Genetic Diversity and Insights into the Distribution of Brown Howler Monkeys (*Alouatta guariba* Group) (Atelidae, Alouattinae)

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Abstract

The assessment of genetic diversity and karyotypes can help to identify new species and taxonomic units and contribute to species conservation. Ecological niche modeling can be used to predict species distribution and to estimate the impact of climate changes on its survival. The *Alouatta guariba* group is one of the most threatened primates in the Americas, with two endemic Atlantic Forest subspecies: *A. g. guariba* and *A. g. clamitans*, referred to as Northern and Southern populations, respectively. Although extensively studied, the distribution and the genetic diversity of these subspecies remains unclear. We assessed species relationships and genetic diversity in the *A. guariba* group and evaluated the potential future distribution of populations. We sequenced the mitochondrial cytochrome b gene of 14 captive and 108 free-living individuals to generate a maximum likelihood phylogenetic tree and median-joining haplotype network and to estimate the nucleotide diversity between the taxa. We conducted neutrality tests to test for population expansion. We also linked ten new karyotypes, obtained from cell cultures from blood samples of captive individuals, with the positions of the clades in the gene tree. Finally, we used ecological niche modeling to predict the potential distribution of the Northern and Southern populations. We found three lineages within the *A. guariba* group: North A and North B associated with *A. g. guariba*, and South C associated with *A. g. clamitans*. The results showed that *A. guariba* group is a species complex, with reduced haplotype and nucleotide diversity within each lineage, compared with other *Alouatta* species. The neutrality tests and median-joining analysis suggest a recent diversification and expansion of each lineage, and the geographical data, based on the localities of the individuals that we analyzed genetically, increase the geographic distribution of *A. g. guariba*, expanding the distribution farther south in southeastern Brazil than previously described. Our findings show the distribution of

the *A. guariba* group and genetic diversity of recently expanding populations, contributing to the assessment of this Critically Endangered species. We also suggest that environmental losses and climate change are major factors in the drastic population decline of the *A. guariba* group.

Introduction

Primates exhibit a considerable level of genetic diversity within genera, and related species may show different levels of genetic diversity (Osada, 2015). Legislative definitions around the world include taxonomic levels of species, subspecies, varieties, and genetically distinct populations, allowing the protection of genetic diversity and evolutionary processes at different levels (Coates *et al*., 2018). Identifying lineages, which may be endemic, rare, or endangered, helps to implement private or public policies to conserve specific areas (Winters *et al*., 2012). Furthermore, the maintenance of genetic diversity is an essential condition for populations to evolve and adapt to environmental change, strengthening the ability of species and populations to survive (Hoban *et al*., 2021). In particular, human-induced climate change presents numerous potential threats to nonhuman primate species, many of which are already threatened by human activities, such as deforestation, hunting, and trade in exotic animals (Chapman *et al*., 2006; Ribeiro *et al*., 2009; Wiederholt & Post, 2010).

Platyrrhines are abundant and very diverse, with high species richness, including around 171 species mainly inhabiting the Amazonia and Atlantic Forest (Estrada *et al*., 2017). Despite being well studied, their genetic diversity is still insufficiently understood, and several new species and subspecies have been described or revalidated in the past 10 years (Boubli *et al*., 2016; Costa-Araújo *et al*., 2021; Dalponte *et al*., 2014; Gualda-Barros *et al*., 2012; Marsh, 2014). The main threats to primates are loss of habitat due to agriculture, followed by logging and wood harvesting, with the Atlantic Forest retaining the largest area of agricultural production (Estrada *et al*., 2017).

The family Atelidae encompasses the largest-sized platyrrhine genera, including howler monkeys of the genus *Alouatta* Lacépède, 1799. Howler monkeys are well-adapted to several morphoclimatic domains, with a wide distribution through southern Mexico to northern Argentina (Rylands, 2000), and the number of recognized species ranges from nine to 15 (Cortés-Ortiz *et al*., 2015a; Gregorin, 2006; Rylands & Mittermeier, 2009). The *Alouatta guariba* group encompasses two subspecies endemic to the Atlantic Forest: *Alouatta guariba clamitans* Cabrera, 1940 and *A. g. guariba* (Humboldt, 1812). The exact geographical distribution of these two subspecies is not well established (Fig. 1). Based on the IUCN Red List distribution, *A. g. clamitans,* also called the Southern Atlantic Forest population, occurs in the southern Atlantic Forest from Rio Grande do Sul
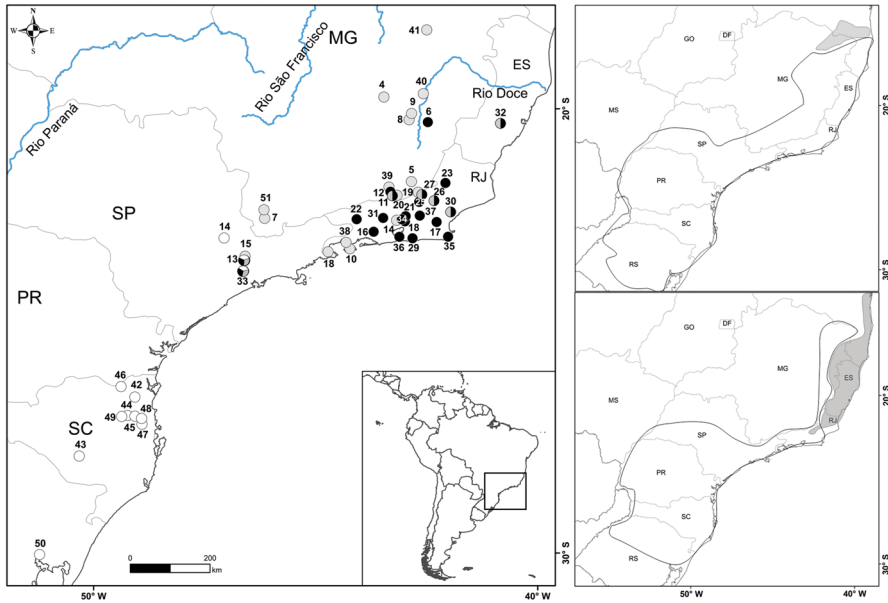
Fig. 1 Maps with localities of the samples used in analyses and the geographic distribution of *A. guariba* group based on IUCN (Top right, Buss *et al*., 2021; Neves *et al*., 2021) and morphological data (Bottom right, Gregorin, 2006). In the top and bottom right maps, the area delimited in grey shows the distribution of *A. guariba guariba*, and white the distribution of *A. guariba clamitans*. Numbers refer to localities listed in Table S1. Brazilian states are Espírito Santo (ES), Rio de Janeiro (RJ), Minas Gerais (MG), São Paulo (SP), Paraná (PR), Santa Catarina (SC), and Rio Grande do Sul (RS), Goiás (GO) and Mato Grosso do Sul (MS), and Federal District (DF). The samples used from ES, RJ, MG, SP, and RS states were obtained in 2013, 2014, 2017, 2018, and 2019.

