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Genetic diversity and structure of red handed howler monkeys assessed by mitochondrial and genotyping by sequencing analyses

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The red-handed howler monkey, *Alouatta belzebul*, has an uncertain taxonomy complex that includes *A. belzebul*, *A. discolor* and *A. ululata*. These primates are endemic to Brazil and are classified as vulnerable or endangered by the IUCN Red List due to population declines over the past 40 years. Damming by hydroelectric power plants is the greatest threat to the taxa. The recent implantation of the fourth largest dam worldwide (Belo Monte Hydroelectric Dam, UHBM) threatens their populations, because its flooding zone covers areas where *A. belzebul* and *A. discolor* may co-occur. Determining species classification is crucial for conservation efforts. Here, we characterized the genetic diversity and structure of howler monkeys rescued before the flooding. We performed Genotyping by Sequencing (GBS) analyses and constructed a mitochondrial-based phylogenetic tree and a haplotype network in specimens identified as *A. belzebul*, based on their coat color, from both banks of the Xingu River and three islands. The study revealed the occurrence of both taxa, indicating high genetic diversity. These findings are valuable for understanding group diversity, providing reference data for monitoring populations in the region and implementing conservation actions, as well as serving as a model for similar studies of other taxa.

Keywords Population genetics, Neotropical primates, Biological conservation, GBS, Genomics, Dloop

Primates face significant threats from human activities, including the overexploitation of natural resources impacting biodiversity[1–4]. Among Neotropical primates, the howler monkeys (*Alouatta*, Atelidae, Platyrrhini) are widely distributed throughout Central and South America, inhabiting the Atlantic Forest, Caatinga, Cerrado and Amazon Forest biomes in Brazil[5,6]. The Brazilian Amazonian has special economic, ecological and environmental relevance, as it holds the largest reserves of freshwater and tropical forest, in addition to the greatest biodiversity in the world[7,8].

Among the Amazonian howler monkeys that have been negatively impacted by anthropogenic activities is the red-handed howler monkey, *Alouatta belzebul* Linnaeus, 1766[6,9]. This taxon has recently been reassessed as a complex of species that embraces two other taxa namely *Alouatta discolor* (Spix, 1823) and *Alouatta ululata*[10]. Nonetheless, the taxonomic status of these taxa varies among authors, since different methods of classification lead to divergent taxonomic conclusions[11–17], depending on the genetic approach or morphological criteria adopted. Thus, some studies have reported *A. ululata* and *A. discolor* as subspecies[11]; *A. belzebul* and *A. discolor* as synonyms[15] or these taxa as a species complex[9,10].

These discordances are largely attributed to phenotypic similarities or the lack of satisfactory morphological data, even when a genetic evidence is pointed out[9]. However, Gregorin[5] described the three taxa at the species level, highlighting differences in the measurements of the cranial and hyoid bones, and in the pelage color pattern, with *A. belzebul* and *A. discolor* showing subtle differences in relation to length and width of the red stripes on their bodies, characterizing a mosaic variation pattern for *A. belzebul*. Previous studies had already pointed out variations in the coat color patterns of *A. belzebul*, *A. discolor* and *A. ululata*[11]. While *A. ululata* presents sexual

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dichromatism, with males and females showing, respectively, black and yellowish-brown coloration; *A. discolor* and *A. belzebul* present both sexes with black or dark brown coats, with red hands, feet and tip of the tail[11,12]. Therefore, despite the differences, distinguishing these two species remains challenging. Thus, their geographic area of occurrence has also been considered a criterion for identifying these taxa[5,11].

The geographic distribution of *A. belzebul*, *A. discolor* and *A. ululata* has been reported in the literature as restricted to Brazil[5,9,11]. According to Gregorin[5], *A. ululata* is limited to the ecotone of the Caatinga to the east, and to the ecotone of Amazon to the south and west; while *A. belzebul* displays a disjunct distribution, occurring in remnants of the northeastern Atlantic Forest and within the Amazon biome, extending from the Xingu River to the Tocantins River. *A. discolor* occurs from the Tapajos River to the Xingu River, also in the Amazon biome, evidencing a possible parapatry with *A. belzebul* in the northernmost area, between the Xingu and Tocantins rivers[5]. Additionally, Povill et al.[10] recently reported that *A. discolor* occurs on the west (left) bank of the Xingu River, while *A. belzebul* occurs on the east (right) bank of the Xingu River. Reductions in habitats and population sizes have been used to define the conservation status of these species, in which *A. ululata* is listed as Endangered (EN)[18], while *A. discolor*[19] and *A. belzebul*[6] are both classified as Vulnerable (VU). Thus, though the *Alouatta* genus has been recognized by its high capacity of survival in human-modified habitats[17,20], habitat fragmentation and/or loss may have as consequence population isolation and/or decline[2,21,22].

One of the most impactful anthropogenic threats to the howler monkeys is the construction of hydroelectric plants. The flooding of the associated forest causes environmental disturbances, affecting the river dynamics and the fauna and flora that depend on or are interrelated with the river and the flooded forest[23,24]. Under this scenario, ecological functions and population dynamics are negatively impacted, due to the loss of habitat and food resources, the increased competition and the impaired dispersion, for example, which increase the risk of local extinctions[25–29]. These events can fragment and isolate populations. Once small and isolated populations, the genetic drift and inbreeding effects will be intensified. This contributes to loss of genetic diversity and fixation of deleterious alleles, compromising fitness and the capacity of response to environmental changes and, consequently, the long-term persistence of populations and species[30–34].

By 2010–2011, the fourth largest dam worldwide - Belo Monte Hydroelectric Plant (UHBM) - began construction on the Xingu River[35], when *A. belzebul* was the only *Alouatta* species recognized in the area. Due to the negative impacts on biodiversity, caused by hydroelectric dams, environmental compensation actions are mandatory for companies responsible for managing dams in Brazil[36,37]. Such actions aim to ensure the reduction of the hazardous consequences, at least on a local scale and animal species, which are rescued and transferred to surrounding areas that have not been flooded[37]. Following these mandatory recommendations, *A. belzebul* from both banks of the Xingu River were rescued from the potential flooding area and transferred to safety areas in their respective river banks. At that time, biological samples of these specimens were collected for further genetic studies.

Considering that *A. belzebul* and *A. discolor* are currently recognized as distinct taxa that inhabit opposite banks of the Xingu River[10], here we focus on studying *Alouatta* specimens rescued in the UHBM inundation area, in the Volta Grande of the Xingu River, in order to investigate the occurrence of both lineages and infer about their genetic diversity. Our hypotheses are (i) the individuals rescued on the right and left banks of the Xingu River may represent *A. belzebul* and *A. discolor*, respectively; (ii) the existence of islands in the region before the flood offered possibilities for dispersion; and (iii) high genetic diversity is expected for both taxa, representing the pristine environmental condition before the construction of the UHBM. In addition to contributions on diversification within *Alouatta*, our results shed light on the genetic diversity represented in the rescued individuals, establishing the baseline for monitoring the remaining populations after the forest flooding, and for the conservation of Amazonian howler monkeys.

