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RESEARCH ARTICLE

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Phylogeographic evidence for two species of muriqui (genus *Brachyteles*)

Paulo B. Chaves[1,2] | Tielli Magnus[3] | Leandro Jerusalinsky[4] | Maurício Talebi[5,6] | Karen B. Strier[7] | Paula Breves[8] | Fernanda Tabacow[9] | Rodrigo H. F. Teixeira[10] | Leandro Moreira[9] | Robson O. E. Hack[11,12] | Adriana Milagres[13] | Alcides Pissinatti[14] | Fabiano R. de Melo[15,16] | Cecília Pessutti[17] | Sérgio L. Mendes[18] | Tereza C. Margarido[19] | Valéria Fagundes[18] | Anthony Di Fiore[20] | Sandro L. Bonatto[3]

[1]Department of Anthropology, New York University, New York, New York

[2]New York Consortium in Evolutionary Primatology (NYCEP), New York, New York

[3]Escola de Ciências, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil

[4]Centro Nacional de Pesquisa e Conservação de Primatas Brasileiros, Instituto Chico Mendes de Conservação da Biodiversidade, João Pessoa, Paraíba, Brazil

[5]Laboratório de Ecologia Aplicada e Conservação (LECON), Departamento de Ciências Ambientais, Universidade Federal de São Paulo, Diadema, São Paulo, Brazil

[6]Instituto Pró-Muriqui, São Paulo, São Paulo, Brazil

[7]Department of Anthropology, University of Wisconsin-Madison, Madison, Wisconsin

[8]Diretoria de Biodiversidade, Sociedade Ecoatlântica, Rio de Janeiro, Rio de Janeiro, Brazil

[9]Muriqui Instituto de Biodiversidade, Caratinga, Minas Gerais, Brazil

[10]Programa de Pós-graduação em Animais Selvagens, Universidade Estadual Paulista (UNESP), Botucatu, São Paulo, Brazil

[11]Divisão de Meio Ambiente, Departamento de Recursos Ambientais, Instituto de Tecnologia para o Desenvolvimento (Lactec), Curitiba, Paraná, Brazil

[12]Programa de Pós-Graduação em Desenvolvimento de Tecnologia, Instituto de Tecnologia para o Desenvolvimento e Instituto de Engenharia do Paraná, Curitiba, Paraná, Brazil

[13]Programa de Pós-graduação em Ecologia Aplicada, Universidade Federal de Lavras, Lavras, Minas Gerais, Brazil

[14]Centro de Primatologia do Rio de Janeiro, Instituto Estadual do Ambiente, Guapimirim, Rio de Janeiro, Brazil

[15]Unidade Acadêmica Especial de Ciências Biológicas, Universidade Federal de Goiás, Goiânia, Goiás, Brazil

[16]Departamento de Engenharia Florestal, Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil

[17]Parque Zoológico Municipal Quinzinho de Barros, Sorocaba, São Paulo, Brazil

[18]Departamento de Ciências Biológicas, Universidade Federal do Espírito Santo, Vitória, Espírito Santo, Brazil

[19]Departamento de Pesquisa e Conservação da Fauna, Prefeitura Municipal de Curitiba, Curitiba, Paraná, Brazil

[20]Department of Anthropology, Primate Molecular Ecology and Evolution Laboratory, The University of Texas at Austin, Austin, Texas

Correspondence
Paulo B. Chaves, PhD, Superintendência de Polícia Técnico-Científica de Goiás, Laboratório de Biologia e DNA Forense, Av. Atílio Correia Lima, 1223, Cidade Jardim, Goiânia, GO 74425-030, Brazil.
Email: pbchaves@gmail.com

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Abstract

The taxonomy of muriquis, the largest extant primates in the New World, is controversial. While some specialists argue for a monotypic genus (*Brachyteles arachnoides*), others favor a two-species classification, splitting northern muriquis (*Brachyteles hypoxanthus*) from southern muriquis (*B. arachnoides*). This uncertainty affects how we study the differences between these highly endangered and charismatic primates, as well as the design of more effective conservation programs. To address this issue, between 2003 and 2017 we collected over 230 muriqui fecal samples across the genus' distribution in the Brazilian Atlantic Forest, extracted DNA from these samples, and sequenced 423 base pairs of the mitochondrial DNA (mtDNA) control region. Phylogenetic and species delimitation analyses of our

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sequence dataset robustly support two reciprocally monophyletic groups corresponding to northern and southern muriquis separated by an average 12.7% genetic distance. The phylogeographic break between these lineages seems to be associated with the Paraíba do Sul River and coincides with the transition between the north and south Atlantic Forest biogeographic zones. Published divergence estimates from whole mitochondrial genomes and nuclear loci date the split between northern and southern muriquis to the Early Pleistocene (ca. 2.0 mya), and our new mtDNA dataset places the coalescence time for each of these two clades near the last interglacial (ca. 120–80 kya). Our results, together with both phenotypic and ecological differences, support recognizing northern and southern muriquis as sister species that should be managed as distinct evolutionarily significant units. Given that only a few thousand muriquis remain in nature, it is imperative that conservation strategies are tailored to protect both species from extinction.

