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Genetic analysis of an insular population of *Sapajus nigritus* (Primates: Cebidae) in Rio de Janeiro state, Brazil

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Abstract

The black-horned capuchin (*Sapajus nigritus*) is a neotropical primate with wide distribution from southeastern Brazil to northeastern Argentina. Although this species has been described with coat pattern variation, even with intrapopulation differences, and characterized as having the greatest genetic diversity among *Sapajus* species, there are still few studies on natural populations that contribute to the knowledge of this intraspecific variability. We examined individuals from an as yet unstudied population of Ilha da Marambaia, Rio de Janeiro (RJ) state, Brazil, compared with published data for *S. nigritus*. We sought to confirm the species through phenotypic and genetic characterization using C-banding and fluorescence in situ hybridization with #11qHe+/21WCP probes for chromosomal constitutive heterochromatin (He+) patterns, and cytochrome c oxidase I and II gene sequences for phylogenetic analysis. The coat presented two color patterns, varying from brown to blackish on the body, yellow to brown on the chest, and white to yellow on the face, besides the presence and shape of the tufts on the head, corresponding to *S. nigritus*. He+ was identified in pairs 4, 12, 13 and 17, and less consistently in pairs 6, 19 and 21, already described for this species. While most *Sapajus* species have a large He+ block, here pair 11 was identified without extracentromeric He+, the same as reported for *S. nigritus* from Argentina. Molecular analysis showed divergence of this population from other *S. nigritus* sequences, reinforcing a trend already demonstrated when samples from RJ are compared with the rest of the distribution, which may represent an evolutionary deviation.

Keywords Capuchin monkeys · C-banding · Fluorescence in situ hybridization · Cytochrome c oxidase I and II · DNA sequencing · Ilha da Marambaia

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10329-020-00880-9>.

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Introduction

The species of *Sapajus* (robust capuchin monkeys) are widely distributed from the north of South America to northeastern Argentina and southern Brazil (Vilanova et al. 2005; Lynch Alfaro et al. 2012a, b). Currently, eight species are considered, namely *S. macrocephalus*, *S. apella*, *S. flavius*, *S. libidinosus*, *S. xanthosternos*, *S. robustus*, *S. cay* and *S. nigritus* (Lynch Alfaro et al. 2012b), with high phenotypic and genetic diversity, throughout several habitats (Rylands et al. 2005; Lynch Alfaro et al. 2012a; Lima et al. 2018; Martins-Junior et al. 2018).

Endemic to the Atlantic Forest, *Sapajus nigritus* (Goldfuss 1809) occurs in southern and southeastern regions of Brazil as well as in northeastern Argentina (Fragaszy et al. 2004; Vilanova et al. 2005). Two subspecies are described, with *S. n. cucullatus* occurring in the Brazilian states of Santa Catarina and Rio Grande do Sul, along with Misiones,

Argentina, while *S. n. nigritus* is restricted to Brazil, in the states of Minas Gerais, Espírito Santo, São Paulo and Rio de Janeiro (Lynch Alfaro et al. 2014). The wide distribution favors the intraspecific diversity reported for *S. nigritus*, be it phenotypic (Silva Jr. 2001; Vieira and Oliveira 2014), molecular (Ruiz-García et al. 2012; Martins-Junior et al. 2018) or cytogenetic (Freitas and Seuánez 1982; Matayoshi et al. 1987; Nieves et al. 2017).

Commonly known as black-horned capuchin, the subspecies of *S. nigritus* has coat coloration ranging from deep brown to black (and white fur mixed with the dark brown in *S. n. cucullatus*), with vague or absent dorsal stripe. The limbs are darker than the rest of the body and the chest has a reddish hue. The face is white in contrast to the rest of the body. The cap is dark, and tufts on the top of the head are evident in adults (Groves 2001; Fragaszy et al. 2004; Rylands et al. 2005). Different patterns of coat coloration may occur, corresponding to the color pattern described for the species, even within a population (Vieira and Oliveira 2014).

Molecular markers have been applied to understand the phylogenetic relationships among species. The mitochondrial cytochrome c oxidase I gene (*MT-COI*) is considered a suitable tool that best reconstructs the taxonomy for neotropical primates (Mello et al. 2018) and has been used in recent phylogenetic studies of *Sapajus* (Ruiz-García et al. 2016; Lima et al. 2017). Using this gene, in association with other mitochondrial regions (cytochrome b and D-loop), the monophyly of *S. nigritus* was confirmed, demonstrating that the species is more related to *S. xanthosternos* and *S. robustus* (Lima et al. 2017). Another mitochondrial gene widely used in phylogenetic studies with primates (Ashley and Vaughn 1995; Collins and Dubach 2000; Ascunce et al. 2002, 2003; Ruiz-García et al. 2010) is cytochrome c oxidase II (*MT-CO2*). Through the use of this gene, it has been identified that *S. nigritus* shows great intraspecific diversity, besides the greater genetic divergence in relation to the other *Sapajus* species, with the highest estimated time of emergence, approximately 357,000 years ago (Ruiz-García et al. 2012). Regarding the great intraspecific diversity observed in *S. nigritus*, results with the mitochondrial cytochrome b showed paraphyletic clustering when a sample from Rio de Janeiro state, Brazil, was included in the analysis (Lynch Alfaro et al. 2012a; Nascimento et al. 2015; Martins-Junior et al. 2018), representing a possible genetic divergence of the populations in this region.

Cytogenetic analysis has shown that the karyotype of *S. nigritus*, as well as the other *Sapajus* species, is $2n = 54$, XX/XY (Matayoshi et al. 1986). The X chromosome is submetacentric, and the Y chromosome has already been described as a small acrocentric (Matayoshi et al. 1986; Mudry et al. 1991; Amaral et al. 2008; Iughetti 2008) and submetacentric chromosome (Freitas and Seuánez 1982).