Results

Dloop data

After the sequence editing and alignment, we obtained fragments with final lengths of 1014 bp, containing 120 polymorphic sites. Of these, 31 sites were classified as singletons and 89 sites were parsimoniously informative, evidencing a total of 19 haplotypes for 24 sampled individuals, that produced Dloop sequences of high quality, in addition to two samples identified as *A. discolor* downloaded from GenBank (https://www.ncbi.nlm.nih.gov/genbank/): TAP 315355 and BM 48342, respectively, from the right bank of the Tapajos River and from the left bank of the Xingu River.

The Bayesian tree topology showed two well supported clades (posterior probability = 1; Fig. 1), indicating the existence of two markable distinct lineages in our sampling. Therefore, although the initial morphological identification indicated all collected samples as being *A. belzebul*, taking this result into account, we named these samples as *A. belzebul* or *A. discolor*. The lineage nominated *A. discolor* joined the studied individuals from the left bank of the Xingu River and from the three sampled islands with the two individuals from the GenBank. On the other hand, the lineage named herein *A. belzebul* clustered all individuals from the right bank, and unexpectedly two individuals from the left bank of the river. The haplotype network also pointed out the two lineages, with no haplotypes shared between *A. belzebul* and *A. discolor* (Fig. 2).

The genetic diversity estimated for both lineages detected in the Xingu River evidenced high haplotype diversity, ranging from 0.927 (*A. discolor*) to 0.949 (*A. belzebul*). The nucleotide diversity varied from 0.0132 to 0.0186 to *A. discolor* and *A. belzebul*, respectively (Table 1). The fixation index value (F_{ST} = 0.73) indicated high differentiation between *A. belzebul* and *A. discolor* lineages. In accordance, pairwise genetic distance between the two lineages, using the Kimura two-parameter model, was 6.2% (standard deviation equal to ± 0.71%), while the mean genetic distance intra-lineage was 1.3% (± 0.003%) for *A. discolor* and 1.9% (± 0.003%) for *A. belzebul.*

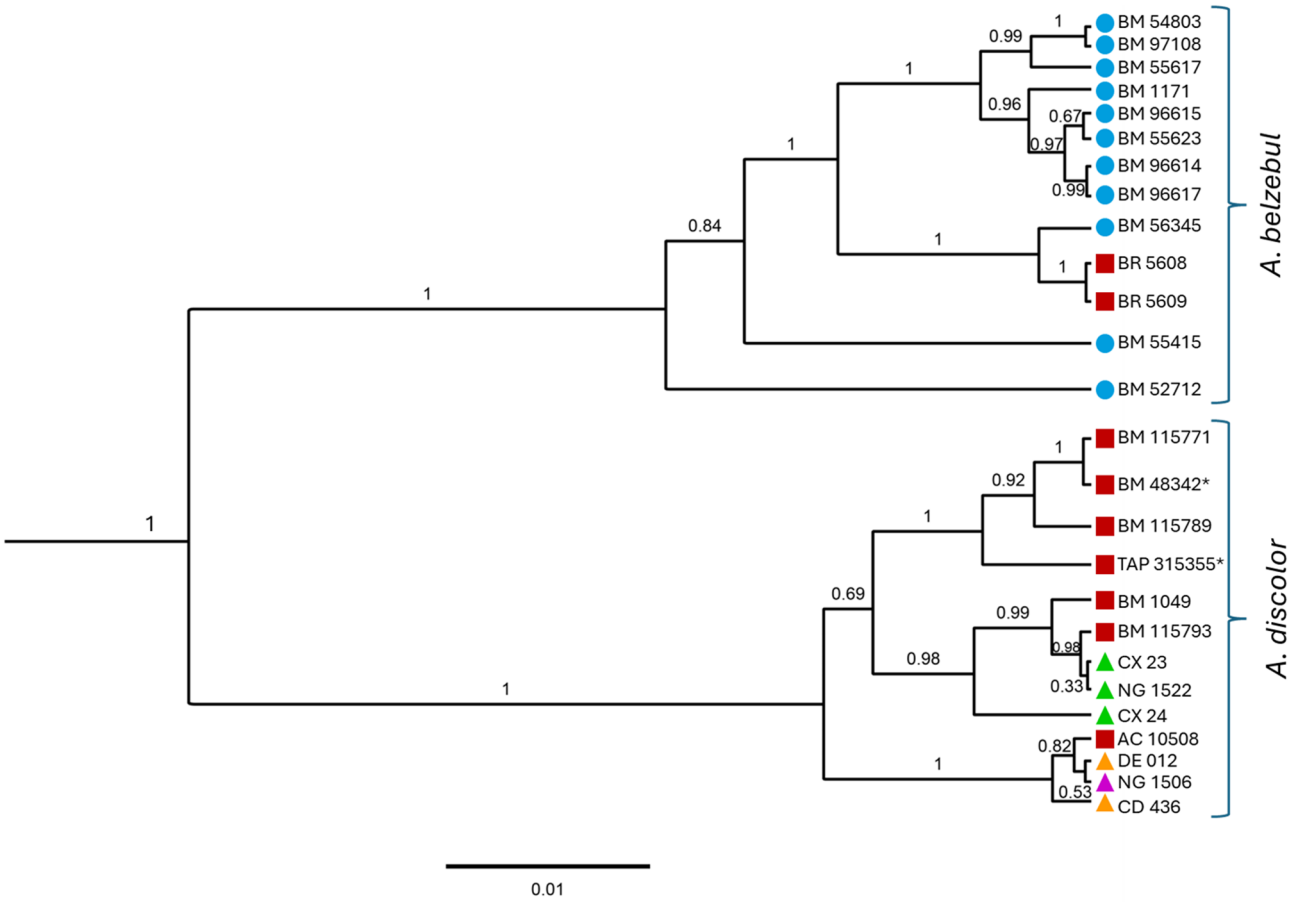

Fig. 1. Bayesian Inference for Dloop data of 24 individuals of *Alouatta* collected in the Xingu River (Vitória do Xingu, PA, Brazil), in addition to two GenBank* samples previously identified as *Alouatta discolor*: BM 4834 and TAP 315355, respectively, from the left bank of the Xingu River and the right bank of the Tapajos River. The symbols and colors represent the five studied locations: the right bank of the Xingu River (blue circle), left bank of the Xingu River (red square), Pimental Island (orange triangle), Maravilha Island (green triangle), and Meio Island (purple triangle).

GBS data

After quality control and data filtering, we obtained 5,753 SNPs for 13 samples. Using a total of 5,071 SNPs, classified as neutral, sNMF analysis evidenced two genetic groups (K = 2), one represented by seven samples from the right bank of the Xingu River (BM 1171, BM 95515, BM 96617, BM 97108, BM 96614, BM 92976 and BM 94383), and another represented by three individuals from the left bank of the river (BM 1026, BM 1049 and BM 1142) and three individuals from the Pimental Island (CD 435, CD 436 and DE 012) (Fig. 3A). The PCA and DAPC corroborated the existence of two genetic lineages, also joining the individuals from the left bank of the Xingu River with the individuals of the Pimental Island (Figs. 3B,C). The F_{ST} values confirmed the results obtained by these previous analyses, indicating low levels of differentiation between individuals from the left bank and the Pimental Island, and pointing to the existence of high genetic differentiation between individuals from the right and the left bank of the Xingu River (Fig. 4).