KEYWORDS
Atelinae, conservation, mtDNA, Platyrrhini, sister species, systematics

1 | INTRODUCTION

The practice of primate taxonomy and systematics has greatly changed in the past two decades (Groves, 2014; Rylands & Mittermeier, 2014). This change has been primarily driven by the increasing use of DNA-sequence data and phylogenetic methods to study the tempo and mode of both species-level and above-species diversifications. Although these advances have improved the way primatologists now see our cousins' tree of life and our understanding of macroevolutionary patterns of diversification in living primates (Perelman et al., 2011; Pozzi et al., 2014), the relationships between closely related species are still poorly resolved for several genera. This is evidenced by the recent identification of cryptic diversity within genera, leading to the recognition of new primate species on different continents, many of which are already found on the brink of extinction (e.g., Fan et al., 2017; Hart et al., 2012; Nater et al., 2017).

Part of the challenge that primate taxonomy faces comes from the numerous species concepts available in the literature and from disagreements between biologists about which concept (if any) and dataset are best able to capture a process ("speciation") that is complex, works due to diverse evolutionary forces acting over an unpredictable length of time, and produces different results in each lineage. To shift away from the "species concept" debate, De Queiroz (2007) have pointed out that although most taxonomists agree that a "species" can be thought of as a separately evolving metapopulation lineage, where they disagree is over the specific criteria used to diagnose these lineages (e.g., reproductive isolation, monophyly, diagnosability). To reconcile this, some researchers have suggested using a unified or generalized species concept, which recognizes that all good and complementary criteria should be accepted to diagnose separately evolving lineages and that congruence between different approaches is desirable (Carstens, Pelletier, Reid, & Satler, 2013). One such approach is to use genetic markers such as mitochondrial DNA (mtDNA) to identify evolutionarily significant units (ESUs), that is, lineages that have been separated long enough to have become reciprocally monophyletic (Moritz, 1994).

Muriquis (genus *Brachyteles* Spix, 1823), the largest extant New World monkeys, represent one such challenge to primatologists (Aguirre, 1971). For decades, taxonomists and conservationists have debated whether *Brachyteles* consists of a monotypic genus or not. Endemic to the Atlantic Forest of Brazil and the sister genus of *Lagothrix* (woolly monkeys), muriquis have two morphological features that vary between populations—the presence of a vestigial thumb and variable skin depigmentation on the face and genitalia in the northern morphotype (*Brachyteles hypoxanthus* (Kuhl, 1820) or the northern muriqui), and completely black facial/genital skin and the absence of a thumb in the southern morphotype (*Brachyteles arachnoides* (Saint-Hilaire, 1806) or the southern muriqui). The utility of phenotypic variation in these traits for assigning full species rank to these different morphotypes has been emphasized in some publications (Groves, 2001; Leigh & Jungers, 1994; Lemos de Sá, Pope, Glander, Struhsaker, & Fonseca, 1990; Vieira, 1944) and challenged elsewhere (Vieira, 1955; Villavicencio, 2016). The original description of a *Brachyteles* specimen is attributed to the French naturalist Étienne Geoffroy Saint-Hilaire at the beginning of the 19th century when it was considered a species of spider monkey, *Ateles arachnoides* (Hill, 1962). Additional specimens provided details on the geographic distribution and on morphological variation in muriquis, prompting both Wied (1820) and Kuhl (1820) to first recognize southern and northern muriquis as separate species within *Ateles* (Garbino & Costa, 2015; Vanzolini, 1996). Three years later, Spix (1823) was the first to classify muriquis into their own genus, assigning both southern and northern forms to the species

Brachyteles macrotarsus. Subsequent assessments tended to support Spix's conclusion, but there was debate over whether to recognize southern and northern forms as a single species, as subspecies, or as distinct species based on the morphological variation in the thumbs and in skin and pelage color as well as differences between northern and southern forms in the degree of canine dimorphism (see Table 1).

An analysis of the genetic diversity within and between northern and southern muriquis could help us to clarify this problem. However, sampling these highly endangered and rare primates has proven difficult. Recent molecular phylogenies using mitochondrial and/or nuclear DNA, and including one exemplar of each form suggest an age for the common ancestor of all *Brachyteles* at 2.0–3.2 million years ago (mya; Di Fiore et al., 2015; Perelman et al., 2011; Springer et al., 2012). However, it is unclear whether these forms represent only one lineage sharing a common history extending back to the Plio–Pleistocene or whether the genus split into additional monophyletic lineages after that. An analysis of allozyme diversity based on the sampling of individuals from a single population of each form showed a significant degree of differentiation ($F_{ST} = 0.413$) between them (Pope, 1998). This result could, however, be explained by the long geographic distance (roughly 600 km) separating the two populations sampled. Thus, we still lack a comprehensive analysis, based on sampling multiple populations across the entire genus' geographic range, to test whether or not southern and northern muriquis are each monophyletic and diagnosable entities evolving independently.

In 2005, a group of stakeholders including scientists, zookeepers, public agents, NGOs, and managers of protected areas began discussions to establish a series of priorities and strategies to protect muriquis from extinction. In 2010, this effort resulted in the National Action Plan for the Conservation of Muriquis which was officially recognized by the Brazilian Government (Jerusalinsky, Talebi, & Melo, 2011; Strier et al., 2017). One of the goals set by this plan was to measure the genetic diversity within and between southern and northern muriquis to test the species boundaries and inform both *in situ* and *ex situ* conservation actions. Here, we present the first phylogeographic study of the muriquis based on sampling extensively across the distribution of both the northern and southern morphotypes. Our goals are to examine genetic diversity within and between these forms under the principles of a generalized species framework (De Queiroz, 2007; Groves, 2014) and test whether our data, together with additional published evidence, support the recognition of southern and northern muriquis as distinct species. We then address the consequences of our findings for *Brachyteles* conservation.