Although the karyotype is the same for the species, there are differences in the presence and amount of extracentromeric constitutive heterochromatin in chromosome pairs, as evidenced by the C-banding. For *S. nigritus*, the extracentromeric heterochromatin pattern presents polymorphisms and has already been described at pairs 3, 4, 5, 6, 12, 13, 17, 19, 20 and 21 (Mudry 1990; Mudry et al. 1991; Ponsà et al. 1995; Steinberg et al. 2014; Nieves et al. 2017). The chromosome pair 11 is particularly characteristic for *Sapajus*, being the larger acrocentric with a large terminal block occupying up to 86% of the q arm for most species (Matayoshi et al. 1987) and a small intercalary block between euchromatin in *S. xanthosternos* (Seuánez et al. 1986). For *S. nigritus*, is described as specific the total deletion of the large terminal block, turning this pair into a small acrocentric, based in analysis of samples from Misiones, Argentina (Mudry 1990; Mudry et al. 1991; Ponsà et al. 1995; Nieves et al. 2011; Steinberg et al. 2014). However, this does not seem to be the unique form of this pair, since the large heterochromatic block has already been described in samples from Rio de Janeiro state (Freitas and Seuánez 1982; Matayoshi et al. 1987) and from captive individuals phenotypically identified as *S. nigritus* (Penedo et al. 2014).

It has been emphasized that in order to understand the diversity within the species, studies are needed that include populations throughout the distribution, mainly in environments subjected to increasing fragmentation (Lynch Alfaro et al. 2014). Groups of capuchin monkeys inhabit the Ilha da Marambaia (Marambaia Island), Mangaratiba city, south of Rio de Janeiro state, Brazil. This region represents an important Atlantic Forest remnant in Rio de Janeiro state (Roncarati and Menezes 2005), and to date there is no information about the capuchin monkey population. According to a survey conducted with the oldest residents of the local population, mostly comprising descendants of enslaved people that have inhabited Ilha da Marambaia since the eighteenth century (Nóbrega 2004), we estimate that these primates represent a natural population present in this area for at least 130 years, when residents' great-grandparents reported seeing them (pers. comm.). Complementarily, a survey carried out with the military personnel who work at Restinga da Marambaia, the only land connection between Ilha da Marambaia and the mainland, made it possible to identify that the capuchin monkeys are isolated and do not have access to the continent (pers. comm.).

Our objective was to confirm the species of the capuchin monkey population from Ilha da Marambaia, RJ, Brazil, and characterize them by phenotype, cytogenetics and molecular genetics. For this, we used classical and molecular cytogenetics, investigating the extracentromeric heterochromatin pattern, especially the form of the chromosome pair 11 and, through *MT-COI* and *MT-CO2* gene sequencing, whether

the divergence already observed in a sample from Rio de Janeiro state would be confirmed.

Methods

Study site

The study was carried out at Ilha da Marambaia ($23^{\circ}04'S/43^{\circ}53'W$), Mangaratiba city, Rio de Janeiro state, southeastern Brazil (Fig. 1). The region is classified as environmental protection area, established by state decree 9.802/87 (Rio de Janeiro 1987), and comprises 42 km² of Atlantic Forest remnant (Conde et al. 2005). Ilha da

Marambaia is almost connected to the continent by the Restinga da Marambaia, an east–west 40 km-long peninsular-like sandbank extension, remaining separated only by a small channel, called Canal do Bacalhau (Conde et al. 2005; Roncarati and Menezes 2005).

Captures and biological sampling

Captures were performed using Tomahawk traps. The animals were sedated with dissociative anesthetic (ketamine hydrochloride and midazolam, 15 mg/kg and 0.05 mg/kg, respectively), and identification was performed by a subcutaneous microchip (2 × 12 mm). The blood for genetic analysis was collected from the femoral vein, after asepsis