Assuming the existence of the two genetic lineages, we estimated genetic diversity parameters using two independent datasets of neutral SNPs, from which 12,943 neutral SNPs were identified for *A. belzebul* (samples from the right bank of the river) and 3,999 neutral SNPs were identified for *A. discolor* (samples from the left bank and Pimental island). Although the number of individuals sampled were quite similar, the number of alleles was very distinct among both taxa. This result may be related to differences among the datasets from both lineages likely due to the quality of the samples. For one sample of *A. discolor* we obtained only 754,143 reads, before SNP catalog prospection. In sum, we obtained a minimum-maximum total reads equal to 1,619,555–3,247,033 for *A. belzebul* and 754,143–3,603,121 for *A. discolor*. Such differences possibly resulted in a smaller number of neutral SNPs for *A. discolor*. Despite this, allelic richness, expected and observed heterozygosity, and inbreeding coefficient values were similar for both lineages (Table 2). These results indicate a relatively high genetic diversity, and, although the values of inbreeding coefficients were negative, they were near to zero and not significant (i.e., within confidence interval) (Table 2).

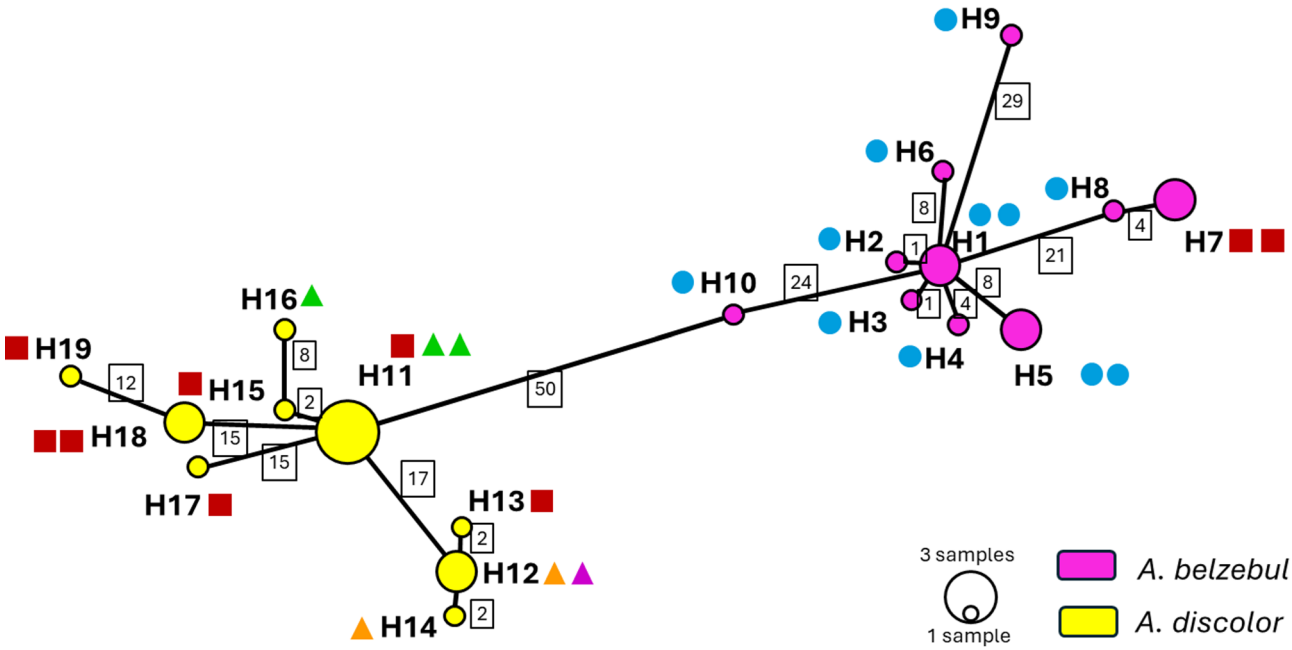

Fig. 2. The randomized minimum-spanning haplotype tree from Dloop data, using an infinite-sites model for 24 individuals of *Alouatta* from the Xingu River (Vitória do Xingu, PA, Brazil), in addition to two samples from GenBank previously identified as *A. discolor*: BM 48342 (H18) and TAP 315355 (H19), respectively, from the left bank of the Xingu River and the right bank of the Tapajos River. The symbols and colors represent the individuals of the five studied locations: individuals from the right bank of the Xingu River (blue circle), left bank of the Xingu River (red square), Pimental Island (orange triangle), Maravilha Island (green triangle), and Meio Island (purple triangle). The number of mutation steps between haplotypes is indicated within squares.

Discussion

The joint analyses from the Dloop and SNP data both suggest the existence of two lineages of howler monkeys in the region of Vitória do Xingu: one of them composed of individuals from the left bank of the Xingu River and individuals from the nearby islands, and another composed mostly of individuals from the right bank. According to the sample localities (see Fig. 5; Table 3), the individuals from the islands are geographically nearer to the left bank of the river, with a medium distance of about 450 m among islands and from the islands to the left bank, justifying the obtained results and suggesting the role of the Xingu River acting as a potential geographic barrier to the gene flow in the past. The haplotype network, in addition to the Bayesian and principal component analyses based on GBS data, indicates the existence of two distinct Evolutive Significative Units (ESU), reinforcing that these lineages should be treated as two conservation units[38].

The number of species within *Alouatta* have been widely debated mostly due to the phenotypic similarity among the taxa and/or the lack of satisfactory morphological data to resolve taxonomic uncertainties into the genus[39–42]. Regarding the complex *A. belzebul*, Gregorin[5] proposes that *A. belzebul*, *A. ululata* and *A. discolor* be considered distinct taxonomic units at the level of species. Even though some authors treat this group as a complex of subspecies and/or do not recognize *A. discolor* as a valid unit different from *A. belzebul*[9,43,44]. However, considering the existence of two well-differentiated genetic lineages in our study, combined with recently published data that consider the geographic distribution of howler monkeys to define the taxa[10], we support the separate lineages *A. discolor* (left bank and islands of the Xingu River) and *A. belzebul* (right bank of the Xingu River).

Although we detected a clear separation between the two lineages related to their occurrence on the left or the right bank of the Xingu River, the Dloop analysis grouped two individuals from the left bank (BR 5608 and BR 5609, both males, see Table 3) with individuals from the right bank, i.e., *A. belzebul*. Unfortunately, these individuals were not analyzed by GBS data, due to insufficient parameters of DNA quality and quantity. However, the river stretches between the islands and the riverbanks, next to the collection sites of these two individuals, not exceeding 250 m, increasing the possibility that these animals have crossed the Xingu River, originally from the right bank to the left bank. Therefore, we suggest that these two males might have migrated from the right bank to the left bank of the river, likely crossing the river by swimming.