2 | MATERIALS AND METHODS

2.1 | Sampling, DNA extraction, polymerase chain reaction, and sequencing

Fecal samples ($N = 235$) were collected between 2003 and 2017 either actively or opportunistically throughout the muriquis' natural geographic range in the Brazilian Atlantic Forest (Figure 1). Immediately after defecation, samples were transferred to a vial containing RNAlater or nucleic acid preservation (NAP) buffer (Camacho-Sanchez, Burraco, Gomez-Mestre, & Leonard, 2013), silica gel, 70–100% ethanol, or sodium chloride (NaCl). For the liquid preservatives (RNAlater, NAP buffer, and 70–100% ethanol), a minimum 1:1 ratio of feces to preservative was used. After collection, samples were stored at −20°C as soon as possible. Both feces and blood of captive animals, collected by trained personnel, complemented the set of southern muriqui samples (Table S1). This study complied with protocols approved by the Instituto Chico Mendes de Conservação da Biodiversidade and adhered to the legal requirements of Brazil and to the American Society of Primatologists' Principles for the Ethical Treatment of Primates.

DNA was extracted from feces using the QIAamp Stool Mini Kit (Qiagen) according to the manufacturer's protocol with the following modifications to maximize DNA yield: (a) roughly 250 µl of fecal slurry or 180 mg of dry feces was incubated with agitation in ASL buffer at 56°C for at least 2 hr and no more than 24 hr; (b) AL buffer incubation was carried out for 30 min instead of 10 min; and (c) the elution step was done with 60–100 µl, instead of 200 µl, of elution buffer. DNA was extracted from blood using a standard phenol-chloroform protocol (Sambrook, Fritsch, & Maniatis, 1989).

A 463-base-pair (bp) fragment containing the mtDNAs hypervariable region I (HVI) was amplified via the polymerase chain reaction (PCR) using the primers Mono1 and Mono2 (Fagundes et al., 2008). We used primers specifically designed for *Brachyteles* and high initial annealing temperature (see below) to reduce the risk of amplifying nuclear DNA insertions (*numts*). PCR reactions contained 5 µl of 2X AccuStart PCR SuperMix (Quantabio), 0.5 µl of each primer at 10 µM, 2.0–4.0 µl of DNA template, and ddH_2O to yield a final volume of 10 µl. Mock PCR blanks, which included 2.0 µl of ddH_2O instead of template DNA, were included in all PCR reactions to monitor contamination.

Thermal cycling conditions were as follows: initial denaturation at 94°C for 3 min, 10 touchdown cycles of 94°C for 30 s, initial annealing at 60°C, with a decrease of 1°C/cycle, for 30 s, and extension at 72°C for 1 min; followed by 25 additional cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min. A final extension step was carried out at 72°C for 1 min. Five microliters of PCR products were run on 1.0% agarose gels stained with GelRed (Biotium) to check amplification success.

PCR products that presented a single DNA band at the expected size on the agarose gel were then purified using an Exo-SAP protocol before sequencing. Purification reactions contained 5.0 µl of PCR product, 0.5 µl (10 units) of exonuclease I, and 10 µl (1.0 unit) of FastAP Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific). The purification mix was incubated on a thermal cycler at 37°C for 15 min followed by 85°C for 15 min for inactivation. PCR amplicons were then sequenced in both the forward (Mono1) and reverse (Mono2) directions on an ABI 3730 DNA Analyzer at the DNA Sequencing Facility at The University of Texas at Austin. Each submitted mix contained 1.0 µl of the 1:3 PCR dilution, 1.0 µl of either the F or R primer at 10 µM, and 10.0 µl of ddH_2O to a final

TABLE 1 Relevant historic information on *Brachyteles* taxonomy (adapted from Villavicencio, 2016)

Authors	Justification	Arrangement		
E. G. Saint-Hilaire (1806)	Morphology[a]	*Ateles arachnoides*		
Wied (1820)	Presence or absense of thumb	*Ateles arachnoides*	*Ateles hypoxanthus*	
Kuhl (1820)	Morphology	*Ateles arachnoides*	*Ateles hypoxanthus*	
Spix (1823)	Morphology		*Brachyteles macrotarsus*	
E. G. Saint-Hilaire (1827)	Presence or absense of thumb	*Ateles arachnoides*	*Ateles hypoxanthus*	
I. G. Saint-Hilaire (1828)	Morphology	*Eriodes arachnoides*	*Eriodes hemidactylus*	*Eriodes tuberifer*
Gray (1843)	Morphology	*B. arachnoides*	*B. hypoxanthus*	*Brachyteles frontatus*
Vieira (1944)	Morphology	*Brachyteles arachnoides arachnoides*	*Brachyteles arachnoides hypoxanthus*	
Vieira (1955)	Morphology	*B. arachnoides*		
Hill (1962)	Morphology	*B. arachnoides*		*Brachyteles brasiliensis*
Lemos de Sá, Pope, Struhsaker, and Glander (1993)	Degree of canine dimorphism	*Brachyteles arachnoides arachnoides*	*Brachyteles arachnoides hypoxanthus*	
Leigh and Jungers (1994)	Presence or absense of thumb	*Brachyteles arachnoides arachnoides*	*Brachyteles arachnoides hypoxanthus*	
Rylands, Mittermeier, and Rodriguez-Luna (1995)	Morphology	*B. arachnoides*	*B. hypoxanthus*	
Pope (1998)	Allozyme differentiation	*Brachyteles arachnoides arachnoides*	*Brachyteles arachnoides hypoxanthus*	
Groves (2001)	Morphology	*B. arachnoides*	*B. hypoxanthus*	
Villavicencio (2016)	Morphology	*B. arachnoides*		
This study	mtDNA	*B. arachnoides*	*B. hypoxanthus*	