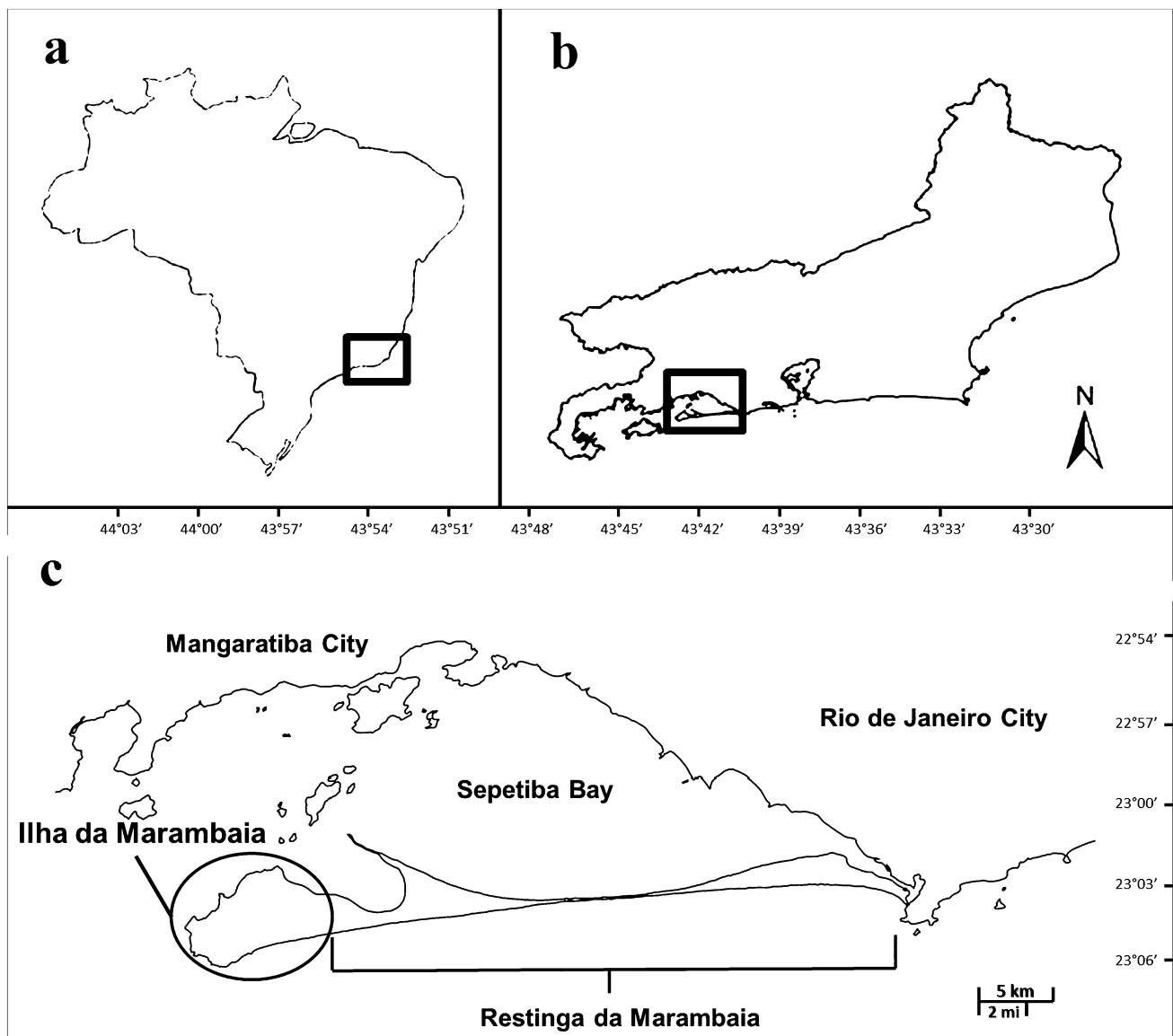


Fig. 1 Map with the location of Ilha da Marambaia, Mangaratiba city. **a** Brazil; **b** Rio de Janeiro state; **c** in detail, Ilha da Marambaia at Sepetiba Bay. The east–west peninsular-like extension of sand that connects the Ilha da Marambaia to Rio de Janeiro city is called Restinga da Marambaia

of the puncture site with 70% alcohol, and transferred to sterile tubes with heparin for cytogenetic analysis and with EDTA for molecular genetic analysis.

This research was authorized by the Biodiversity Information and Authorization System (SISBIO/IBAMA) and adhered to the guidelines of the Animal Ethics Committee of the Instituto de Ciências Biológicas e da Saúde of Universidade Federal Rural do Rio de Janeiro and to the American Society of Primatologists principles for the ethical treatment of primates.

Phenotypic analysis

The animals were photographed with a digital camera from ventral and dorsal decubitus, as well as the head in detail. The coloration of the back, the presence of a dorsal stripe, and the coloration of the chest, limbs, tail, top of head and face, including the presence and shape of tufts of fur on top of the head, were evaluated and then compared with that described for *S. nigritus* (Groves 2001; Fragaszy et al. 2004; Rylands et al. 2005). We classified the individuals as adult, subadult and juvenile using phenotypic characteristics (Izawa 1980; MacKinnon 2013), as detailed in Online Resource 1.

Cytogenetic analysis

Metaphase chromosomes were obtained by peripheral blood lymphocyte culture according to Moorhead et al. (1960). The C-banding technique was implemented following Sumner (1972) to reveal extracentromeric heterochromatic patterns, and G-banding was implemented according to Seabright (1971) for the identification of chromosome pairs. Following Nieves et al. (2017), variability in the C-banding patterns was determined in terms of heteromorphisms (absence/presence of a C+ band on one or both homologues and differences in heterochromatic block size between homologues) and polymorphisms (duplication and inversions of C+ bands).

Fluorescence in situ hybridization (FISH) was performed for confirmation of pair 11 and those with extracentromeric heterochromatin described by C-banding, using two specific probes following Steinberg et al. (2014): the #11qHe+ SCY specific for extracentromeric heterochromatin of capuchin monkeys and the human 21WCP (whole chromosome painting) probe that hybridizes with the proximal euchromatic region of pair 11 (Nieves et al. 2005). This combination of probes enables the correct identification of this pair as a small acrocentric chromosome for *S. nigritus* (Nieves et al. 2008, 2011).

DNA extraction, *MT-CO1* and *MT-CO2* gene amplification and sequencing

DNA was extracted using the QIAamp[®] DNA Blood Mini Kit (Qiagen), following the manufacturer's instructions, and quantified by spectrophotometry using a NanoDrop[®] 2000 spectrophotometer (Thermo Scientific).

Polymerase chain reaction (PCR) for *MT-CO1* gene amplification was performed using the primers Fish1SapF (5'-TCAACTAACCATAAGGATATTGGTAC-3') and Fish1SapR (5'-TATACCTCTGGGTGACCAAAAAATCA-3'), described by Lima et al. (2017). *MT-CO2* gene amplification was performed using the primers A7552 (5'-AACCATTTCATAACTTTGTCAA-3') and B8321 (5'-CTCTTAATCTTAACTTAAAG-3'), developed by Ruvolo et al. (1991). The reactions were prepared in a 25 µL volume, containing 1X buffer (GoTaq[®] Colorless Reaction Buffer, Promega[®]), 3 mM MgCl₂, 0.2 mM dNTPs, 2 µM of each primer, 2U Taq DNA polymerase (GoTaq[®] DNA polymerase, Promega[®]) and DNA (20 ng/µL). The volume was completed with ultrapure water. The PCR was performed in a ProFlex[™] PCR System thermocycler (Applied Biosystems[®] Thermo Fisher Inc.). For *MT-CO1*, temperature cycles were applied according to Lima et al. (2017), with initial temperature of 95 °C for 5 min, followed by 30 cycles of 94 °C for 45 s, 60 °C for 45 s and 72 °C for 1 min, with a final extension at 72 °C for 5 min. For *MT-CO2*, temperature cycles described by Ruiz-García et al. (2012) were used, with initial temperature of 95 °C for 5 min, followed by 35 cycles of 95 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min, with a final extension at 72 °C for 5 min.