(RS) to the south of Bahia (BA) (Buss *et al*., 2021), and *A. g. guariba*, also called the Northern Atlantic Forest population, occurs from the south of Bahia state to northeastern Minas Gerais state (Neves *et al*., 2021). However, the last taxonomic revision of the Brazilian species of *Alouatta* (Gregorin, 2006), based on morphological features, extended the distribution of *A. g. guariba* to Espírito Santo and northern Rio de Janeiro states, and narrowed the distribution of *A. g. clamitans* in the southeast region (Fig. 1). Although Gregorin (2006) elevated both subspecies to the level of species, most studies and the IUCN Red List (Buss *et al*., 2021; Neves *et al*., 2021), continue to refer to them as subspecies.

Previous studies hypothesized that *A. guariba* is a species complex based on karyotypes (de Oliveira *et al*., 1998; Steinberg *et al*., 2017), molecular markers (de Mello Martins *et al*., 2011; Harris *et al*., 2005), and morphological data (Gregorin, 2006), without a clear geographic distribution of each taxon. Like some other *Alouatta* species, *A. guariba* males have one fewer chromosome than females due to the translocation of the Y chromosome to an autosome (Koiffmann & Saldanha, 1974). The *A. guariba* group has two karyotypes, with a diploid number (2n) of 45 for males and 46 for females in the Southern population (de Oliveira *et al*., 2000; Steinberg *et al*., 2017), and 49 for males and 50 for

females in the Northern population (de Oliveira *et al.*, 1998, 2000). These differences in karyotype have led some authors to assume that the populations are reproductively isolated (de Oliveira *et al.*, 2000; Steinberg *et al.*, 2017). Studies based on phylogenetic analyses of mitochondrial markers proposed that the two clades obtained for the *A. guariba* group are linked to *A. guariba guariba* and *A. guariba clamitans* (Harris *et al.*, 2005; de Melo Martins *et al.*, 2011). Among the morphological characters that differentiate *A. g. guariba* from *A. g. clamitans*, pelage coloration is the most conspicuous, with homogeneous coloration without sexual dichromatism in *A. g. guariba*, and presence of sexual dichromatism in *A. g. clamitans* (Gregorin, 2006).

Alouatta guariba guariba is considered to be among the 25 most-threatened primates (Mittermeier *et al.*, 2022; Neves *et al.*, 2021) due to habitat loss, hunting, traffic, and deaths during outbreaks of yellow fever, which reduced both populations considerably: the South mainly in 2008/2009 (Almeida *et al.*, 2012; de Almeida *et al.*, 2019; Estrada *et al.*, 2018) and the North in 2017/2021 (Agostini *et al.*, 2017; Sacchetto *et al.*, 2020). The last outbreaks may have moved *A. g. guariba* from Vulnerable to Endangered (Bicca-Marques *et al.*, 2017; Estrada *et al.*, 2018). The Atlantic Forest is considered a world biodiversity hotspot, suffering from long-term deforestation, and is completely fragmented, with approximately 12% of its original coverage remaining (Hansen *et al.*, 2013; INPE, 2020; Rezende *et al.*, 2018; Ribeiro *et al.*, 2009).

We assessed species relationships and genetic diversity in the *A. guariba* group and evaluated the potential future distribution of the Northern and Southern populations. To do this, we: (i) assessed the cytochrome b and karyotype diversity in *A. guariba* group, concentrating on the Northern population, which is less studied and sampled than the Southern population, and (ii) evaluated the potential future distribution of populations from the northern and southern Atlantic Forest to shed light on the long-term conservation prospects for the group.

Methods

Ethical Note

We obtained blood samples from free-living and captive *Alouatta* in accordance with methods and protocols approved by the Institutional Ethics Committee for Animal Experimentation (Protocol CEUA/IOC-029/2016, license L-037/2016), the Brazilian Ministry of the Environment (SISBIO = System of Authorization and Information in Biodiversity, license number 54707–4 to R. Lourenço-de-Oliveira), and Rio de Janeiro's Environment agency (INEA 012/2016 and 019/2018). Veterinarian staff collected blood samples from captive animals at Centro de Primatologia do Rio de Janeiro (CPRJ) and Zoo Parque de São Paulo (RTM). Sample collection and management of free-living animals are described in two recent publications (Abreu *et al.*, 2019; Teixeira *et al.*, 2022). Live, free-living animals were released at the same location as they were caught. We also obtained muscle tissue from roadkill animals under licenses IBAMA for operation 1187/2013 and ABIO 514/2014 to C.

Bueno (deposited in Mammal collection of Museu Nacional), as well as from individuals that died during the yellow-fever outbreak from samples held in the mammalian collection of the Centro de Coleções Taxonômicas of Universidade Federal de Minas Gerais (UFMG). We obtained samples in accordance with the ethical treatment guidelines of Conselho Nacional de Controle de Experimentação Animal (CONCEA), normative resolution n. 28 (Pissinatti *et al*., 2016).

Sampling

We obtained blood and muscle samples for DNA analysis from 122 howler monkeys, 14 in captivity and 108 free-living, including animals killed by yellow fever and found run over during road monitoring. The samples included 117 animals from the Northern population (Espírito Santo, Rio de Janeiro, Minas Gerais, São Paulo states) and five from the Southern population (Rio Grande do Sul; Fig. 1; Table S1). We stored muscle samples in a freezer at −20 °C. We stored blood samples in a refrigerator at 8 °C and processed them within 2 days.