Hernandez-Camacho and Cooper[45] have already reported that howler monkeys possess capacity for swimming, and some individuals were more than once observed crossing water bodies with average widths varying between 200 and 300 m. In *Alouatta palliata* and *Alouatta seniculus*, field observations have reported that both species can swim without human stimulation[46,47]. In Pavelka et al.[48] the swimming ability of *Alouatta* was exemplified to discuss the riverine barrier hypothesis, taking into account previous observations for *Alouatta macconnelli* in the Rupununi River (Guyana) and for *A. palliata* in the Panama Canal (Panama). The capacity of these monkeys to cross rivers also supports the conclusions of Janiak et al.[49] that rivers may not be clear

Samples	H	S	h	Hd	Pi
Alouatta belzebul		65	10	0.949	0.0186
BM 55623, BM 96615	H1				
BM 96614	H2				
BM 96617	H3				
BM 1171	H4				
BM 54803, BM 97108	H5				
BM 55617	H6				
BR 5608, BR 5609	H7				
BM 56345	H8				
BM 55415	H9				
BM 52712	H10				
Alouatta discolor		33	8	0.927	0.0132
BM 115793, CX 23, NG 1522	H11				
DE 012, NG 1506	H12				
AC 10508	H13				
CD 436	H14				
BM 1049	H15				
CX 24	H16				
BM 115789	H17				
BM 115771	H18				

Table 1. Genetic diversity indices from Dloop data for 24 individuals of *Alouatta belzebul* ($N = 13$) and *Alouatta discolor* ($N = 11$) from the Xingu river (Vitória do xingu, PA, Brazil). H: Haplotypes; S: Number of polymorphic sites; h: Number of haplotypes; Hd: Haplotype diversity; Pi: Nucleotide diversity.

barriers to howler monkeys. Thus, other evolutionary mechanisms involved with parapatric speciation must be important for the diversification of the group[50,51]. However, evidence of ecological, morphological or behavioral aspects playing a clear role as an effective barrier to gene flow, acting as a reproductive barrier in the taxa studied here, is absent in the literature. On the other hand, the reproductive behavior of howler monkeys, which includes mechanisms of intra- and inter-sexual selection, can promote the migration of males across the river to access females, due to higher levels of intra and inter-group competition[51,52]. Thus, although considered a rare event in Platyrrhini[46], the ability of *Alouatta* species to swim to adjacent areas, particularly if they are on islands, is already well known[53,54], supporting our suggestion that both *A. belzebul* individuals rescued from the left bank of Xingu River were migrants from the right bank.

According to Milton[55], howler monkeys are travel minimizers, energetically limited due to their diet, which is generally low in obtaining ready energy from some portion of the foliage. In addition, changes in their behavior, including decreased vocalization and increased locomotion, can occur in more spatially disturbed locations[56]. Therefore, despite the ability for swimming observed in *Alouatta* species may be associated with natural events, such behavior may be also related to environmental disturbances promoted by anthropogenic actions[56,57], which can favor an increase in dispersion for searching proper areas for food or reproduction. This migration, however, may not result in effective gene flow between distinct populations or lineages, due to possible events that favor parapatric speciation, which prevent reproduction, such as sexual selection[58].

Although we are not able to identify the true reason that could be allowed some *A. belzebul* individuals crossing the river, we cannot disregard the huge environmental disturbances caused by the intense human interference in the Volta Grande of the Xingu River region, during the construction of the UHBM[59–61], which began before the rescue of the howler monkeys and their transfer to safe forest fragments adjacent to the flooded area. It is well known that the contact of distinct evolutionary lineages and/or genetically structured populations, caused by human influence, promotes the emergence of artificial hybridization zones, originating hybrid lineages[62,63], which may present lower fitness, or, conversely, genetic superiority or hybrid vigor, thus threatening the pure lines that originated them[64,65]. On the other hand, the separation of specimens belonging to the same evolutionary lineage can reduce the genetic diversity due to the occurrence of genetic drift and founder effect, in addition to facilitate an increase in inbreeding due to eventual significant population size reduction[62,64–66].

Thus, considering the existence of some individuals of *A. belzebul* in a potentially historic distribution area of *A. discolor*, this co-occurrence may favor hybridization and, on the other hand, the loss of genetic diversity. Although our sampling does not indicate evidence of hybridization or low genetic diversity in the studied area, prior to the construction of the dam, these data serve as a reference for both taxa in a pristine condition. Therefore, based on that, the scientific community and stakeholders must establish decision-making that prioritizes maintaining the integrity of populations, lineages and/or species in terms of retention of genetic diversity and adaptive and evolutionary potential[38,67].

Overall, the *A. discolor* and *A. belzebul* populations studied herein evidenced relatively high genetic diversity, showing a similar number of Dloop haplotypes and similar values of haplotype and nucleotide diversity. However, we found differences between the number of polymorphic sites, with *A. belzebul* showing a higher