Abbreviation: mtDNA, mitochondrial DNA.
[a]Morphology: Taxonomic arrangement based on a constellation of more than one specific morphological trait.

volume of 12.0 µl, as recommended by the facility. Some sequences were also produced at Universidade Federal do Espírito Santo and the Pontifícia Universidade Católica do Rio Grande do Sul with similar conditions.

2.2 | Sequence alignment and model selection

The chromatograms were inspected by eye, and consensus DNA sequences between the forward and reverse reads for each sample were assembled in the software Geneious R9 (Kearse et al., 2012). Primers were trimmed from each sequence read to obtain the final 423-bp DNA fragment. Each consensus sequence was then searched against the BLAST nucleotide database (nr/nt) to check for similarities with other *Brachyteles* sequences in the database and to exclude contamination. The new DNA sequences generated in this study were complemented by 60 additional *Brachyteles* sequences downloaded from GenBank (Chaves et al., 2011; Collins & Dubach, 2000; Di Fiore et al., 2015; Schrago, Menezes, Moreira, Pissinatti, & Seuánez, 2012). When necessary for particular analyses, one *Lagothrix* sequence was used as outgroup (GenBank Accession number KC757398). Consensus sequences were aligned in Geneious R9 and exported into various formats for subsequent analyses. The model that best described substitution parameters within the dataset was selected using jModelTest2 (Darriba, Taboada, Doallo, & Posada, 2012; Guindon & Gascuel, 2003) according to the Bayesian information criterion. Phylogenetic and phylogeographic analyses were preferentially run on the CIPRES science gateway server (Miller, Pfeiffer, & Schwartz, 2010) whenever possible or on personal computers.

2.3 | Network and population genetics

A median-joining haplotype network (Bandelt, Forster, & Röhl, 1999) using all *Brachyteles* sequences was inferred using PopART with the epsilon parameter set to zero (Leigh & Bryant, 2015). Arlequin 3.5 (Excoffier & Lischer, 2010) was used to calculate haplotype diversity (Hd) and nucleotide diversity (π) and to perform analysis of molecular variance (AMOVA, Excoffier, Smouse, & Quattro, 1992). The alignment was trimmed to overlap the 366 bp sequence length from Chaves et al. (2011) to make genetic estimators of diversity consistent and comparable across studies. For each of the three of the populations surveyed (RPPN-FMA, SMJ, and PECB), samples from over 30 individuals were sequenced. To minimize the effect of overrepresentation of these populations on our genetic analyses, a

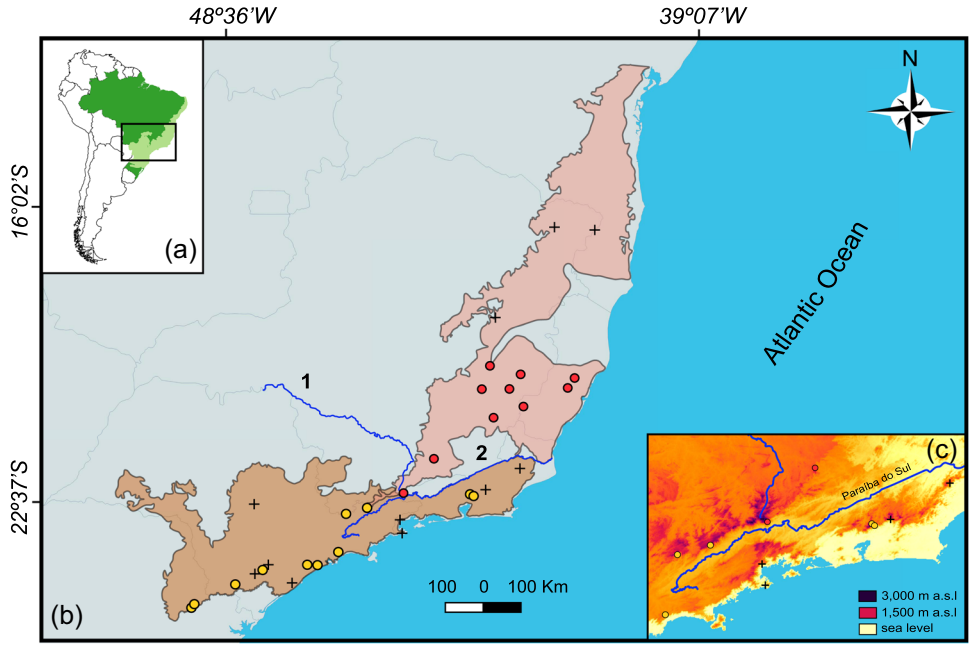

FIGURE 1 Map showing key geographic elements mentioned in the text. (a) Inset of South America highlighting Brazil (green), the Atlantic Forest domain (light green), and an approximated region where muriquis are found (rectangle). (b) Historic distribution of *B. hypoxanthus* (pink polygons) and *B. arachnoides* (brown polygon) according to (Ingberman et al., 2016; Strier et al., 2017). Yellow dots mark sampling sites for northern and southern muriquis, respectively, and crosses mark unsampled muriqui sites based on recent surveys (Jerusalinsky et al., 2011 and references therein). Rivers, shown in dark blue, are Rio Grande (1) and Rio Paraíba do Sul (2). (c) Inset depicting high and low elevation zones near the species distribution limits. Darker zones north of the Rio Paraíba do Sul are the Serra da Mantiqueira mountain chain and the high elevation zones south of the Paraíba do Sul are the Serra do Mar mountain chain