We isolated DNA from 21 muscle samples using the phenol–chloroform protocol (Sambrook & Russel, 2001). We isolated DNA from 101 blood samples with a saline protocol (Miller *et al*., 1988), Promega® Wizard Genomic DNA Purification Kit®, or QIamp® DNA Blood mini kit according to the kit or protocol available when we obtained the sample.

We karyotyped ten of the animals from the Northern population: seven from Rio de Janeiro state: (a) Parque Estadual da Serra dos Órgãos – CPRJ3622, (b) Cabo Frio - CPRJ2799 (locality 35 in Fig. 1), (c) Niterói – CPRJ3723 (locality 36), (d) Macaé – CPRJ3636, (e) Parque Estadual dos Três Picos – CPRJ2443 (locality 37), (f) Itaocara – CPRJ3216 (locality 23), (a-f) housed in captivity in Centro de Primatologia do Rio de Janeiro/CPRJ, (g) Angra dos Reis – CPRJ140 (locality 38), and three from São Paulo state (h) Parque Estadual das Fontes do Ipiranga/PEFI—RTM1518, RTM1526, RTM1542 (locality 33), housed in Zoo Parque de São Paulo.

Molecular and Statistical Analyses

We amplified the MT-CYB (ca.1140 bp) of 122 samples using polymerase chain reactions with primers L14725 (Irwin *et al*., 1991) and Cytb rev 5 '-GAATATCAG CTTTGGGTGTTGRTG-3'. Nomenclature of the mitochondrial cytochrome b protein-coding gene followed the HGNC Database, HUGO Gene Nomenclature Committee for the use of the MT-CYB abbreviation.

We used the same primers plus the internal primers CB-in1 5'-GAATATCAGCTT TGGGTGTTGRTG-3' and CB-in2 5`-TGAGGACAAATATCATTYTGAG-3` for sequencing. The amplification reaction mixture contained 1.0 μl of DNA template, 2.5 μl of 10×buffer, 2.0 μl of MgCl2 (25 mM), 1.0 μl of forward primer 1 (10 mM), 1.0 μl of reverse primer 2 (10 mM), 0.4 μl of dNTPs (10 mM), 0.3 μl of Platinum® Taq DNA Polymerase (Invitrogen), and Ultrapure water for a total volume of 25 μl. PCR amplification conditions consisted of an initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s, extension at

72 °C for 90 s, and the final extension at 72 °C for 5 min. We obtained amplicons using a ProFlex™ 3 thermal cycler×32-well Sample Block by Life Technologies, and purified them with PureLink TM Quick Gel Extraction & PCR Purification Combo kit by Thermo Fisher Scientific following the manufacturer's recommendations. We performed the sequencing in the Applied Biosystems 3730xl DNA Analyzer platform using the reagents of BigDye™ Terminator v3.1 Cycle Sequencing Kit. The sequencing reaction mixture in each well contained 2–4 μl of DNA, 1 μl of Ready Reaction kit, 1.5 μl of Big-Dye sequencing buffer (5x), 1 μl of oligonucleotide, and ultrapure water to a volume of 10 μl. The sequencing conditions consisted of an initial denaturation at 96 °C for 1 min, followed by 20–40 cycles of denaturation at 96 °C for 15 s, annealing at 50 °C for 15 s, and extension at 60 °C for 4 min. We assembled MT-CYB in sequence contigs using Chromas PRO version 2 (TechnelysiumPtyLtd) and aligned the sequences using Muscle in Mega X (Kumar *et al.*, 2018). We checked for possible nuclear mitochondrial DNA pseudogenes (NUMTs) by translating the nucleotide sequence into protein and verifying the length and the presence of stop-codons in the wrong position. We did not identify any sequences as potential NUMTs. The phylogenetic positions in the analyses were as expected, suggesting that the inclusion of NUMTs was not an issue.

In addition to the sequences that we obtained, we added sequences available in Gen-Bank with at least 800 base pairs (bp) identified as *A. guariba clamitans* (AF289986-987, AY065898-899, HQ385483-494, HQ385496-501, DQ679770-775, DQ679777-784, KY202428, KR528422), *A. belzebul* (DQ387044), *A. caraya* (DQ350637), *A. seniculus* (EU232713), *A. macconnelli* (AJ489759), *A. sara* (AY065887), corresponding to the South American *Alouatta* clade, and *A. pigra* (AY065885) and *A. palliata* (AY065880) from the Mesoamerican clade (Cortés-Ortiz *et al.*, 2003). As outgroups, we used available sequences of *Ateles geoffroyi* (KR902382) and *Ateles belzebuth* (KR202369). To check the phylogenetic relationship and identify geographic structure within the samples obtained for the *A. guariba* group, we performed a maximum likelihood (ML) gene tree estimation using Iqtree (Nguyen *et al.*, 2015) based on MT-CYB. The best-fit model of nucleotide substitution was TN+F+G4, selected with ModelFinder (Kalyaanamoorthy *et al.*, 2017). We estimated branch support using SH-LRT (Shimodaira-Hasegawa-like procedure; Guindon *et al.*, 2010) and UFBoot (ultra-fast bootstrap approximation; Hoang *et al.*, 2018).

To confirm the grouping of the samples obtained in the ML tree and understand the mutational steps separating each lineage, we produced a haplotype network. First, we identified the haplotypes with DNASP program version 6 (Rozas *et al.*, 2017) and drew the median-joining (MJ) network analyses using Network program version 10 (fluxus-engineering.com; Bandelt *et al.*, 1999). To identify haplotypes, we excluded sites with gaps and missing data to avoid biasing the haplotype distribution pattern, because missing data may represent poorly sequenced nucleotides (Joly *et al.*, 2007). We used 690 sites to generate the haplotype network. MJ analysis is commonly used to infer an unrooted branching diagram from intraspecific data and to visualize the relationships among DNA sequences within populations or species, where alternative branching may represent potential ancestral sequences. We also used DNASP to calculate indices of nucleotide (π) and haplotype diversity to estimate the level of genetic diversity.

We carried out neutrality tests of Tajima's D and Fu's FS based on the null hypothesis of constant population size and neutral mutations with 1,000 replications (Table I) using Arlequin version 3.5 (Excoffier & Lischer, 2010).