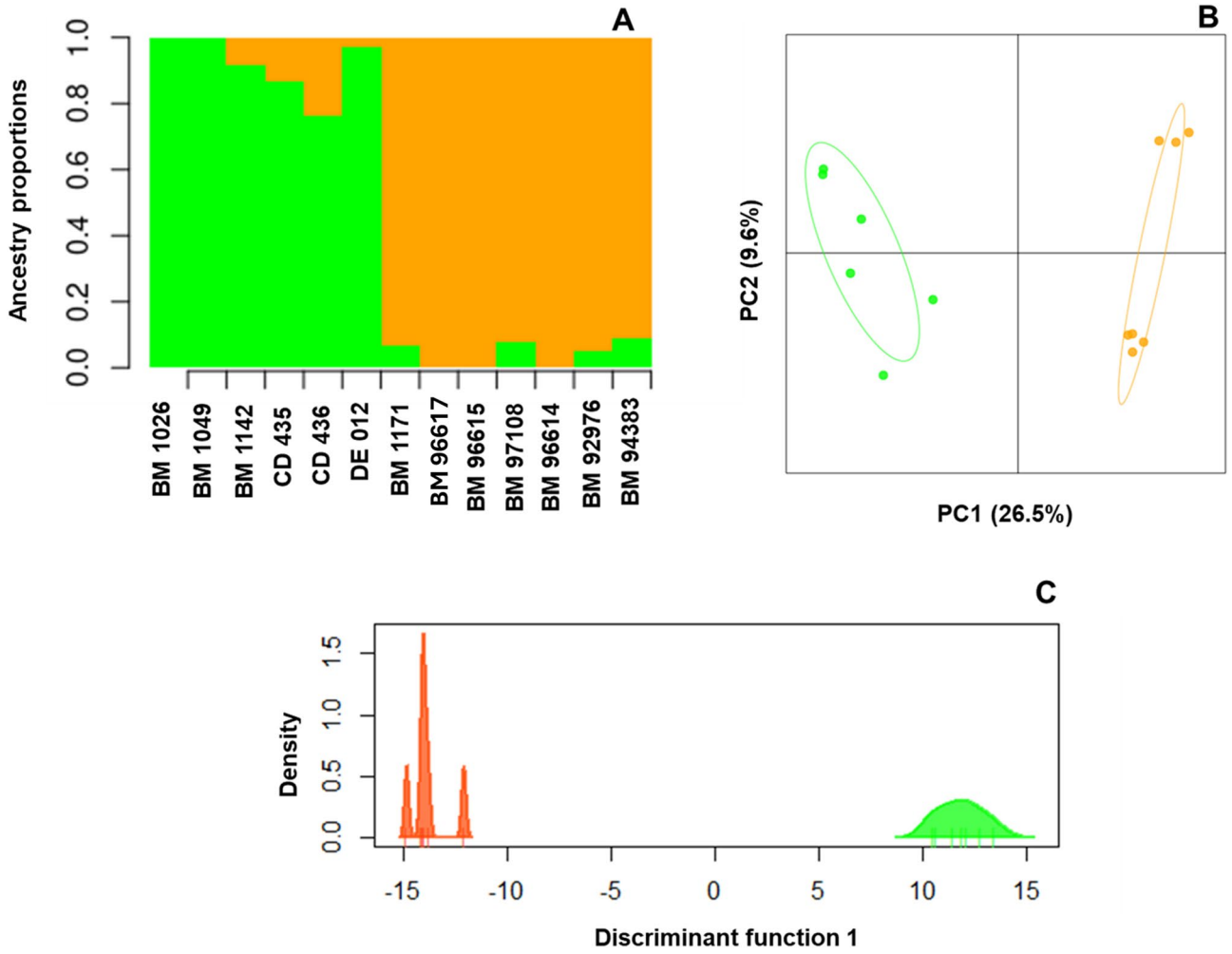

Fig. 3. Estimates of individual admixture proportions inferred with sNMF (**A**), Principal Component analysis, PCA (**B**) and Discriminant Analysis of Principal Components, DAPC (**C**) for *Alouatta* specimens from the Xingu River (Vitória do Xingu, PA, Brazil) using SNP data. Green and orange colors highlight, respectively, *Alouatta discolor* (left bank of the Xingu River and Pimental Island) and *Alouatta belzebul* (right bank of the Xingu River) lineages.

score. Regarding the SNPs, both lineages evidenced similar values of expected and observed heterozygosity, with non-significant excess or deficit of heterozygotes, indicating no evidence of inbreeding, confirmed by the negative inbreeding coefficients, which showed confidence intervals within the expected.

The high genomic diversity and haplotype and nucleotide diversity scores for the *Alouatta* lineages studied herein, as well as the high number of mutational steps observed in some Dloop haplotypes, may be attributed to the possibility of the previous existence of a great and stable population in the past, which diverged from isolation promoted, likely, by a geographic barrier. In this context, Boubli et al.[62] reinforce the idea that the primates reached the Neotropical region by the late Oligocene, that is, before the final stage of uplifting of the Andean Cordillera and the subsequent reorganization of the Amazon drainage system[68–74]. In this way, the constitution of new rivers throughout time was an important vicariance factor among and within species[68].

Changes in the Amazon hydrography and forest cover during the Quaternary period may have also been decisive for the diversification and the current disposition of the genetic status of *Alouatta* and other Amazon fauna, as mentioned in Ribas et al.[74] and Ferreira et al.[75]. In turn, the hypothesis that *A. discolor* and *A. belzebul* have evolved separately leading to a higher variation takes into account the river acting as a restriction to the gene flow, which consequently could corroborate genetic drift[76]. Nonetheless, several characteristics of the rivers may affect their potential as barriers, such as meander loops, streamflow, presence of islands, time of formation and time of river establishment. In this sense, studies have shown that taxa occurring in river mouths exhibit less similarity than those in headwater[76].

The recent connection through the northeastern Atlantic Forest, related to the Pleistocene climatic cycles, have probably created gallery forests that acted as a connection between the Amazon and the Atlantic Forest, allowing the maintenance of gene flow between these two biomes[77,78]. In this context, *A. belzebul* within the genus *Alouatta* has the most ancient demographic expansion, which can explain the largest distribution and its occupation in both Amazon and Atlantic Forest biomes, while congeneric taxa such as *A. discolor* is endemic to

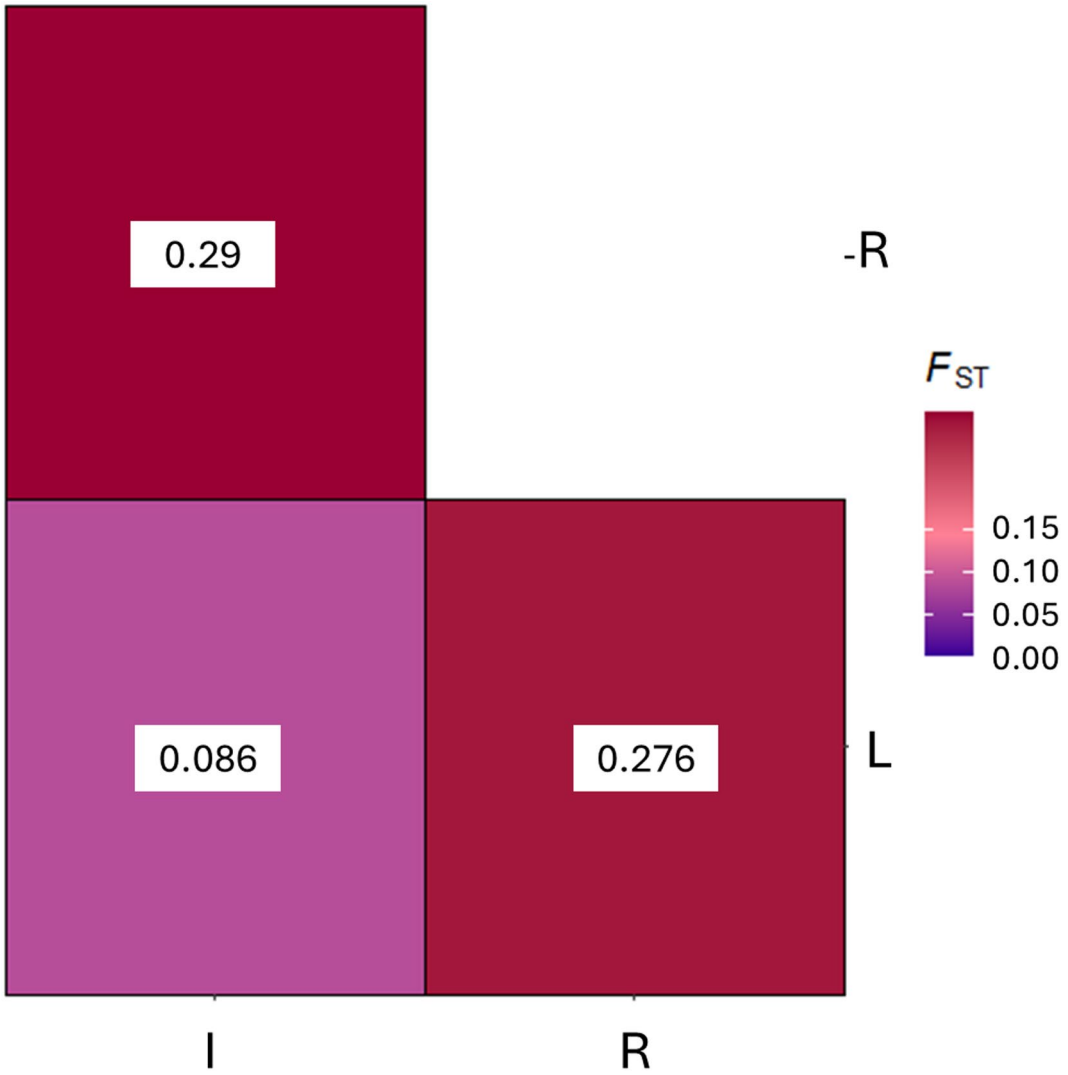

Fig. 4. Plot for the values of F_{ST} for *Alouatta* from the Xingu River (Vitória do Xingu, PA, Brazil), evidencing the sample localities and the color scale pattern for the genetic differentiation level using SNP data. Range color varies from purple (lower level of genetic differentiation) to red (higher level of genetic differentiation). White indicates absence of genetic differentiation. R: right bank; L: left bank; I: islands.