new alignment was constructed based on randomly selecting a subset of 20 samples from each of these populations. These 20 samples were selected such that each haplotype had approximately the same frequency as in the total population sample. Our AMOVA analysis was constructed to test how genetic diversity is apportioned between the two muriqui morphotypes; for this analysis, then, samples of muriqui individuals were assigned to their respective groups ("northern" vs. "southern") based on phenotype (i.e., presence or absence of facial depigmentation in the population).

2.4 | Species delimitation

To test for one versus two species of muriqui, we used the method implemented in the Automatic Barcode Gap Discover (ABGD) protocol (Puillandre, Lambert, Brouillet, & Achaz, 2012). ABGD uses the so-called "barcode gap" as a criterion to propose species limits. The barcode gap is a break in pairwise genetic distances between two putative species relative to the within-species distances. The algorithm scans a range of prior intraspecific divergence (from P_{min} to P_{max}, with P steps as parameters) to infer from the data a model-based one-sided confidence limit for intraspecific divergence. It then specifies the barcode gap as the first significant break beyond this intraspecific limit and uses it to partition the data. The limit and gap detection are then recursively applied to previously obtained groups to identify finer partitions until no further partitioning according to the gap criterion is possible. If there is no significant overlap between intra- and interspecies genetic distances given the parameters applied, the algorithm suggests splitting the genetic groups into two or more species. ABGD was run with $P_{min} = 0.01$, $P_{max} = 0.1$, $X = 1.5$ (relative barcode gap), and $P = 10$ steps using Kimura-2-parameter genetic distances with an expected transition/transversion ratio = 2.0.

2.5 | Phylogenetics and demographic history

Bayesian phylogenetic analyses were run in BEAST 2 (Bouckaert et al., 2014) to infer the phylogenetic tree for the complete set of *Brachyteles* control region sequences and the age of monophyletic groups within the genus. The tree topology analysis was set up in BEAUti 2.4.7 using an alignment containing *Lagothrix* as the outgroup. BEAST 2 was run for 30,000,000 generations, sampling trees every 3,000 generations, and using a set of priors following Drummond and Bouckaert (2015). The consensus tree and clade posterior probabilities were summarized in TreeAnnotator 2.4.7 (after discarding the first 10% of the sampled trees as burn-in) and were visualized in FigTree 1.4.3. After confirming that the BEAST 2 tree retrieved the same monophyletic groups suggested by our network, AMOVA, and species delimitation analyses, a second analysis was run with the same parameters as above but using an alignment without *Lagothrix*. This was done to reduce the variance introduced by poor homology inference between the ingroup and outgroup taxa and the effect of long branches, which can make Bayesian sampling and dating more

difficult (Drummond & Bouckaert, 2015). For this analysis, one log-normal calibration point was used as a prior for the most recent common ancestor (MRCA) of all *Brachyteles* sequences with a mean of 1.98 mya and a 95% confidence interval between 1.29 and 2.87 mya. This root prior was extracted from a whole mitochondrial genome phylogeny of platyrrhine primates that used six fossil calibrations (Di Fiore et al., 2015). Although direct fossil calibrations are preferred in most phylogenetic analyses due to improved precision (Schenk, 2016); in this case, there are no good fossils to date splits within the genus *Brachyteles*. Using more distantly related fossils could circumvent this limitation; however, aligning these noncoding and highly variable HV1 sequences among different genera is problematic, as explained above. Thus, to estimate the coalescence time of each muriqui lineage, we set two MRCA priors; one consisting of all the northern muriqui samples and the other including all the southern muriqui samples.

To infer historical changes in population sizes, Bayesian skyline plots (BSPs; Drummond, Rambaut, Shapiro, & Pybus, 2005) were constructed for both northern and southern muriquis independently in BEAST 2. The coalescence analysis was run with the same conditions as above. A normal prior was used for the age of the MRCA of each of the northern and southern muriquis clades, based on the dates obtained from our phylogenetic analysis. To check for convergence between runs and for appropriate prior choice, BEAST 2 analyses were run three times using different starting seeds, and log files were inspected in Tracer 1.6.0 (Rambaut, Suchard, Xie, & Drummond, 2014). Results were averaged over all runs using LogCombiner 2.4.7.

3 | RESULTS

The HVI sequences produced with the primers Mono1 and Mono2 were similar to and clustered with those from full mtDNA genomes of *Brachyteles* sequenced after long-range PCR and massively parallel sequencing protocols (Di Fiore et al., 2015; Schrago et al., 2012), indicating the absence of older paralogous nuclear DNA insertions in our dataset that might complicate phylogenetic analysis. The complete mtDNA alignment was 424 bp long and contained a total of 249 HVI sequences, of which 189 are new. Most of the sequences ($N = 235$) came from wild animals with known geographic origin. Only a small portion of them ($N = 14$) came from captive animals, of which most had known approximate geographic provenance ($N = 11$). Sixty-four sequences were attributed to specimens sampled within the southern muriqui historical range and 185 were sampled from northern muriqui sites. The newly generated sequences were deposited in GenBank under accession numbers MG365921-MG366113. The reduced alignment (after subsampling 20 sequences each from the RPPN-FMA, SMJ, and PECB populations) comprised a total of 152 sequences, 48 from southern muriquis and 104 from northern muriquis. Southern muriquis presented 22 haplotypes (Hd = 0.906; $\pi = 0.015$) while northern muriquis presented 24 haplotypes (Hd = 0.932; $\pi = 0.013$).