Karyotype Preparation

We cultured each of ten peripheral samples from captive individuals attributed to the northern Atlantic forest (CPRJ3622, CPRJ2799, CPRJ3723, CPRJ3636, CPRJ2443, CPRJ3216, RTM1518, RTM1526, RTM1542, RJ140) in 8 ml of RPMI 1640, 2 ml of fetal calf serum, 20 μl of lectin, 0.5 ml of L-Glutamine, and 60 μl of heparin for 70 h. Then, we added 0.5 ml of colchicine $10^{-5\,M}$ for 2 additional hours. We added a solution containing KCl (0.075 M) for hypotonic shock, and a Carnoy solution (3 methanol: 1 acetic acid) for fixation. After culturing the lymphocytes, we performed conventional staining with Giemsa 2%, producing visible karyotypes with condensed chromosomes. We photographed metaphases with a QImaging Retiga™ 3000 camera coupled to a Zeiss Axio Imager. We also performed C-banding (Sumner, 1972), a technique used to show heterochromatic bands, which are areas of chromosomes that typically remain condensed during interphase and are typically found as blocks surrounding centromeres (Sumner, 1972). However, only one sample (CPRJ3636) showed a suitable result. We assembled the karyotypes in descending order, based on chromosome size and number of arms (de Oliveira *et al.*, 1998).

Predicted Potential Distribution

We used occurrences available at Global Biodiversity Information Facility (GBIF, 2021) and in a dataset of occurrences of primates in the Atlantic Forests (Culot *et al.*, 2019) for the *Alouatta guariba* group, which included localities in

Table I Neutrality statistical tests (Tajima's D and Fu's FS) showing genetic variability of *Alouatta guariba* group of lineages A, B, and C from Northern and Southern clades, based on samples obtained in the years of 2013, 2014, 2017, 2018, and 2019 in the Brazilian states of Rio de Janeiro, Minas Gerais, São Paulo, Espírito Santo, Paraná, Santa Catarina, Goiás, Mato Grosso do Sul and Rio Grande do Sul

MT-CYB of the *A. guariba* group	Northern clade		Southern clade
	Lineage A	Lineage B	Lineage C
Number of transitions	10	11	6
Number of transversions	3	7	1
Sample size	74	57	30
S	12	17	7
Tajima's D	− 0.818	− 2.083	− 0.725
Tajima's D *p*-value	0.226	0.008*	0.251
Fu's FS	− 2.646	− 3.822	0.136
Fu's FS p-value	0.125	0.025*	0.549

*(Significant *p*-value); S (number of segregating sites)

nine Brazilian states (Rio de Janeiro, Minas Gerais, Espírito Santo, São Paulo, Bahia, Santa Catarina, Paraná, Rio Grande do Sul and Mato Grosso do Sul), and ten sites from the province of Misiones, Argentina. We did not include individual records from São Paulo that we could not allocate to the Northern or Southern populations with certainty. We also included records available in studies that identified specimens from Northern and Southern population using morphology, karyotype, or molecular analysis (Cardoso Coimbra, 2015; de Oliveira *et al*., 2000; Gifalli-Iughetti, 2008; Gregorin, 2006; Machado, 2011; Steinberg *et al*., 2017).

We gathered the geo-referenced occurrences for Northern ($n = 207$; Table S2) and Southern ($n = 259$; Table S3) populations, defined South America as the background extent for species distribution models (SDM), and removed duplicate records of each grid cell for analysis. We used Maxent (version 3.4.4) to generate SDM. Maxent uses presence data based on georeferenced points of occurrence and environmental variables to estimate the occurrence of a species realized niche and model their potential distribution (Phillips *et al*., 2006). We conducted all SDM analyses in R (version 4.0.4), using the "raster" (Hijmans *et al*., 2015), "dismo" (Hijmans *et al*., 2017), and "rJava" (Urbanek, 2021) packages. We used the following parameters for all models: logistic output format (habitat suitability measured on a scale of 0–1), 80% of records used as training data and 20% for testing, maximum 500 iterations, 5 replicates, 10,000 background points (Phillips *et al*., 2006). We defined the threshold for the distribution of suitable habitat as the tenth percentile training presence (TPTP) threshold superimposed on the model training area (Pearson *et al*., 2007).

We modeled potential species distribution using 19 bioclimatic variables available at WorldClim 2.1 (Fick & Hijmans, 2017; Hijmans *et al*., 2005), which represent recent (years 1970 to 2000) and future conditions (years 2081 to 2100). We also included current altitude data that was used to produce WorldClim 2.1 as a variable in the present and future models. We used anthropogenic emissions quantification data for two main future scenarios (SSP2-4.5 and SSP5-8.5) of greenhouse gas concentration. We used the mean projections from eight global climate models (GCMs): BCC-CSM2-MR, CNRM-CM6-1, CNRM-ESM2-1, CanESM5, IPSL-CM6A-LR, MIROC-ES2L, MIROC6, and MRI-ESM2-0, available at WorldClim 2.1 (Fick & Hijmans, 2017; Hijmans *et al*., 2005). We projected all scenarios using a spatial resolution of 2.5 arc-minutes (~ 4.5 km; Fick & Hijmans, 2017; Hijmans *et al*., 2005). We assessed the proportional contributions (%) of each environmental variable to each model by using jackknife tests (Phillips *et al*., 2006).

To estimate the predictive accuracy of the potential habitat models, we used area under the curve (AUC) metrics, based on the area under the receiver operating characteristic (ROC) curve (Elith *et al*., 2006; Swets, 1988). AUC assesses how well the model's predictions discriminate between places where observations are present and absent and is one of the most widely used threshold independent evaluators of the model's discriminatory power. AUC values above 0.9 indicate high model performance, whereas AUC values close to 0.5 indicate models equal to or worse than random (Peterson *et al*., 2011). For comparative purposes, we reclassified the images resulting from each model (with continuous values from 0 to 1) into four classes

of environmental suitability: unsuitable (UNS; 0, value TPTP), moderately suitable (MOS; value TPTP, 0.5), highly suitable (HIS; 0.5–0.75), and very highly suitable (VHS; 0.75–1). We plotted a final map with the occurrences and the final distribution model in ArcGIS. For more details of the methods, localities of occurrences, results of the AUC, and Jackknife test, see Appendix S1.

