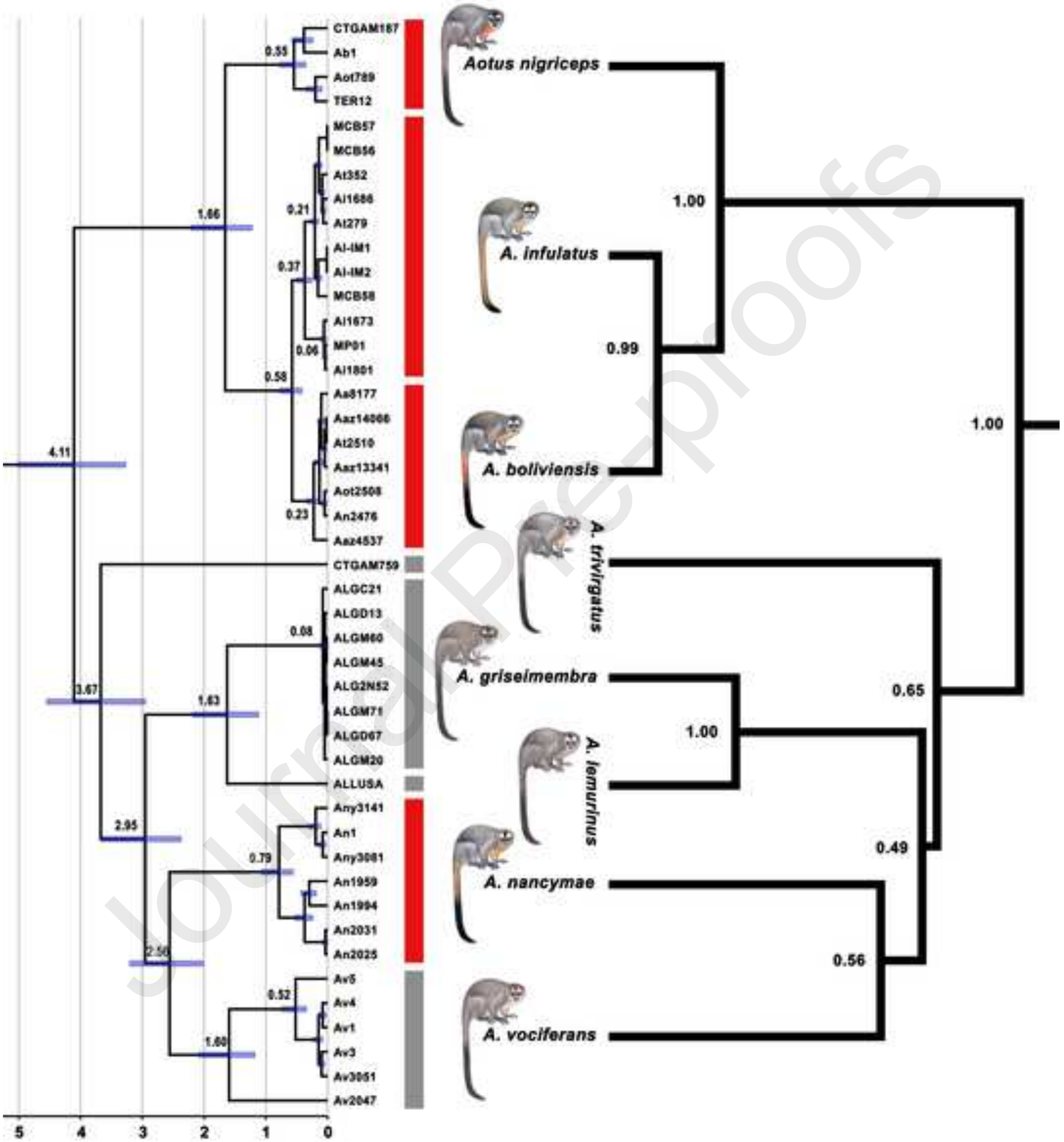

Species Distribution
zonalis/lemurinus
miconax/nancymae
boliviensis/nigriceps
Aotus zonalis
Aotus trivirgatus
Aotus vociferans
Aotus nigriceps
Aotus nancymae
Aotus miconax
Aotus lemurinus
Aotus griseimembra
Aotus brumbacki
Aotus azarae
Aotus boliviensis
Aotus infulatus
80° W
70° W
60° W
50° W
40° W
10° N
0°
10° S
20° S
N
0
380
760
1,140
Km