F_{ST} calculated between the set of southern and northern muriqui samples indicates a strong and significant apportionment of the genetic structure based on morphotype ($F_{ST} = 0.89$; $p < .001$). This result is easily visualized on the haplotype network (Figure 2), which shows 35 substitution steps between southern and northern muriquis and only a few substitution steps (1–4) between haplotypes within each putative species. The southern muriqui is more genetically diverse and shows slightly more evidence of within-taxon genetic structure than the northern muriqui, which shows a very shallow haplotype network, probably due to its very recent demographic history (Chaves et al., 2011).

In the ABGD analysis, when two well-established species are analyzed, the distribution of pairwise differences between all sequences of the alignment typically shows a conspicuous gap when the mode of the distribution of intraspecific divergence is lower than the mode of interspecific divergence. This is exactly the pattern observed in muriquis (Figure 3), with intraspecific divergences ranging from 0% to 5% (mean = 1.4%) and interspecific divergences ranging from 10.5% to 15% (mean = 12.7%). Consequently, ABGD recovered two partitions most of the time (8 times out of 10 runs with different p values across the range tested). Thus, it correctly allocated southern and northern muriqui samples within their respective groups with the prior maximal distance $p \geq .0028$. The software also recovered 47 groups twice. These groups were defined by very small intergroup maximal genetic distances ($p < .0017$) and are consistent with the partition of mtDNA sequences into groups defined by each haplotype.

The Bayesian tree with the outgroup confirms the analyses above by recovering northern and southern muriquis in reciprocally monophyletic groups with high posterior probability support (Figure S1). While the two lineages diverged from one another about 1.98 mya (Di Fiore et al., 2015), the estimated coalescence time within northern muriquis (median = 82 thousand years ago [kya]; 95% HPD = 25–171 kya) and within southern muriquis (median = 126 kya; 95% HPD = 42–264 kya) is both inferred to be much more recent in time. Based on our BSP analyses, northern muriquis seemingly went through a marked population decline during the Holocene. Southern muriquis, on the other hand, are inferred to have experienced an increase in population size after the last glacial maximum, followed by a weak and very recent population decline (Figure 4). We acknowledge, however, that BSPs inferred with a single locus such as the mtDNA should be interpreted with caution as these plots have been shown to be affected by factors other than demographic change itself, including the level of DNA-sequence polymorphism, population structure, sampling scheme, sample size, natural selection, and estimates of mutation rate (Grant, 2015).

4 | DISCUSSION

We found that multiple substitutions separate northern and southern muriquis in their mtDNA control region and phylogenetic analyses strongly indicate they belong to two reciprocally monophyletic lineages. The divergence between these lineages is matched by a

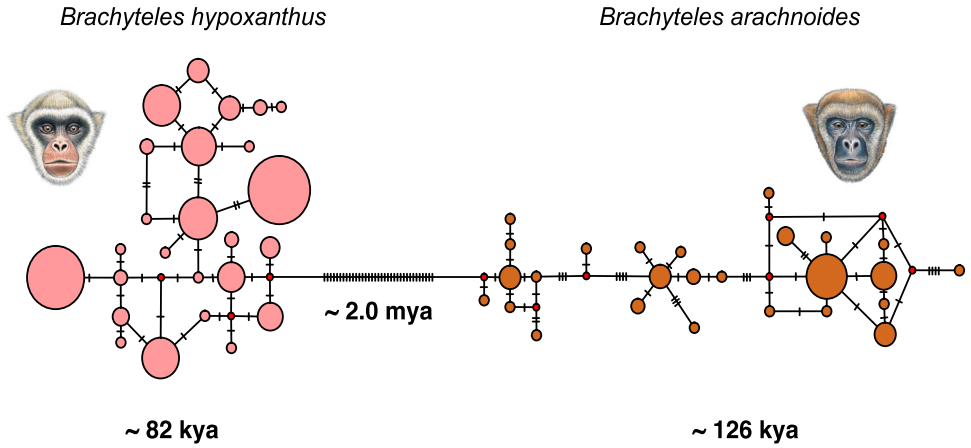

FIGURE 2 Median-joining network of all 250 *Brachyteles* HVI sequences analyzed in the present study. *B. hypoxanthus* and *B. arachnoides* haplotypes are separated by 35 mutation steps (hatch marks) in this analysis. These sets of haplotypes form monophyletic groups with high posterior probability support in a Bayesian tree (see Figure S1) separated by roughly 2.0 mya and coalesce around 82 kya and 126 kya, respectively, in northern and southern muriquis. The southern muriquis' genetic diversity is slightly higher and more structured than the northern muriqui's. *Brachyteles* illustrations courtesy of Dr Stephen D. Nash/IUCN SSC Primate Specialist Group 2001. HVI, hypervariable region I; kya, thousand years ago; mya; million years ago