Results

Phylogenetics, Genetics, and Median-Joining Analyses

The maximum likelihood analysis divided the clade containing the *A. guariba* group into two major clades and three lineages: lineages A and B (Northern Clade) (94.1 SH-aLRT, 90 UFBoot); and lineage C (Southern Clade) (88.8 SH-aLRT, 91 UFBoot) (Fig. 2).

The MJ network analysis (Fig. 2) with 37 variable sites and 161 sequences showed 26 haplotypes (h), and haplotype diversity (Hd) of 0.8359. The MJ network analysis showed star-like haplogroups corresponding to lineages A, B, and C. Haplogroups A and B were separated by at least six mutations and haplogroups B and C by at least seven mutations. The nucleotide diversity (π), estimated for the dataset according to the haplotypes and haplogroups recovered in ML and MJ, was 0.00367 for lineage A (Northern Clade), 0.00309 for lineage B (Northern Clade), and 0.00306 for lineage C (Southern Clade).

Karyotypes

Karyotypes of lineage A (Northern Clade) from Rio de Janeiro (CPRJ3636 and RJ140) and São Paulo (RTM1526 and RTM1542) are all males with 2n=49 (Fig. 3). The autosome complement of these four males is composed of seven two-armed pairs, two large, one medium, and four small, and 16 medium to small one-armed pairs. The male sexual chromosome is translocated to an autosome, and the X is a medium biarmed chromosome. Pair numbers 18 and 20 showed a secondary constriction in these four samples, and the karyotype of sample CPRJ3636 also presented a heteromorphic 14th pair (Fig. 3).

Karyotypes of males from lineage B (Northern Clade) from Rio de Janeiro (male CPRJ2443) and São Paulo (male RTM1518) also showed 2n=49 and a chromosome morphology similar to males of lineage A (Northern Clade). The CPRJ2443 karyotype presented a heteromorphic 20th pair, and the CPRJ3723 karyotype presented a heteromorphic 15th pair (Fig. 3).

The karyotypes of females from lineage B (Northern Clade) from Rio de Janeiro (females CPRJ2799, CPRJ3622, CPRJ3723, CPRJ3216) showed 2n=50 (Fig. 3). The autosome complement is composed of eight two-armed pairs, two large, one medium, and five small, and 16 medium to small pairs with one arm. The X sexual chromosome is a medium biarmed chromosome. Females also showed two pairs of chromosomes with secondary constrictions.
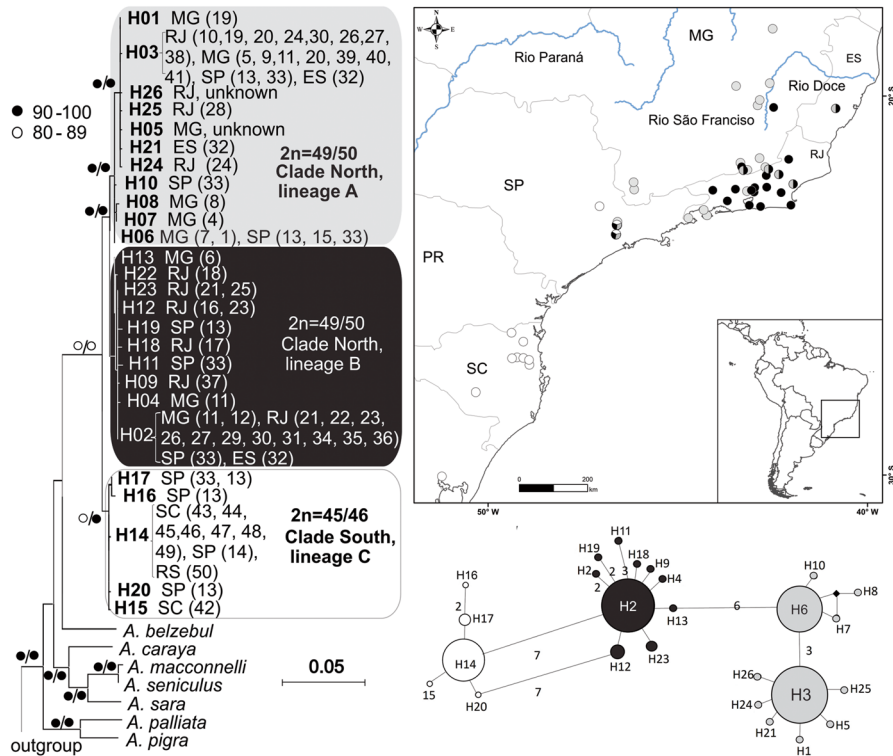
Fig. 2 Left, maximum likelihood topology of the *Alouatta guariba* group, with haplotypes, locality numbers (corresponding to map in Fig. 1 and Supplementary Table 1) in parentheses for each haplotype. Values close to the nodes are SH-aLRT and UFBoot supports. Brazilian states are Bahia (BA), Espírito Santo (ES), Goiás (GO), Minas Gerais (MG), Paraná (PR), Rio de Janeiro (RJ), Rio Grande do Sul (RS), São Paulo (SP), and Santa Catarina (SC). Top right, map with sampling localities according to the color of the clades and haplogroups found in the maximum likelihood and median-joining network (MJ) analyses, gray circles represent lineage A (Northern Clade), black circles the lineage B (Northern Clade), and white circles the lineage C (Southern Clade). Bottom right, the MJ network where circles are haplotypes with dimensions proportional to the number of shared sequences, numbers in the lines connecting the circles are nucleotide substitutions and diamond the median vector. The samples used here from ES, RJ, MG, SP, and RS states were obtained in 2013, 2014, 2017, 2018, and 2019.