The *MT-CO1* fragments were sequenced by Macrogen Inc. (South Korea), and *MT-CO2* was sequenced using an ABI 3130 automated DNA sequencer (Applied Biosystems[®] Thermo Fisher Inc.). The electropherogram analysis was performed using SeqScape v2.7 and Sequencing Analysis v5.4 software (Applied Biosystems[®] Thermo Fisher Inc.), and manually checked. The sequences obtained were deposited in GenBank (*MT-CO1* BankIt: MT521517 to MT521527; *MT-CO2* BankIt: MN712194 to MN712201).

Phylogenetic analysis

Molecular analysis was performed in Mega v10.1.7 software (Kumar et al. 2018). Phylogenetic relationships were inferred using the maximum likelihood method, for each gene, following the Tamura-Nei model (Tamura and Nei 1993). The bootstrap method (Felsenstein 1985), based on 1000 replications, was used to support each clade. Genetic divergence values were obtained considering all nucleotide substitutions, for each gene, based on the Tamura-Nei method (Tamura and Nei 1993).

Sequences of the *MT-CO1* gene from GenBank for all eight *Sapajus* species and one of *Cebus kaapori* (outgroup), and of *MT-CO2* from *S. nigritus*, *S. cay*, *S. xanthosternos* and *Cebus capucinus* (outgroup) were used in phylogenetic analysis, as detailed in Table 1. Only *S. nigritus* sequences were used in genetic divergence analysis.

Results

Twelve individuals of capuchin monkeys were captured at Ilha da Marambaia, including eight males (five adults and three subadults) and four females (two adults and two juveniles).

Table 1 Sequences of *MT-CO1* and *MT-CO2* genes of *Sapajus* species obtained from GenBank and used in this study for comparison

Species	Identification	GenBank codes	Geographical origin	References
<i>MT-CO1</i> gene sequences				
<i>Sapajus nigritus</i>	S nigritus_01	KY173124	São Paulo, Brazil	Lima et al. (2017)
	S nigritus_02	KY173125		
	S nigritus_03	KY173126	Paraná, Brazil	
<i>S. robustus</i>	S robustus_01	KY173127	No information	
<i>S. macrocephalus</i>	S macrocephalus_01	KY173121	Rondônia, Brazil	
	S macrocephalus_02	KY173122		
	S macrocephalus_03	KY173123	Amazonas, Brazil	
<i>S. xanthosternos</i>	S xanthosternos_01	KY173128	Bahia, Brazil	
	S xanthosternos_02	KY173129		
	S xanthosternos_03	KY173130		
	S xanthosternos_04	KY173131		
<i>S. libidinosus</i>	S libidinosus_01	KY173096	Goiás, Brazil	
	S libidinosus_02	KY173097		
	S libidinosus_03	KY173098		
<i>S. flavius</i>	S flavius_01	KY173086	Paraíba, Brazil	
	S flavius_02	KY173087		
	S flavius_03	KY173088		
<i>S. cay</i>	S cay_01	KY173084	Mato Grosso, Brazil	
	S cay_02	KY173085		
<i>S. apella</i>	S apella_01	KY173081	Rondônia, Brazil	
	S apella_02	KY173082		
	S apella_03	KY173083		
<i>Cebus kaapori</i>	Cebus kaapori	KY173039	Maranhão, Brazil	
<i>MT-CO2</i> gene sequences				
<i>Sapajus nigritus</i>	S nigritus_01	AF181088	Argentina (captivity)	Ascunce et al., 2002
	S nigritus_02	*	Argentina	Ruiz-García et al. (2012)
	S nigritus_03	*		
<i>S. cay</i>	S cay_01	AF181089	Paraguay (captivity)	Ascunce et al., 2002
	S cay_02	*	Paraguay	Ruiz-García et al. (2012)
	S cay_03	*	Brazil	
<i>S. xanthosternos</i>	S xanthosternos_01	*	Brazil	
<i>Cebus capucinus</i>	Cebus capucinus	JF735258	Colombia	Ruiz-García et al. (2011)

Note: The species, the identification used in the analysis, the GenBank reference codes, the geographical origin of the samples and the references of the works where they were used are shown

*Sequences not published on GenBank and provided by Manuel Ruiz-García

Phenotype

All 12 individuals presented a black cap covering the top of the head, extending down the sides and connecting at the jaw (Fig. 2a). The face was contoured by white to yellow fur covering part of the forehead and cheeks (Fig. 2b–d).

Five adults (three males and two females) presented the back with uniform brown pelage (with no dorsal stripe), also covering the upper arms. The forearm, pelvic limbs and tail were black (Fig. 2e). The belly had yellowish fur (Fig. 2f). One male presented small tufts turned to the sides (Fig. 2b), while in a second one it was more prominent and erect (Fig. 2c). In the third male there was no tuft of hair on the head. The two females presented erect tufts (Fig. 2d).