substantial number of differences in the two morphotypes' mitochondrial genomes (Di Fiore et al., 2015) and in their nuclear DNA as well (Perelman et al., 2011). Although the presence of a vestigial thumb appears to be a polymorphic trait in the genus *Brachyteles* (Villavicencio, 2016), to our knowledge, and consistent with previous accounts (de Assumpção, 1983; Nishimura, 1979), *B. hypoxanthus* adults all show some degree of facial and genital skin depigmentation during adulthood, while *B. arachnoides* individuals do not. This phenotypic trait maps precisely onto the species boundaries suggested by the mitochondrial DNA (Figure 1). Together, these data support recognizing *B. hypoxanthus* and *B. arachnoides* as independent ESUs (Moritz, 1994) and as distinct species. The same taxonomic conclusion has been reached in other well-accepted primate sister species after analysis of similar mtDNA data, including chimpanzees (*Pan troglodytes*) and bonobos (*P. paniscus*; Gonder et al., 2011), and Peruvian yellow-tailed woolly monkeys (*Lagothrix flavicauda*) and common woolly monkeys (*L. lagotricha;* Chaves & Di Fiore, 2019; Di Fiore et al., 2015).

In addition to their genetic and phenotypic differences, *B. hypoxanthus* and *B. arachnoides* populations may be adapted to different climate conditions, as indicated by a recent species distribution model analysis (Ingberman, Fusco-Costa, & de Araujo Monteiro-Filho, 2016). While *B. hypoxanthus*' geographic distribution is best predicted by a combination of temperature seasonality, isothermality, and altitude, *B. arachnoides*' distribution is better predicted by precipitation, isothermality, and mean diurnal range. As a consequence, these species may exclude one another, if their ranges could overlap, based on ecological adaptations to different climate conditions. These differences may also explain behaviors observed in one species but not the other. For instance, Talebi, Beltrão-Mendes, and Lee (2009) reported an intracommunity lethal coalitionary attack in southern muriquis in which both males and females bit an adult male of their own group, causing injuries that led to the male's death. In northern muriquis, by contrast, aggressive behaviors are rare (Strier, Carvalho, & Bejar, 2000) and lethal confrontations have never been reported in over 30 years of behavioral study (Strier & Mendes, 2012).

In agreement with recent reports that muriquis found at the Parque Nacional do Itatiaia (PNI) have mottled faces (Aximoff, 2015), the sample analyzed in the present study from this population clusters clearly within the northern muriqui group. In fact, the haplotype found at PNI is identical to one found at the Parque Estadual da Serra do Brigadeiro population, which is roughly 290 km north of PNI. For this reason, the PNI population now represents the southern-most record within the *B. hypoxanthus* distribution range. While the northern-most *B. arachnoides* population overlaps *B. hypoxanthus* latitudinally at Parque Nacional da Serra dos Órgãos

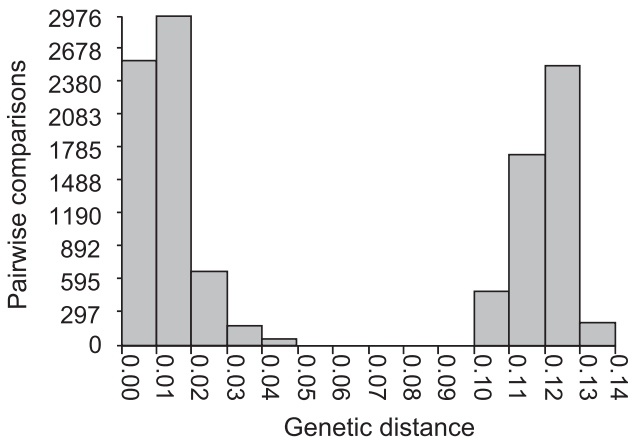

FIGURE 3 ABGD analysis histogram showing the distribution of genetic distances within northern and southern muriquis (left) and between them (right). Note the gap between intraspecific and interspecific genetic distances, as expected when the taxa analyzed belong to different species. ABGD, Automatic Barcode Gap Discover

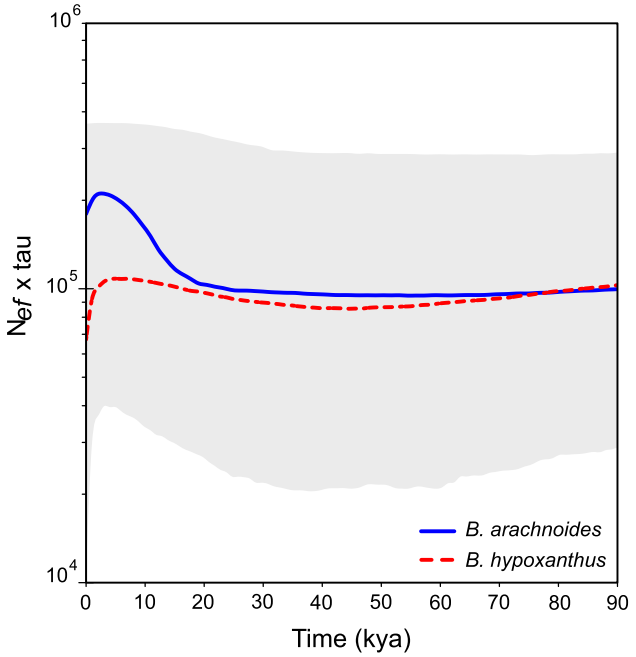

FIGURE 4 Bayesian skyline plot (BSP) depicting northern (red dotted line) and southern (solid blue line) muriqui median population sizes over time. The *x*-axis is in thousand years in the past (kya). The *y*-axis shows the product of female effective population size (N_{ef}) and generation length (tau). The gray background delimits the combined 95% HPD interval around both estimates. HPD, highest posterior density