Predicted Potential Distribution

After removing duplicates from each grid cell, we used 249 presence records to model the potential distribution of the Southern Clade and 197 to model that of Northern Clade. The mean training AUC for the replicated runs was 0.982 (SD = 0.001) for the Southern population habitat suitability model and 0.985 (SD = 0.001) for the Northern population habitat suitability model, meaning that the model performance was high (Figure S1). The jackknife test of variable importance for the Southern and Northern population habitat suitability models showed that the environmental variable that best explained the population distributions when used alone is BIO4 (Temperature Seasonality), which is a measure of the change
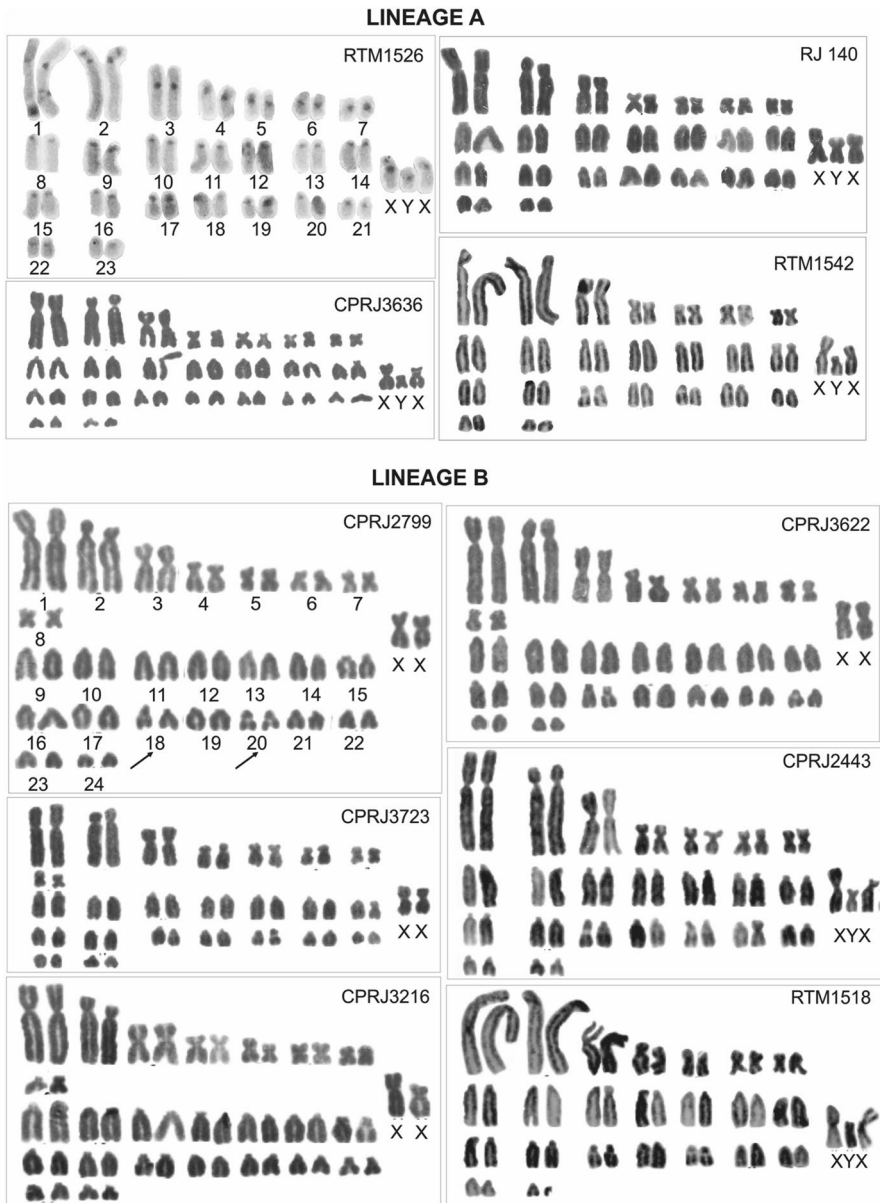
Fig. 3 Karyotypes of the Northern Clade: lineage A (Northern Clade) with C-banding pattern (RTM1526 from São Paulo state), and Giemsa coloration (CPRJ3636 and RJ140 from Rio de Janeiro state, and RTM1542 from São Paulo state); lineage B (Northern Clade) with Giemsa coloration (females CPRJ2799, CPRJ3622, CPRJ3723, and CPRJ3216 from Rio de Janeiro and males CPRJ2443 from Rio de Janeiro state and RTM1518 from São Paulo state). X and Y are sexual chromosomes. Arrows indicate chromosome pairs with secondary constrictions. The samples from Rio de Janeiro state were obtained in 2018 and the samples from São Paulo in 2019, Brazil.

in temperature throughout the year (Figure S2). For the Southern population, the second environmental variable that best explained population distribution is BIO11 (Mean Temperature of Coldest Quarter). This index measures mean temperatures during the three coldest months of the year. The second variable that best explained the distribution of the Northern population is BIO3 (Isothermality), which quantifies how much day-to-night temperatures oscillated relative to annual oscillations.

Based on the TPTP cutoff criteria, SDM identified regions in Brazil suitable for the occurrence of Southern and Northern populations (Fig. 4), mainly in Atlantic Forest areas. In the assessment of the recent potential distribution (years 1970 to 2000) for the two species, the geographic range of the Southern population had an area of 464,912.37 km and the Northern population had an area of 492,811.88 km. When assessing the potential distribution for two greenhouse gas emission scenarios (a lower-emission scenario (SSP245) and a worse scenario (SSP585)) in 60 to 79 years (2081–2100), the ideal climatic conditions for the two populations were greatly reduced in geographic coverage. The Southern population had a reduction in geographic coverage of 52% (227,420.43 km) in the most optimistic scenario, and 81% (94,104.18 km) in the worst scenario. The Northern population had a reduction in geographic coverage of 72% (141,025.01 km) in the most optimistic scenario, and 92% (46,944.69 km) in the worst scenario (Fig. 4).