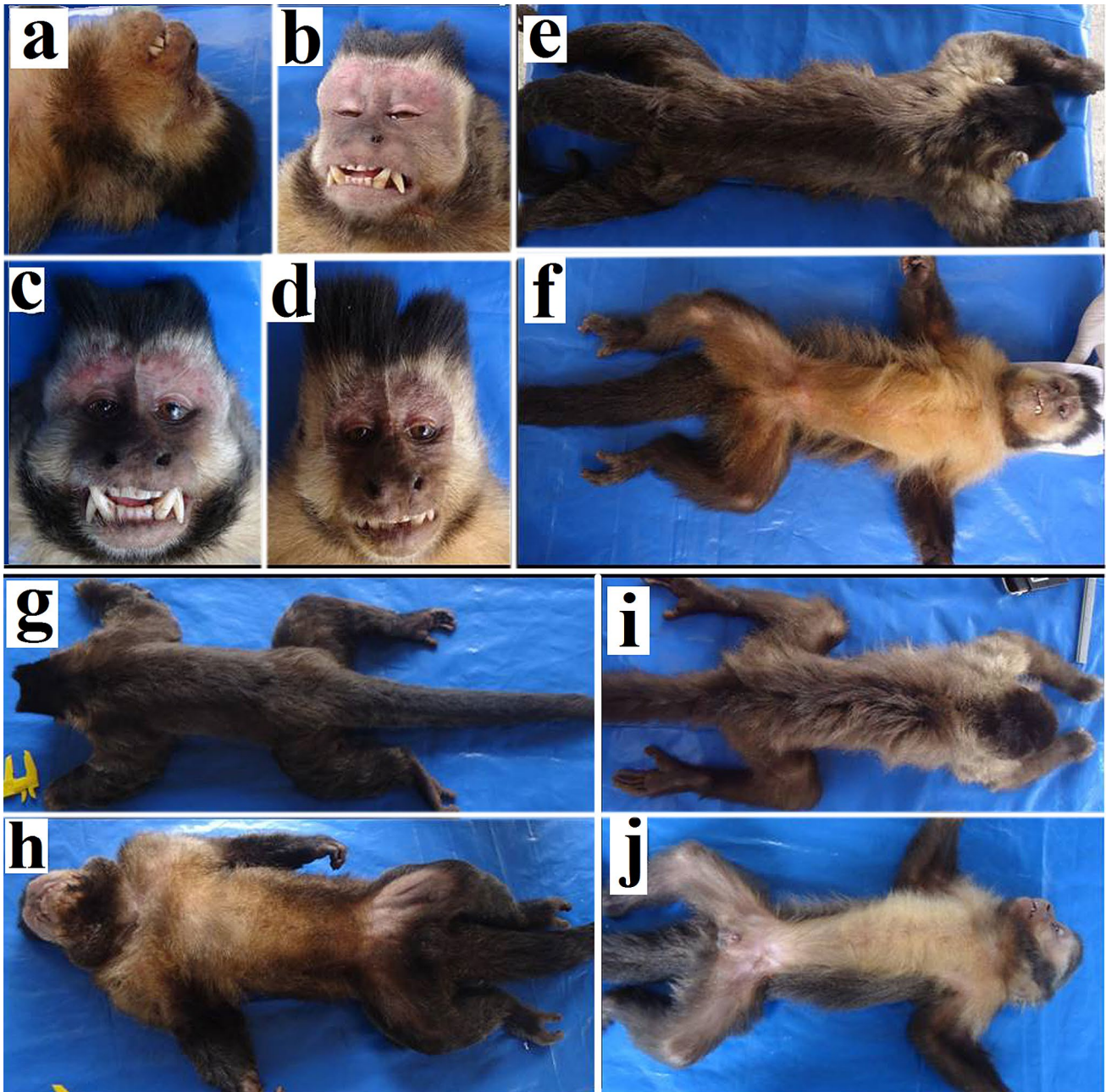


Fig. 2 Coat patterns presented by individuals of capuchin monkeys from Ilha da Marambaia, Rio de Janeiro state, Brazil. (a–d) Fur coloration of the face and shapes of tufts on top of the head; (e and f)

five adults with lighter coat pattern; (g and h) two adult males with darker coloration; (i and j) three subadults and two juveniles with coat coloration like the first group, but presenting a dorsal stripe

Two adults (males) presented a slightly different phenotypic pattern, with a blackish color covering the back, limbs and tail (Fig. 2g). The belly was darker than that observed in the previously described adult group, with brown coloration (Fig. 2h). Both individuals presented small tufts turned to the sides.

Three subadult males and two juvenile females presented a fur pattern like the first five adults, except for the lighter belly (light yellow) and the presence of a dorsal stripe extending from the cap to the base of the tail (Fig. 2i and j). Tufts were not observed on these individuals.

Cytogenetic data

The 12 individuals presented $2n = 54$, XX/XY, with ten biarmed and 16 acrocentric pairs. Sex chromosomes were submetacentric, with the Y chromosome being the smallest of the karyotype.

Extracentromeric heterochromatin, intercalar to euchromatin, was identified on chromosome pairs 4, 12, 13 and 17 in all individuals, along with pairs 6, 19 and 21 less consistently (Fig. 3a).

Chromosome pair 13 presented three variants. In six individuals, the heterochromatic band occupied approximately 45% of the q arm, close to the centromere, in homozygosis (Fig. 3a). In the other six individuals, another two variants were observed. In four of them, one chromosome had the same pattern described previously and its homologue was smaller, showing a more distal and reduced band, occupying approximately 30% of the arm (Fig. 3b). The other two individuals presented similar patterns, although the chromosome

with proximal C-banding presented reduced size, with the band occupying approximately 30% of the arm (Fig. 3c).

FISH with the 21WCP probe confirmed pair 11 as a small acrocentric without an extracentromeric heterochromatic band (Fig. 4 in red). The #11qHe+ probe confirmed the C-banding results, revealing constitutive heterochromatic regions in chromosome pairs 4, 6, 12, 13, 17, 19 and 21, all intercalar to euchromatin (Fig. 4 in green).

Molecular data

Sequences were obtained of the *MT-CO1* gene ranging from 636 to 648 bp for 11 individuals and of the *MT-CO2* from 555 to 695 bp for eight individuals. One haplotype was identified in the *MT-CO1* sequences. Four haplotypes were observed in the *MT-CO2* gene, diversified by one base deletion m.500delA in an individual (sequence MN712197), a base substitution m.182C > A in another individual (MN712199), and a base insertion m.635_636insA for a third individual (MN712200).