(PARNASO) and Parque Estadual do Desengano in Rio de Janeiro (Cunha, Grelle, & Boubli, 2009), the species are nonetheless separated by approximately 160 km to both the east (PARNASO) and south (São Francisco Xavier, SP). Given the geographic proximity between southern and northern muriqui populations both in Rio de Janeiro and São Paulo—and given the large extent of forest remnants in this region—it is still plausible that a small contact zone exists between the two species in this region (Strier et al., 2017), a possibility that is also suggested by species distribution models (Ingberman et al., 2016). Future research in the region near the PNI is warranted to better characterize this population, primarily focusing on a possible hybrid zone.

Southern and northern muriqui lineages split around the transition between the Pliocene and Pleistocene (ca. 2.6 mya), when the Earth's temperatures were dropping, which probably affected species distributions across the globe. In South America, several cladogenetic events of platyrrhine genera date to this time (e.g., species-level divergences within *Cebus*, *Sapajus*, *Ateles*, and *Lagothrix*) suggesting a common phenomenon influencing speciation during this epoch (Kiesling, Yi, Xu, Sperone, & Wildman, 2015; Perelman et al., 2011). Around the same time, *Sapajus* also experienced its north-south diversification into different species in the Atlantic Forest (Lima et al., 2017), with little morphological divergence (Wright et al., 2015), just as we see in *Brachyteles*. However, it was only more recently that current northern (ca. 82 kya) and southern (ca. 126 kya) mtDNA diversity diversifies from their MRCA.

The southern and northern atlantic Forest domains are recognized as distinct centers of species endemism (Costa, Leite, da Fonseca, & da Fonseca, 2000), and multiple nonexclusive mechanisms of genetic isolation probably contributed to biotic diversification in these regions. Given the coincidence between the geographic position of the Paraíba do Sul and Grande Rivers with the phylogenetic break in the muriquis' mtDNA, it is possible that this riverine system played a prevalent role in shaping muriqui genetic differentiation. This region has been used to define broader northern and southern primate biogeographic domains in the Atlantic Forest (Kiesling et al., 2015; Kinzey, 1982), emphasizing its role as a biogeographic barrier for Atlantic Forest primates. The high and irregular nature of Serra da Mantiqueira and Serra do Mar terrain in this region likely contributed to genetic isolation too, especially during episodes of climatic cooling that led to the expansion of grasslands at a higher elevation and restricted gene flow between these "forest islands." Several studies suggest that the Atlantic Forest has probably expanded and contracted multiple times following warm and cool glacial cycles during the Pleistocene, favoring the expansion of open grasslands and isolating populations in forest islands (or refugia; Kinzey, 1982). The cessation of migration between these refugia is argued to have contributed to genetic diversification in these regions (Carnaval, Hickerson, Haddad, Rodrigues, & Moritz, 2009).

In sum, *B. hypoxanthus* and *B. arachnoides* differ genetically, geographically, phenotypically, ecologically, and possibly behaviorally. These differences are sufficient to warrant their recognition as distinct taxa according to most contemporary criteria used to define a "species" (De Queiroz, 2007), although we suggest that data from nuclear loci, in addition to our mtDNA data, should also be analyzed to further evaluate this conclusion. Regardless of whether one accepts them as distinct "species" or not, northern and southern muriquis demonstrably belong to two reciprocally monophyletic mitochondrial lineages and, from a conservation perspective, should be managed as such. Thus, decisions about translocating animals between isolated areas and for designing captive breeding programs must take this phylogenetic distinctiveness into account. In particular, the relevant stakeholders need to be conscientious of the distinct conservation needs of the two taxa (Jerusalinsky et al., 2011; Strier & da Fonseca, 1997). Moreover, all management actions should seek to (a) maintain a "healthy" degree of genetic variation in captive populations of both taxa (i.e., similar to the variation observed in larger natural populations) and (b) avoid interbreeding southern and northern muriquis to prevent the deleterious effects of outbreeding depression and the genetic swamping of natural populations (Weeks et al., 2011).

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

The DNA sequences that support the findings of this study are openly available in GenBank (see text for accession numbers) at https://www.ncbi.nlm.nih.gov/genbank/.

ORCID

Paulo B. Chaves http://orcid.org/0000-0003-1491-1449
Leandro Jerusalinsky http://orcid.org/0000-0003-0744-1987
Maurício Talebi http://orcid.org/0000-0001-6783-2715
Karen B. Strier http://orcid.org/0000-0003-2520-9110
Rodrigo H. F. Teixeira http://orcid.org/0000-0001-8219-0845
Leandro Moreira http://orcid.org/0000-0001-6283-6151
Robson O. E. Hack http://orcid.org/0000-0001-8678-6646
Adriana Milagres http://orcid.org/0000-0003-4531-3097
Fabiano R. de Melo http://orcid.org/0000-0001-9958-2036
Valéria Fagundes http://orcid.org/0000-0001-9501-0751
Sérgio L. Mendes http://orcid.org/0000-0002-7287-6058
Anthony Di Fiore http://orcid.org/0000-0001-8893-9052
Sandro L. Bonatto http://orcid.org/0000-0002-0064-467X

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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