Discussion

Genetic Diversity of the *A. guariba* Group Lineages

Our MT-CYB gene tree divided the *A. guariba* group into two major clades, one with individuals from the South and another with individuals from the North,
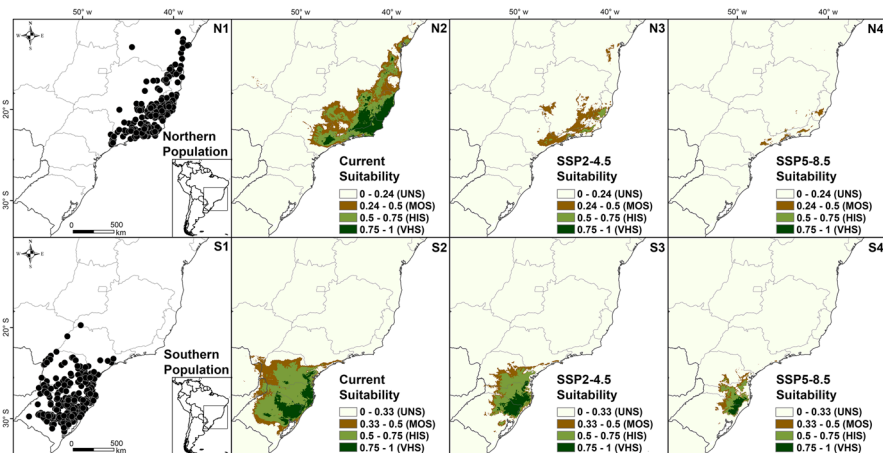

Fig. 4 Southern (S) and Northern (N) population habitat suitability model of the *A. guariba* group as predicted by MaxEnt: (N1 and S1) presence records, (N2 and S2) current distribution suitability, years 1970 to 2000, and two future distribution suitability in 2081–2100, (N3 and S3) in the better scenario SSP2-4.5, and (N4 and S4) in the worse scenario SSP5-8.5.

similar to previous studies (de Mello Martins *et al.*, 2011; Harris *et al.*, 2005; Machado, 2011; Steinberg *et al.*, 2014). Our findings reinforce the association of the Southern Clade with *A. g. clamitans* and the Northern Clade with *A. g. guariba*. The Northern Clade was further divided into two clades, named lineage A and lineage B, and we named the Southern Clade, named lineage C. Lineage A (Northern Clade) occurs mostly in the state of Rio de Janeiro, and Lineage B (Northern Clade) in Minas Gerais state, but we found areas of overlap between lineages A and B, and A, B, and C. Although mitochondrial DNA evolves faster than single-copy nuclear DNA and can be used to clarify taxonomic ambiguities and evolutionarily significant units, it still shows some of the limitations associated with using data from a single locus to infer phylogenetic history. Mitochondrial DNA only represents the maternal side and records scant evidence of recent events. As a result, it does not accurately represent the species tree (Wan *et al.*, 2004). Although biparental markers should be taken into consideration for future research with the *Alouatta guariba* group, mitochondrial DNA is still a powerful tool in conservation genetics, because its shows a strong phylogenetic signal at specific and population level.

In the MJ network and MT-CYB analyses, Southern and Northern populations showed a low number of haplotypes compared to the number of sequences used, and a low nucleotide diversity estimate, even when we analyzed lineages A and B (Northern Clade) separately, a pattern similar to previous studies based on MT-CYB (Bonvicino *et al.*, 2015; Machado, 2011). The greater sample size and the number of sites that we analyzed for samples in the Northern Clade allowed us to better understand the geographic limits of these populations and find instances of sympatry between lineages A and B in the states of Minas Gerais and Rio de Janeiro. These areas of overlap represent an expansion of lineage B (Northern Clade) as shown by neutrality tests, for which the negative value deviated significantly from neutrality, suggesting an excess of rare nucleotide site variants. It is likely that lineages A and B separated recently and accumulated nucleotide differences, allowing us to observe these two isolated lineages. Evidence of expansion was found in the Southern population ca. 5,000 years ago (Machado, 2011), right after a postglacial period (Nascimento *et al.*, 2005), in which the populations emerged from a state of isolation to start expanding. Based on the observed star-like pattern in each lineage, we suggest that the three lineages were likely recently isolated and are expanding. The areas of overlap between lineages A and B and between the three lineages in São Paulo may be associated with the expansion of these three lineages.

A recent study based on mitochondrial marker MT-COI (Petit-Marty *et al.*, 2021) estimated that the nucleotide diversity (π) of threatened mammalian species can be much lower, ranging $\pi = 0.0044$–0.0102, than that of nonthreatened mammalian species, ranging $\pi = 0.0128$–0.0165. Compared with other *Alouatta* species, such as Amazon and Atlantic Forest populations of *A. belzebul*, analyzed by using the same mitochondrial marker (Nascimento *et al.*, 2005, 2007; Povill *et al.*, 2022), the *A. guariba* group had fewer haplotypes and low nucleotide diversity (Table S4). *Alouatta caraya* showed low MT-CYB nucleotide diversity as showed for *A. guariba* group, but more haplotypes (Ascunce *et al.*, 2007; Nascimento *et al.*, 2007). This finding may be related to the large distribution of *A. caraya*, the strong geographic structuring of the populations, and its limited dispersal, which occurs only along

forest corridors (Nascimento *et al.*, 2007). Threatened species, such as *A. pigra* and *A. palliata mexicana*, that inhabit areas of continuous forest reduction (Dunn *et al.*, 2014; James *et al.*, 1997; Melo-Carrillo *et al.*, 2020) showed very low mitochondrial nucleotide diversity (Dunn *et al.*, 2014). *A. palliata mexicana* showed lower genetic diversity than any other *Alouatta* species. Some species of *Alouatta* from South America or the Cis-Andean species, such as *A. palliata*, have low nucleotide diversity (Table S4). *Alouatta caraya*, *A. belzebul* (Amazon population), *A. palliata mexicana*, and the *A. guariba* group occur in very different environments. Both *A. guariba* group and *A. palliata* are species complexes that inhabit small patches, in which the genetic diversity was lower than the other species. The low genetic nucleotide diversity observed in the *A. guariba* group may result from genetic drift in small and isolated populations, or habitat fragmentation and climatic variation.

Karyotype Analyses Support the Hypothesis that Northern and Southern Populations are Distinct Taxa

We attributed the Northern Clade to *A. guariba guariba* and the Southern Clade to *A. guariba clamitans*, based on previous studies and our data for karyotype and phylogenetic relationships, and the geographic distribution of *A. guariba guariba* (Gregorin, 2006; Kinzey, 1982). However, the type locality of *A. guariba guariba* in its original description was defined as Brazil by Humboldt in 1812 and later was restricted to Rio Paraguaçu, Bahia state (Cabrera, 1958), a contested action not considered in later publications (Gregorin, 2006) due to the lack of plausible explanations for this restriction. The type locality of *A. guariba clamitans* was referred to as São Sebastião in São Paulo state, based on the lectotype (Gregorin, 2006).