Through phylogenetic analysis (Fig. 5), the sequences of Ilha da Marambaia were not grouped with those of *S. nigritus* from São Paulo, Paraná (Brazil) and Argentina, being more conspicuous for the *MT-CO1*. Using this gene, the samples from Ilha da Marambaia were more closely related to *S. xanthosternos*, although the bootstrap support was not high. The other *Sapajus* species were grouped in polytomy, without definition except for *S. flavius*, which had a more consistent clustering. Using the *MT-CO2* gene, the closest proximity to *S. xanthosternos* was not observed as in the *MT-CO1*. Even so, there was no grouping with the samples of

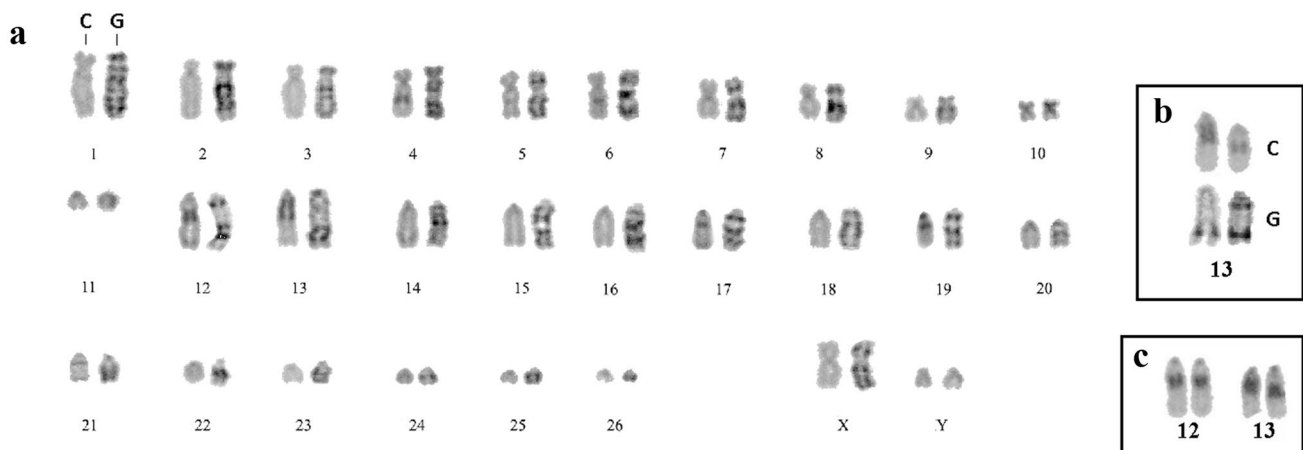


Fig. 3 Karyotype ($2n = 54, XY$) with C- and G-banding of the capuchin monkey population from Ilha da Marambaia, Mangaratiba, Rio de Janeiro, Brazil. **a** Chromosome complement of a male individual with C-banding (left) and G-banding (right) for each pair, demonstrating all pairs observed with extracentromeric constitutive heterochromatin. Pair 13 presents a large block close to the centromere, in homozygosis, for six individuals. **b** in detail, pair 13 polymorphism

presented by four individuals, in C- and G-banding, with one of the homologues having a proximal block, and for the other (small) it is more distal and reduced; **c** pair 13 polymorphism presented by two individuals, with C-banding, showing a proximal block in one chromosome and a distal in the other, both with conserved size compared to pair 12

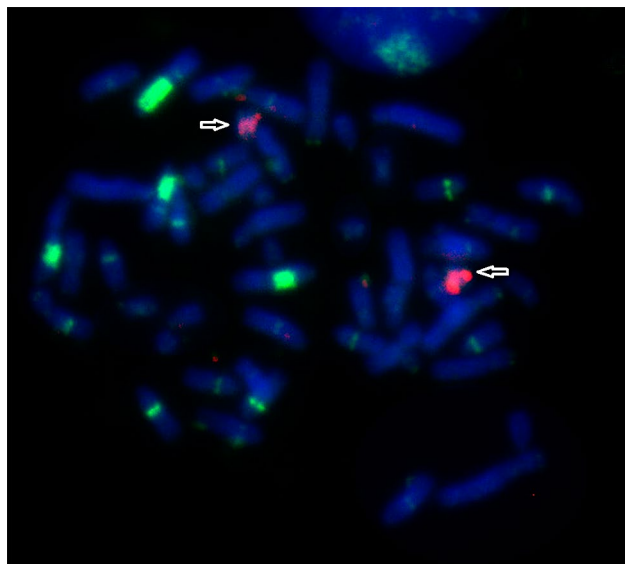


Fig. 4 Metaphase chromosomes obtained with fluorescence in situ hybridization (FISH) for the capuchin monkey population from Ilha da Marambaia, Mangaratiba, Rio de Janeiro, Brazil. The extracentromeric constitutive heterochromatin regions of pair 11 identified by the #11qHe+ probe are marked in green, and the euchromatic region of pair 11 identified using the 21WCP probe is shown in red (indicated by arrows), demonstrating this pair as a small acrocentric

S. nigritus from Argentina, which were more closely related to *S. cay*.

In terms of intrapopulation divergence, a null mean value ($d=0.0000$) was obtained for the *MT-CO1* and $d=0.0010$ for the *MT-CO2*, for the Ilha da Marambaia sequences. Comparing the sequences from Ilha da Marambaia with those published for *S. nigritus* (Table 2), the genetic divergence values of *MT-CO1* varied from $d=0.0375$ to 0.0408 , while the divergence values obtained with *MT-CO2* varied from $d=0.0531$ to 0.0615 .

Discussion

A better understanding of the diversity of the New World primates requires an approach that involves traditional morphology, biogeography and cytogenetics, in combination with molecular genetic analysis (Seuáñez et al. 2005). The molecular and cytogenetic data associated with coat coloration obtained for the robust capuchins from Ilha da Marambaia allowed us to characterize this population and aggregate important data for *Sapajus nigritus* diversity.