Genetic Diversity	*Alouatta discolor*	*Alouatta belzebul*
N	6	7
Number of alleles	7970	25,886
Allelic richness	1.993	1.977
Ho	0.3816	0.3510
Hs	0.3638	0.3260
F_{IS}	-0.049	-0.077
CI	-0.067 to -0.031	-0.086 to -0.067

Table 2. Genetic diversity parameters using SNP data for *Alouatta* specimens from the Xingu river. N: number of samples; number of alleles: total number of alleles; ho: observed heterozygosity; hs: expected heterozygosity; F_{IS}: inbreeding coefficient; CI (confidence interval for F_{IS}).

Amazon, probably having maintaining more restricted gene flow throughout its history[10]. Additionally, the high levels of genetic diversity observed herein could be attributed to the occurrence of secondary contact between distinct lineages that were previously allopatric[79–81].

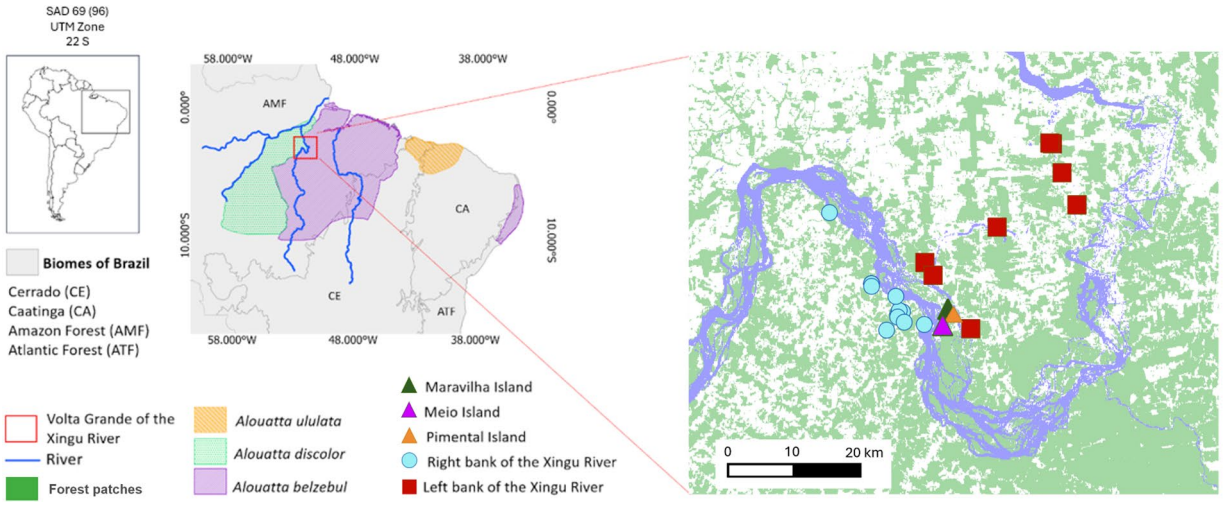

Fig. 5. Map indicating the zone of distribution of *Alouatta discolor*, *Alouatta belzebul* and *Alouatta ululata* and the respective biomes; and the howler monkey samples from the Xingu River in the region of Volta Grande of the Xingu River in the state of Pará, Brazil. The map was generated by the author using the software Qgis v.3.34.14. (https://qgis.org).

Doyle et al.[82], in turn, have used multiple nuclear genomic regions, allied with lognormally distributed fossil-based priors, to obtain the minimal age defined by a fossil and avoid underestimate divergence dates. The authors indicated that the divergence of *A. belzebul* has occurred approximately 9 million years ago (MYA) from other South American species, along with a subdivision occurring in this clade 2.5 MYA. Some approaches have estimated the divergence of Mesoamerican and South American howler monkeys at 13.2 MYA, coinciding with the latest height of the Andes (~ 14 MYA) and with the formation of the land connection by the Isthmus of Panama[83]. Such assessments provide the split of the Mesoamerican howler monkeys moving northwards, and the South American howler monkeys moving southwards.

Although the relatively high estimated genetic diversity for *Alouatta* using genomic data obtained on a large scale is a novel result for the group, analyses using microsatellite loci have already reported similar results in the *A. belzebul* complex. High levels of expected heterozygosity (He), absence of geographic barriers, and the evidence of populations in expansion found in neutrality were reported for populations of *A. belzebul* (He = 0.78–0.86) from both banks of the Tocantins River in the flooding area of the Tucuruí Hydroelectric Dam[84]. In addition, mitochondrial analysis of the Cytochrome Oxidase gene (Cytb) were also performed in the *A. belzebul* complex in the same area[85], showing high haplotype diversity (Hd = 0.93–0.96) and no evidence of the river acting as a barrier to gene flow between populations. Moreover, the indication of the Tocantins river formation in the Pleistocene period reinforces the idea that it does not act as a zoogeographical barrier between the riverbanks, since the existence of islands in the area, formed in the same period, brings the possibility of migration of individuals from one bank to another[85].

For populations from Xingu river, genetic analyses are restricted to mitochondrial analyses of the Cytb gene[10], which evidenced lower levels of haplotype diversity in populations from the left bank (west) of the Xingu River (Hd = 0.766; *A. discolor*) in relation to populations from the right (east) bank (Hd = 0.901; *A. belzebul*). In this study, the authors also assessed haplotype diversity for populations from Tocantins river (Hd = 0.966), which showed similar values to those reported by Nascimento et al.[78] and in the study herein. Phylogenetic analyses using complete mitochondrial genomes were also recently conducted in *Alouatta*, including few specimens of *A. discolor* from the left (west) bank of the Tapajos River and the left (west) bank of the Xingu River, in order to test the hypothesis of riverine barrier in Platyrrhini. The study, however, concluded that large rivers do not seem to act as a barrier for *Alouatta* species[49].

In our study we found *A. discolor* and *A. belzebul* taxa in the region of Volta Grande of the Xingu River, both showing high genetic diversity levels. These results are of major importance with regards to the distribution of the two *Alouatta* lineages and their populations in the study area, since these howler monkeys were rescued and transferred to areas nearby to their original living-sites, before the inundation of the forest during the installation of the UHBM. Worthy of note is that all rescued animals were assumed to be *A. belzebul*, but have now been recognized as two distinct lineages, with the possibility of contemporary contact between them, indicating the need for further studies to investigate signs of eventual hybridization.