We attribute the karyotype 2n = 49–50 to *A. guariba guariba* (Northern Clade) based on previous publications (de Oliveira *et al.*, 2000; Steinberg *et al.*, 2014) and our karyotype results. We attribute the karyotype 2n = 45–46 to *A. guariba clamitans* (Southern Clade) based on previous karyological (de Oliveira *et al.*, 2000; Steinberg *et al.*, 2014, 2017) and molecular (de Mello Martins *et al.*, 2011; Harris *et al.*, 2005) studies. Analysis of a possible hybrid with karyotype 2n = 48 using fluorescence in situ hybridization showed the presence of chromosomes from lineages from Southern Clade (2n = 45–46) and Northern Clade (2n = 49–50) (Cardoso Coimbra, 2015). The single specimen with 2n = 48, among roughly 65 specimens analyzed with 2n = 45–46 or 2n = 49–50, and the absence of offspring from this hybrid suggests a postzygotic barrier between the lineages from the north and south (Cardoso Coimbra, 2015; Gifalli-Iughetti, 2008) that causes hybrid sterility due to cumulative negative interactions between loci. Evidence of hybridization also has been observed between two Mesoamerican species of *Alouatta*: *A. pigra* and *A. palliata* (Cortés-Ortiz *et al.*, 2015b; Kelaita & Cortés-Ortiz, 2013). The differences in diploid number among Northern and Southern clades of *A. guariba* group and the differences we found in the number of chromosomal arms present in the autosomal complement of a somatic cell in the Northern Clade support the

hypothesis that the *A. guariba* group is in a process of chromosomal differentiation or speciation (Gifalli-Iughetti, 2008).

Predicted Potential Distributions

In the current potential distribution areas, the two populations are sympatric in a small area in the southeastern part of São Paulo state, and this overlap is lost in the SDM worst scenario for the future. Temperature seasonality (BIO4) was the most important environmental variable to explain the distribution of the northern and southern populations. For the southern population, the second variable (BIO11), related to the coldest quarter in the year, appears to affect the distribution of this lineage, whereas for the northern population, the variable Isothermality (BIO3), related to temperature fluctuations during the year, could influence its distribution (O'Donnell & Ignizio, 2012).

According to the models, the northern and southern populations will lose a large percentage of their suitable distribution areas in the future (2081–2100) due to changes in climate, in both the most optimistic or pessimistic scenarios. The SDM results showed fluctuations in temperature throughout the year and the colder months as the most influential variables in the Northern and Southern *A. guariba* population distributions. Another factor reinforcing the SDM scenario is a likely reduction in the Atlantic Forest territory, as a result of forest being replaced by savanna (Salazar *et al*., 2007), caused mainly by the direct effect of the change in land use (Ribeiro *et al*., 2009). Other studies of the potential distribution of *Alouatta* suggest that populations are affected by both deforestation and climate changes (Freire Filho & Palmeirim, 2020; Holzmann *et al*., 2015), supporting the scenario that we suggest, that fluctuations in temperature and the decrease in the Atlantic Forest territory are major threats to the *Alouatta* populations.

Variations in effective population size may be caused by climate change, as shown in the potential distribution model of the recent past and the future scenarios for the Northern and Southern clades. Another factor affecting population size is pandemic diseases (Harding *et al*., 2002), which are strong enough to remove the effect of species abundance on mtDNA diversity in mammals (Nabholz *et al*., 2008). *Alouatta guariba* lineages are endemic and mostly adapted to the Atlantic Forest, a very fragmented morphoclimatic domain where outbreaks of yellow fever are increasingly frequent. These outbreaks are considered the most important threat to this species in Misiones province, Argentina (Agostini *et al*., 2017), and are responsible for population declines in other platyrrhine primates from the Atlantic Forest, such as titi monkeys (Berthet *et al*., 2021). The recent devastating yellow fever epizootics are one of the reasons that the *A. guariba* group was considered one of the most threatened primates of the world (Estrada *et al*., 2018; Mittermeier *et al*., 2022). Outbreaks of yellow fever are more frequent as a result of climate change and forest alterations that also influenced the resilience of Northern and Southern populations of *A. guariba*. Yellow fever and environmental modifications together influence their future predicted distributions, indicating very pessimistic outcomes.

Conclusions

Our results add to our understanding of the current and future species distribution of the *Alouatta guariba* group and reveal low genetic diversity in both Northern and Southern populations. The distribution limits for *A. guariba guariba* and *A. guariba clamitans*, based on differences between the Northern (*A. guariba guariba*) and Southern (*A. guariba clamitans*) populations in karyotype, morphology, and phylogenetics, differ from those established by IUCN. The status of *A. guariba guariba* and *A. guariba clamitans* are well supported by molecular, karyotype, and morphologic analyses. Despite being widely distributed, the Northern and Southern clades, designated here as *A. g. guariba* and *A. g. clamitans*, respectively, are restricted to small patches in the southeast. To better understand the genetic diversity of the *A. guariba* group, studies should assess their nuclear DNA diversity, such as levels of heterozygosity. The low MT-CYB nucleotide diversity is an additional concern for these populations, and the high fragmentation of Atlantic Forest and recurrent outbreaks of yellow fever represent potential threats to these taxa. These data reinforce the IUCN Red List categories of *A. guariba clamitans* as Vulnerable (Buss *et al*., 2021) and *A. guariba guariba* as Critically Endangered (Neves *et al*., 2021).

Author Contributions CP conceived (lead), designed (lead), performed the experiments (lead), analyzed the data (lead), writing, review and editing (lead). CRB conceived (equal), funding acquisition (lead), writing review & editing (equal). MBO analyzed and wrote spatial distribution (lead), writing review and editing (equal). FVSA performed the experiments (equal). FVSA, RLO, ES, FAP, CB, CM, AP, CRB got the samples, writing review and editing (supporting), contributed to the final version (supporting).

Data Availability The datasets generated and analyzed during the present study were uploaded to NCBI (Accession OP985658—OP985779).

Declarations

Conflict of Interest The authors declare that they have no conflicts of interest.

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