The coat coloration patterns and the shapes of the tufts on the top of the head observed in the individuals from Ilha da Marambaia correspond to that described for *Sapajus nigritus nigritus* (Groves 2001; Fragaszy et al. 2004; Rylands et al. 2005), which is consistent with the geographic origin in Rio

de Janeiro state (Lynch Alfaro et al. 2014). The intrapopulation variation observed also corresponds to that described for *S. nigritus* in a population from São Paulo state, Brazil (Vieira and Oliveira 2014), although the reddish color on the belly and back and the white fur on the belly, described in that population, was not observed. These color differences may be related to the subspecies, once the population from São Paulo (city of Presidente Prudente) is probably *S. n. cucullatus*. The intrapopulation coat variation observed in both populations of *S. nigritus* should be investigated along its occurrence, in order to verify whether this characteristic is present in the entire distribution of the species.

The diploid number corresponds with that described for *Sapajus* species (Matayoshi et al. 1986), as well as the submetacentric form of the Y chromosome, reported by Freitas and Seuáñez (1982).

Despite the high variability in the distribution of C-banding reported for *S. nigritus* (Nieves et al. 2017), the presence of extracentromeric heterochromatin in pairs 4, 6, 12, 13, 17, 19 and 21, observed in the studied population with different frequency of presence/absence, is the same as that already described for the species in Argentina (Mudry 1990; Mudry et al. 1991; Ponsà et al. 1995; Steinberg et al. 2014; Nieves et al. 2017). The absence of C-banding in pair 11 described for *S. nigritus* from Argentina (Mudry 1990; Mudry et al. 1991) is also present at Ilha da Marambaia, confirming that it is not a geographical polymorphism, as previously mentioned (Ponsà et al. 1995; Penedo et al. 2014). Although it may be a species-specific polymorphism of *S. nigritus*, we cannot establish that this is a characteristic for the entire distribution, since samples have already been reported with a large heterochromatic block in pair 11 (Freitas and Seuáñez 1982; Penedo et al. 2014).

Although euchromatic regions are well conserved, constitutive heterochromatin rearrangements are common and part of the evolutionary process of Platyrrhini species (Garcia et al. 1983; Nieves et al. 2005). Nieves et al. (2017) reviewed the observations related to polymorphic variants that support the hypothesis of highly polymorphic chromosomal pairs, especially for *S. nigritus* and *S. cay* from the southernmost distribution. The polymorphisms observed in pair 13 in the individuals from Ilha da Marambaia probably resulted from paracentric inversion, the most common intraspecific rearrangement in *Sapajus* (Matayoshi et al. 1987; Ponsà et al. 1995; Garcia et al. 2002; Martinez et al. 2004; Ruiz-Herrera et al. 2004), besides duplication.

According to the maximum likelihood trees, the samples from Ilha da Marambaia did not group with *S. nigritus*. With the *MT-CO1* gene, the closest proximity to *S. xanthosternos* is consistent with the finding of Martins-Junior et al. (2018), in which a sample from Rio de Janeiro, Brazil, groups with those from *S. xanthosternos* in a more ancestral clade and not with two others samples of *S. nigritus* from São Paulo