Overall, our findings provide useful information for a better understanding of the group diversification, as well as for monitoring both taxa and their populations, gathering reference data for evaluating eventual impacts in these populations by comparing genetic diversity and structure before and after the UHBM installation, and for the development of appropriate management actions in conservation plans. The study is also helpful for conservation programs of other taxa, serving as a model for similar studies.

ID	Original locality	Capture coordinates	Release coordinates	Collection date	Dloop AC	GBS AC	Sex
AC 10508	Left Bank	X:414,625 Y:9,644,101	X: 415,516 Y: 9,647,542	2015-03-25	PQ438768	-	M
BR 5608	Left Bank	X:416,876 Y:9,639,102	X: 411,198 Y: 9,653,302	2015-04-04	PQ438766	-	M
BR 5609	Left Bank	X:416,876 Y:9,639,102	X: 411,198 Y: 9,653,302	2015-04-04	PQ438767	-	M
BM 1026	Left Bank	X:393,200 Y:9,630,480	No data available	2013-05-06	-	PRJNA1170355	-
BM 1049	Left Bank	X:394,450 Y:9,628,490	No data available	2013-06-04	PQ438770	PRJNA1170355	-
BM 1142	Left Bank	X:400,210 Y:9,620,161	No data available	2013-08-31	-	PRJNA117035	-
BM 115789	Left Bank	X:412,792 Y:9,648,715	No data available	2015-05-26	PQ438769	-	-
BM 115771	Left Bank	X:413,175 Y:9,648,600	No data available	2015-05-26	PQ438765	-	F
BM 115793	Left Bank	X:412,792 Y:9,648,715	No data available	2015-05-26	PQ438771	-	F
BM 1171	Right Bank	X:392,989 Y:9,620,899	No data available	2013-08-20	PQ438758	PRJNA117035	-
BM 96617	Right Bank	X:384,865 Y:9,627,307	No data available	2016-01-20	PQ438761	PRJNA117035	M
BM 96615	Right Bank	X:384,865 Y:9,627,307	No data available	2016-01-20	PQ438759	PRJNA117035	M
BM 97108	Right Bank	X:378,476 Y:9,638,367	No data available	2016-01-20	PQ438756	PRJNA117035	M
BM 96614	Right Bank	X:384,865 Y:9,627,307	No data available	2016-01-20	PQ438760	PRJNA117035	-
BM 92976	Right Bank	X:384,875 Y:9,626,900	No data available	2016-01-20	-	PRJNA117035	-
BM 94383	Right Bank	X:387,125 Y:9,620,100	No data available	2016-01-20	-	PRJNA117035	M
BM 54803	Right Bank	X:389,625 Y:9,622,900	No data available	2015-05-26	PQ438754	-	F
BM 55617	Right Bank	X:388,875 Y:9,622,500	No data available	2015-05-26	PQ438755	-	F
BM 55623	Right Bank	X:389,125 Y:9,623,100	No data available	2015-05-26	PQ438762	-	F
BM 56345	Right Bank	X:388,875 Y:9,622,100	No data available	2015-05-26	PQ438757	-	F
BM 52712	Right Bank	X:388,625 Y:9,625,300	No data available	2015-05-26	PQ438763	-	F
BM 55415	Right Bank	X:389,875 Y:9,621,300	No data available	2015-05-26	PQ438764	-	F
NG 1506	Meio Island	X:395,854 Y:9,620,715	X: 399,749 Y: 9,626,463	2015-07-14	PQ438774	-	M
NG 1522	Maravilha Island	X:396,694 Y:9,623,241	X: 399,749 Y: 9,626,463	2015-07-18	PQ438777	-	F
CX 23	Maravilha Island	X:396,694 Y:9,623,241	X: 399,749 Y: 9,626,463	2015-07-18	PQ438776	-	F
CX 24	Maravilha Island	X:396,694 Y:9,623,241	X: 399,749 Y: 9,626,463	2015-07-18	PQ438775	-	F
CD 435	Pimental Island	X:397,542 Y:9,622,005	X: 402,263 Y: 9,623,144	2015-02-19	-	PRJNA117035	F
CD 436	Pimental Island	X:397,542 Y:9,622,005	X: 402,263 Y: 9,623,144	2015-02-19	PQ438773	PRJNA117035	M
DE 012	Pimental Island	X:396,875 Y:9,622,500	X: 400,742 Y: 9,623,841	2015-02-19	PQ438772	PRJNA117035	M

Table 3. Data for *Alouatta* specimens collected in the Volta Grande of the Xingu river (PA, Brazil), describing individual identification (ID), original locality, coordinates for the capture sites and for the release sites, date of capture, and the methodology applied in this study. GBS: genotyping by sequencing analyses. Dloop: mitochondrial analyses of the control region. M: male; F: female. Accession Number (AC) for Dloop and GBS data. Data not available (–).

Methods

Legal and ethical permits

This study is reported in accordance with ARRIVE guidelines (https://arriveguidelines.org) and the Code of best practices for field Primatology[86]. All experimental protocols were approved by the Ethic Committee on Animal Use and Experimentation of the Federal University of São Carlos (CEUA #6618060320), and the Biodiversity Authorization and Information System of the Ministry of the Environment of Brazil (SISBIO #71555 and #49643). We also obtained the due authorization from the National System of Genetic Patrimony Management and Associated Traditional Knowledge (SisGen #A411359 and #AF14F2E) of the Ministry of the Environment of Brazil to access genetic information. All methods were carried out in accordance with relevant guidelines and regulations.

Study area and biological sampling

The biological samples of the specimens analyzed in the present study come from the Volta Grande region of the Xingu River, located between the municipalities of Vitória do Xingu and Altamira (03°24'43"S, 51°57'56"W), in the southwest of the state of Pará, where *A. discolor* and *A. belzebul* may co-occur (Fig. 5). In total we sampled 29 howler monkeys from this area, in which nine were from the left bank of the Xingu River, 13 were from the right bank of the Xingu River, three were from the Pimental Island, one was from the Meio Island and other three were from the Maravilha Island (Fig. 5; Table 3).

The samples of howler monkeys collected here are from individuals that needed to be rescued and translocated to non-flooded areas, by the Project for Rescue and Scientific Use of Fauna (PRSUF), before the installation of the UHBM. During the field expedition (2015), all rescued specimens were morphologically identified as *A. belzebul*, using coat color criterion following Bonvicino et al.[11] and Gregorin[5], despite the geographic distribution suggesting that sampling could include *A. discolor*[10]. The procedures for biological sampling were performed by

veterinarians following all recommendations proposed by the American Society of Primatologists (ASP) and the International Primatological Society (IPS). The howler monkeys were anesthetized by direct induction using an inhalation equipment calibrated with Isoflurane (2–5%) and Oxygen (2 L/min). Approximately 200–500 µl of peripheral blood per individual were collected by venipuncture, transferred immediately into EDTA tubes and stored at − 20 °C. After biological collections the sampled animals were safely released in the wild.

In addition to the samples collected here, we downloaded, from GenBank (https://www.ncbi.nlm.nih.gov/genbank/), Dloop sequences for two specimens named *A. discolor*, as follows: BM 48342 (Accession Number OM328950.1) from the left bank of the Xingu River (X: 391727 and Y: 9627257) and TAP 315355 (Accession Number OM328889.1) from the right bank of the Tapajos River (X: 695871 and Y: 9620044). These samples were then used in the phylogenetic tree and haplotype network.