Figure 2


80° W
70° W
60° W
50° W
40° W
10° N
0°
10° S
20° S
NS
IM
GH
NW
IN
RN
EA
CP
BO
N
0
280
560
870
Km
NW
NWNS
NS
IM
RNIM
RN
RNIN
EA
EACP
BO
CP
NWIM
Aotus vociferans
INNW
Aotus nancymae
NS
Aotus lemurinus
NS
Aotus griseimembra
IMGH
Aotus trivirgatus
RNBO
Aotus boliviensis
EACP
Aotus infulatus
RNIN
Aotus nigriceps
Pliocene
Pleistocene
4
3
2
1
0
Mya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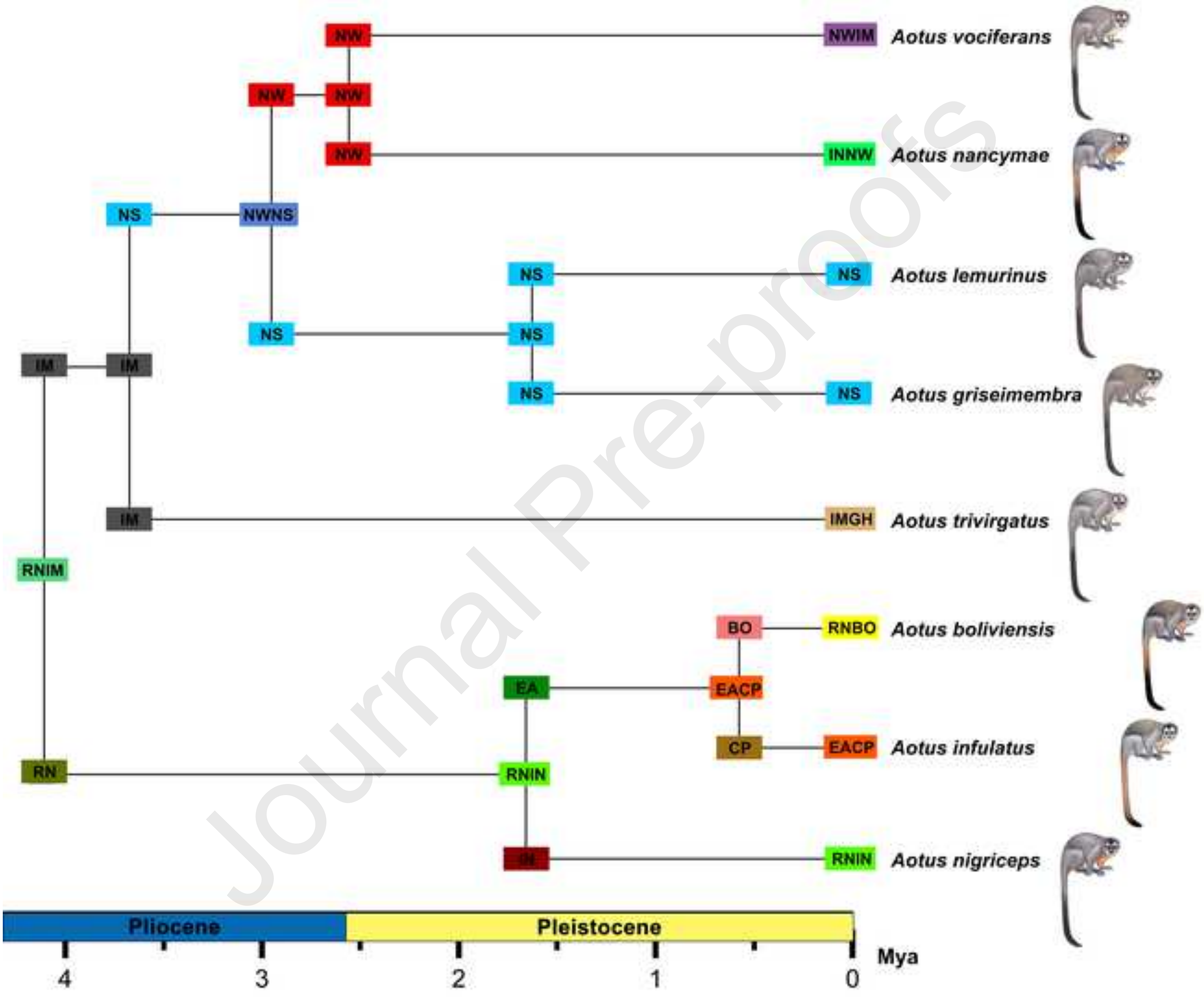
Ab1
Aot789
TER12
MCB57
MCB56
At352
Al1686
At279
Al-IM1
Al-IM2
MCB58
Al1673
MP01
Al1801
Aa8177
Aaz14066
At2510
Aaz13341
Aot2508
An2476
Aaz4537
CTGAM759
ALGC21
ALGD13
ALGM60
ALGM45
ALG2N52
ALGM71
ALGD67
ALGM20
ALLUSA
Any3141
An1
Any3081
An1959
An1994
An2031
An2025
Av5
Av4
Av1
Av3
Av3051
Av2047
0.55
1.66
0.21
0.37
0.06
0.58
0.23
4.11
3.67
0.08
1.63
2.95
0.79
2.56
0.52
1.60
5
4
3
2
1
0
Aotus nigriceps
A. infulatus
A. boliviensis
A. trivirgatus
A. griseimembra
A. lemurinus
A. nancymae
A. vociferans
1.00
0.99
1.00
1.00
0.65
0.49
0.56


NW
NWIM
Aotus vociferans
NW
NW
NW
INNW
Aotus nancymae
NS
NWNS
NS
NS
Aotus lemurinus
NS
NS
IM
IM
NS
NS
Aotus griseimembra
IM
IMGH
Aotus trivirgatus
RNIM
BO
RNBO
Aotus boliviensis
EA
EACP
CP
EACP
Aotus infulatus
RN
RNIN
IN
RNIN
Aotus nigriceps
Pliocene
Pleistocene
4
3
2
1
0
Mya

Highlights

- Multilocus analysis do not corroborate morphology-based groups.
- All species studied were recovered as monophyletic.
- The uplift of the Andes in the Pliocene was crucial for the origin of *Aotus*.
- Night monkeys emerged in the Central Amazon Basin.
- Most of the Amazonian rivers were not geographical barriers for night monkeys.

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