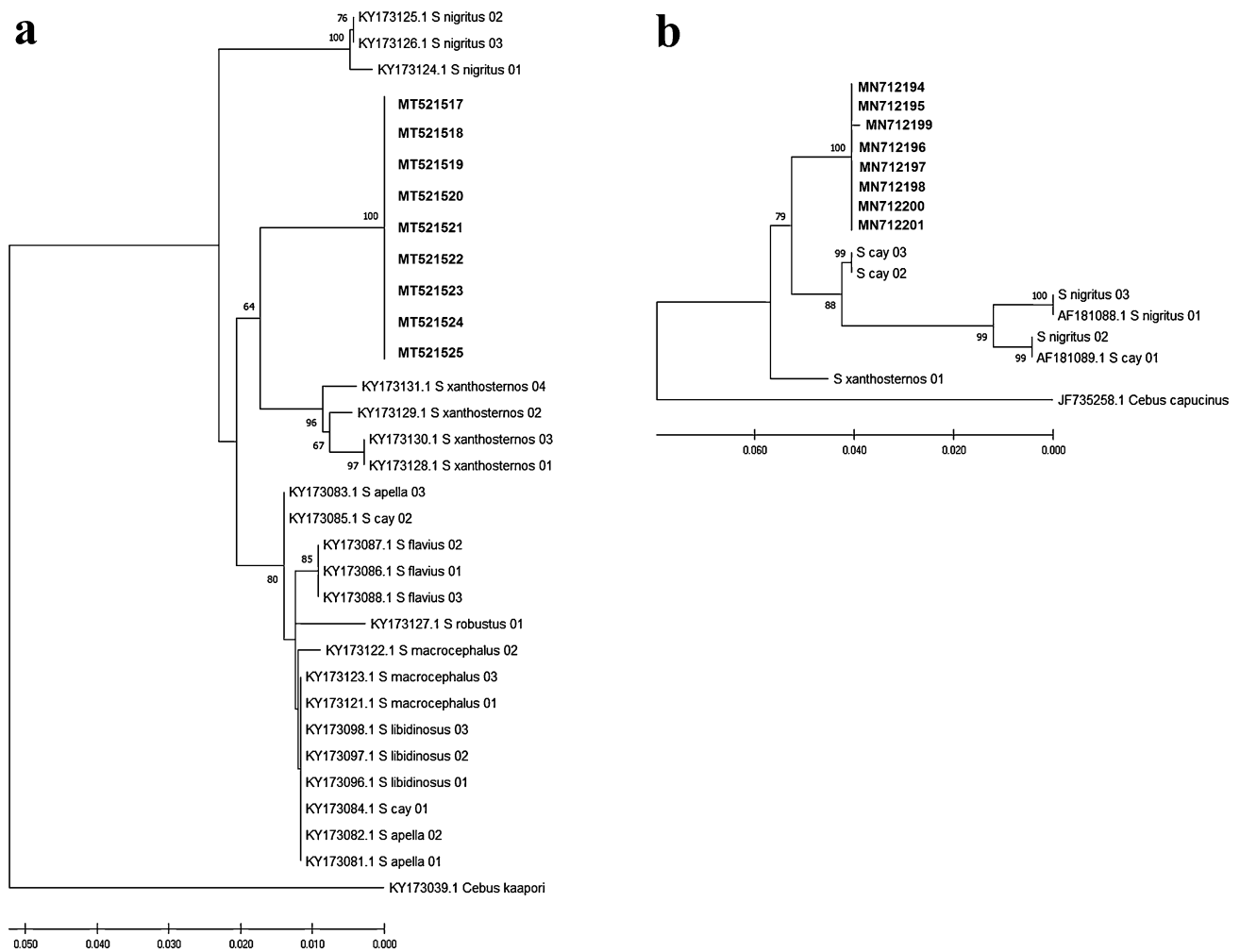


Fig. 5 Phylogenetic trees based on the maximum likelihood method using the Tamura-Nei model (Tamura and Nei 1993) for comparison of the mitochondrial cytochrome c oxidase I (a) and II (b) gene sequences demonstrating the relationship of *Sapajus nigritus* from Ilha da Marambaia, Rio de Janeiro state, Brazil, with species of the same genus obtained in the literature. The percentage of replicate

trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein 1985). The scale represents the genetic divergence values (d). The codes from MT521517 to MT521525 and from MN712194 to MN712201 represent the *MT-CO1* and *MT-CO2* GenBank code sequences, respectively, of *S. nigritus* from Ilha da Marambaia

Table 2 Genetic divergence values

<i>MT-CO1</i> (d value)	
Ilha da Marambaia	
KY173124.1_S_nigritus_01	0.0408
KY173126.1_S_nigritus_03	0.0391
KY173125.1_S_nigritus_02	0.0375
<i>MT-CO2</i> (d value)	
Ilha da Marambaia	
AF181088.1_S_nigritus_01	0.0615
S_nigritus_03	0.0607
S_nigritus_02	0.0531

Comparison of mitochondrial cytochrome c oxidase I and II gene sequences from Ilha da Marambaia, Mangaratiba city, Rio de Janeiro state, Brazil, with those of *Sapajus nigritus* from the literature, using the Tamura-Nei model (Tamura and Nei 1993)

(Brazil) and Argentina. In our findings with *MT-CO1*, however, the *S. nigritus* samples from the literature (from São Paulo and Paraná, Brazil) formed a more ancestral clade, followed by the clade with samples from Ilha da Marambaia and *S. xanthosternos*. The grouping of the *S. nigritus* from both Ilha da Marambaia and the literature is consistent with the high phylogenetic proximity of this species to *S. xanthosternos* (Lima et al. 2018). The most derived clade, formed by the other species in polytomy, was the same as previously observed (Lynch Alfaro et al. 2012a; Nascimento et al. 2015; Lima et al. 2017, 2018; Martins-Junior et al. 2018). Regarding the *MT-CO2*, the proximity of capuchin monkeys from Ilha da Marambaia with *S. xanthosternos* was not observed, with this species being in a more ancestral clade. The samples from Ilha da Marambaia did not show a significant

cluster, probably due to the low number of sequences available in the literature for this gene, being close to the group formed by *S. nigrurus* from Argentina and *S. cay*.

The low average genetic divergence values observed among the individuals of Ilha da Marambaia using the *MT-CO1* and *MT-CO2* genes supports a common origin for the population. The *MT-CO1* genetic divergence values between these individuals and the sequences of *S. nigrurus* from the literature are within the range expected for this gene between distinct subspecies, $d=0.0378 \pm 0.0118$ (Kartavtsev 2011), and higher than those observed, for example, in various *Saimiri* species (Ruiz-García et al. 2014), with maximum value of $d=0.0277$ (*S. peruviansis* x *S. macrodon*). The *MT-CO2* genetic divergence values were even higher, being close to the average expected between species, $d=0.0582$ (Ascunce et al. 2003; Collins and Dubach 2000; Ruiz-García et al. 2016). For this gene, the values obtained were higher than, for example, those observed between *S. nigrurus* and *S. cay* from Argentina, with $d=0.0175$ (Martinez et al. 2002), four species of *Ateles*, with maximum value of $d=0.0470$ (Collins and Dubach 2000), *Saimiri* species, with values $d=0.02$ and $d=0.031$, and *Aotus* species, with values ranging from $d=0.046$ to $d=0.06$ (Ascunce et al. 2003). These results may indicate a high genetic diversity of *S. nigrurus* along the Argentina-Brazil distribution, favoring the divergence among the samples observed.

The possibility of introducing individuals from another locality as the origin for the black-horned capuchins at Ilha da Marambaia seems improbable, according to the surveys carried out with residents and military personnel in the region. Thus, one possible explanation for the presence of the capuchins at this region is its isolation by the last phenomenon of marine transgression, characterized by the sea level rise occurred approximately 7000 years ago, which covered Atlantic Forest regions present where there are today the Sepetiba Bay (Belo et al. 2002; Pereira 2010), isolating the Ilha da Marambaia.

Considering our findings with *MT-CO1* and *MT-CO2* for Ilha da Marambaia and those with cytochrome b for a sample from Rio de Janeiro (Lynch Alfaro et al. 2012a; Nascimento et al. 2015; Martins-Junior et al. 2018), there may be a greater divergence in the capuchin monkeys from this region to the rest of *S. nigrurus* distribution that need to be investigated for a larger number of populations.

This was the first study with the black-horned capuchins from Ilha da Marambaia and one of the few studies with *S. nigrurus* from Brazil that associated a significant variety of methodologies. Our view about *S. nigrurus* is still fragmented and lacks studies that better contemplate their entire occurrence area in order to understand how the genetic diversity is distributed.

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Author contributions All authors contributed to the study conception and design. Data collection was performed by DMP, CEDSV, EJDsS and DMN. Phenotypic analyses were performed by DMP and DMN. Cytogenetic analyses were performed by DMP, JLAa, MN and DMN. Molecular analyses were performed by DMP, AMdO and DMN. The first draft of the manuscript was written by DMP and DMN, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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