Mitochondrial DNA analyses

Amplification and sequencing of the MtDNA control region (Dloop)

We amplified the control region (Dloop) of the mitochondrial DNA (mtDNA) using the primer pair How-RA1 Forward (5' CTACCATCAACACCCAAAGC 3') and How-RA2 Reverse (5' ACCCGTCTAGACATTTT C 3'), described by Ascunce et al.[87] and the Polymerase Chain Reaction (PCR) protocol recommended by the authors. Samples showing satisfactory amplification patterns were purified following the Lis protocol[88], and then sequenced in a single direction on an ABI 3730XL sequencer (Applied Biosystems, Foster City CA, USA). We decided to perform Dloop analyses because recent assessments, on *Alouatta* specimens from the same region studied here, distinguished *A. discolor* and *A. belzebul*[10] using this mitochondrial marker. Thus, we were able to compare the data from our study with the prior sequences available in Genbank.

Population analyses for Dloop data

The Dloop sequences produced in this study and those downloaded from GenBank were aligned and edited using Geneious R7 v.6.1.6[89] and the ClustalW tool[90]. An ultrametric topology was constructed using the Bayesian Inference (BI) method implemented in BEAST v.2.2.1[91]. The nucleotide substitution model was selected based on the Bayesian Information Criterion (BIC) using JModelTest v.2.1.10 (HKY + I)[92]. A strict molecular clock, appropriate for intraspecific data[93], and a Yule model as the tree prior were applied. Three independent Markov chain runs of 10 million iterations each were performed, with trees and parameters recorded every 10,000 generations. Tree and log files were combined using LogCombiner v.1.8[94]. Convergence and effective sample size (ESS ≥ 200) of estimated parameters were assessed in Tracer v.1.7[95]. The final tree was summarized with TreeAnnotator v.1.8[96] and visualized in FigTree v.1.4 (http://tree.bio.ed.ac.uk/). To illustrate phylogeographic relationships among haplotypes, the haplotype network was constructed using the default settings of the haploNet function, based on the infinite sites model, calculating a distance matrix using dist.dna function with "N" model (which excludes sites with missing data), using the R package pegas v.1.3[97].

Genetic diversity parameters, such as number of haplotypes and polymorphic sites, and nucleotide and haplotype diversity, were determined with the program DnaSP[98], for the individuals collected in the Xingu River. DnaSP was also used to calculate the fixation index F_{ST}, and the mean interspecific Kimura two-parameter (K2P) distance was determined using MEGA v.6.04[99].

Genotyping by sequencing (GBS) analyses

Library construction and sequencing

GBS libraries were constructed using the restriction enzyme PstI (New England BioLabs, Ipswich, MA, USA), following the recommendations proposed by Elshire et al.[100]. After digestion of 200 ng of DNA normalized to 5 ng/µl, a ligation reaction of indexing sequences and adapters was performed using T4 DNA ligase. Subsequently, the samples were pooled and purified with magnetic beads (Agencourt AMPure XP, Beckman Coulter, São Paulo, SP, Brazil). The restriction fragments were amplified and purified, and the genomic library quality was verified on a BioAnalyser Agilent 2100 (Agilent Technologies, Santa Clara, California, USA), using a High Sensitivity DNA kit. The genomic library was quantified on a real-time PCR (RT-PCR) with a KAPA Biosystems Quantification kit (Sigma-Aldrich, Merck KGaA, San Luis, Missouri, USA) and diluted to 2 pM. Then, sequencing was performed on a HiSeq 2500 Illumina Platform (Illumina, San Diego, CA, USA).

GBS data processing

The pipeline Stacks v.65[101] was used for quality evaluation, demultiplexing, filtering and identification of Single Nucleotide Polymorphisms (SNPs). First, low quality sequences were eliminated (a raw Phred score of 10, i.e. keeping reads with a 90% probability of being correct). Then, subsequent steps were performed according to the following parameters: minimum sequencing depth (-m) ≥ 3, maximum distance between stacks (-M) = 2, maximum distance between primary and secondary sequences (-N) = 4, and maximum difference between stacks (-n) = 5. The minimum percentage of individuals per locus retained at this stage was 70% of the total sample set, considering only the first locus found in each sequence. An additional step for data quality control was performed using the R package r2vcftools[102].

We removed individuals with more than 15% missing data and filtered the SNPs for excess of heterozygosity based on deviations from Hardy-Weinberg equilibrium (HWE, $p < 0.0001$), and for overall minor allele frequency (MAF) > 0.05%. Also, only the loci present in at least 85% of the samples, and with at least six reads depth, were retained. Then, we implemented a Principal Component Analysis (PCA) using a Mahalanobis distance-based approach with PCAdapt v.4.3.3[103] in R[104]. We assumed that SNPs excessively related to population structure are outliers and potential candidates for differential selection studies. Outlier loci were scored based on q-value corrected p-values, with K set to 2[105,106]. Then, the subset of non-outlier loci was considered neutral loci.

Population analyses for GBS data

Genetic diversity and structure were assessed using only the neutral loci. We evaluated the population genetic structure with the *"snmf"* function from LEA package v.2.0[107], which considers the individual ancestrality and the populational grouping using a non-negative matrix factorization (sparse non-negative matrix factorization - sNMF) and a procedure for optimizing of the minimum squares of the ancestrality coefficients. The "*snmf*" function was used with K = 1–10, with 100 repetitions per K value. The cross-entropy criterion was adopted to determine the value of K that best explained the results. We also performed a multivariate approach based on the Discriminant Analysis of Principal Components (DAPC) using Adegenet package[108]. The optimal number of principal components (PCs) to retain was determined using alpha score optimization, focusing on PCs that explained 80% of the variance. The Bayesian Information Criterion (BIC) was then used to select the appropriate number of discriminant functions for representing the datasets. Finally, we implemented a Principal Component Analysis (PCA), with the Adegenet package[108].

The fixation index F_{ST} was estimated using the package "hierfstat"[109], that calculates the F statistics considering levels of inbreeding and coancestrality from diploid organisms, at different levels of hierarchy[110]. We also used the functions "*basic.stats*", "*nb.alleles*" and "*allelic richness*" to determine, respectively, the allele frequencies and heterozygosity values, the number of alleles, and the allelic richness[111]. All these analyses were conducted in R[104].

Data availability

The mitochondrial sequences generated in this study are deposited in the GenBank repository with the accession numbers PQ438754 – PQ438777. The raw sequence data for GBS analysis are available in the NCBI BioProject and in the Sequence Read Archive repository under accession number PRJNA1170355 (SRA: SRR30914384 – SRR30914396). Any additional information about the data may be requested from the corresponding author.

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Author contributions

J.V.G. and P.D.F. conceived the experiments, V.Y.G. and F.R.M. collected the samples, J.V.G., L.P. and C.C.G. conducted the laboratory procedures. J.V.G., C.C.G., P.M.G.J., A.G. and P.D.F. analysed and discussed the results. J.V.G and P.D.F. led the manuscript writing.All authors reviewed and approved the final version of the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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