Hybridization Effects and Genetic Diversity of the Common and Black-Tufted Marmoset (Callithrix jacchus and Callithrix penicillata) Mitochondrial Control Region

ARTICLE *in* AMERICAN JOURNAL OF PHYSICAL ANTHROPOLOGY · DECEMBER 2014

Impact Factor: 2.51 · DOI: 10.1002/ajpa.22605

11 AUTHORS, INCLUDING:

Joanna Malukiewicz
Arizona State University
6 PUBLICATIONS **18** CITATIONS
SEE PROFILE

Lisieux Fuzessy
Federal University of Minas Gerais
6 PUBLICATIONS **0** CITATIONS
SEE PROFILE

Jeffrey A French
University of Nebraska at Omaha
127 PUBLICATIONS **2,424** CITATIONS
SEE PROFILE

Luiz C. M. Pereira
Universidade Federal do Vale do São Francis…
8 PUBLICATIONS **207** CITATIONS
SEE PROFILE

Hybridization Effects and Genetic Diversity of the Common and Black-Tufted Marmoset (*Callithrix jacchus* and *Callithrix penicillata*) Mitochondrial Control Region

Joanna Malukiewicz,[1]* Vanner Boere,[2] Lisieux F. Fuzessy,[3] Adriana D. Grativol,[4] Jeffrey A. French,[5] Ita de Oliveira e Silva,[6] Luiz C.M. Pereira,[7] Carlos R. Ruiz-Miranda,[4] Yuri M. Valença,[8] and Anne C. Stone[1]

[1]*School of Human Evolution and Social Change, Arizona State University, Tempe AZ, 85287*
[2]*Departamento de Bioquímica e Biologia Molecular, Universidade Federal de Viçosa, Viçosa MG, Brazil*
[3]*Department of Plant Biology, Universidade Federal de Minas Gerais, Belo Horizonte MG, Brazil*
[4]*Ciências Ambientias, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense, Campos dos Goytacazes RJ, Brazil*
[5]*Callitrichid Research Center, University of Nebraska at Omaha, Omaha NE, 68182*
[6]*Departamento de Biologia Animal, Universidade Federal de Viçosa, Viçosa MG, Brazil*
[7]*Centro de Conservação e Manejo de Fauna da Caatinga, Universidade Federal do Vale do São Francisco, Petrolina PE, Brazil*
[8]*Centro de Reabilitação de Animais Silvestres do Santuário dos Três Reinos, Recife PE, Brazil*

KEY WORDS hybridization; population genetics; phylogenetics; New World primates; evolution

ABSTRACT Hybridization is continually documented in primates, but effects of natural and anthropogenic hybridization on biodiversity are still unclear and differentiating between these contexts remains challenging in regards to primate evolution and conservation. Here, we examine hybridization effects on the mitochondrial DNA (mtDNA) control region of *Callithrix* marmosets, which provide a unique glimpse into interspecific mating under distinct anthropogenic and natural conditions. DNA was sampled from 40 marmosets along a 50-km transect from a previously uncharacterized hybrid zone in NE Brazil between the ranges of *Callithrix jacchus* and *Callithrix penicillata*. DNA was also collected from 46 marmosets along a 30-km transect in a hybrid zone in Rio de Janeiro state, Brazil, where exotic marmosets appeared in the 1980s. Combining *Callithrix* DNA sampled inside and outside of these hybrid zones, phylogenetic and network analyses show *C. jacchus* and *C. penicillata* being parental species to sampled hybrids. We expand limited *Callithrix* population genetics work by describing mtDNA diversity and demographic history of these parental species. We show ancient population expansion in *C. jacchus* and historically constant population size in *C. penicillata*, with the latter being more genetically diverse than the former. The natural hybrid zone contained higher genetic diversity relative to the anthropogenic zone. While our data suggest hybrid swarm formation within the anthropogenic zone due to removed physical reproductive barriers, this pattern is not seen in the natural hybrid zone. These results suggest different genetic dynamics within natural and anthropogenic hybridization contexts that carry important implications for primate evolution and conservation. Am J Phys Anthropol 000:000–000, 2014.

Hybrid zones offer many opportunities to examine important evolutionary processes such as speciation, adaptation, and genetic introgression (Shurtliff, 2013). Here, we define hybridization as successful mating between members of populations (typically at the subspecies or species level) possessing distinct heritable traits (modified from Arnold, 1997), and this phenomenon has been documented in several primate taxa (e.g., baboons: Phillips-Conroy and Jolly, 1981; macaques: Bonhomme et al., 2009; howler monkeys: Kelaita and Cortes-Ortiz, 2013). While hybridization in wild primates is shaped by both anthropogenic and natural elements (e.g., Detwiler et al., 2005), the role of each factor in driving hybridization in primates and other animals is still debatable (Mallet, 2005). Further, effects on biodiversity may differ between these two hybridization conditions and they can be difficult to differentiate from one another (Allendorf et al., 2001). These observations carry important conservation and evolutionary implications for primate taxa as incidence of hybridization seems to be on the rise, which necessitates a stronger understanding of the frequency and signature of hybridization within clear anthropogenic and natural contexts.

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: National Science Foundation DDIG #proposal; Grant number: 1061508; Grant sponsor: Fulbright Fellowship to Brazil, ASU SOLS FIGG grant, ASU GPSA Jump Start and Research Grants, ASU Chapter of Sigma Xi, and an International Primatological Society Research Grant (to JM).

*Correspondence to: Joanna Malukiewicz, School of Human Evolution and Social Change, Arizona State University, PO Box 872402, Tempe, AZ 85287, USA. E-mail: jmalukie@asu.edu

Received 5 January 2014; accepted 13 August 2014

DOI: 10.1002/ajpa.22605
Published online 00 Month 2014 in Wiley Online Library (wileyonlinelibrary.com).

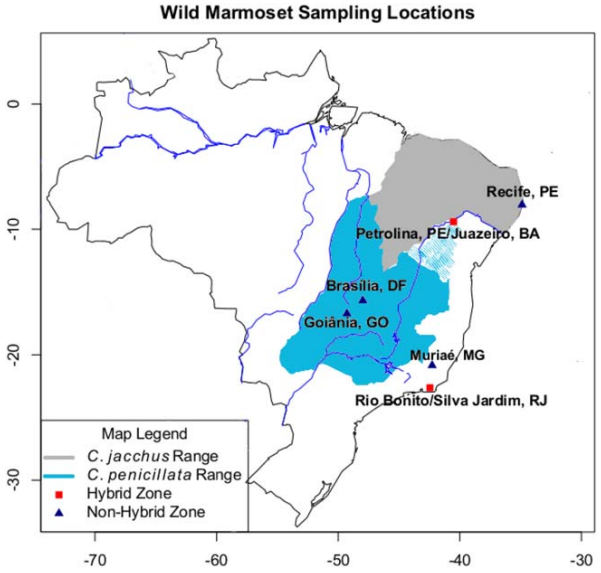

Fig. 1. Overview of approximate natural ranges of *C. jacchus* and *C. penicillata*as well as the locations of individuals sampled inside and outsideof hybrid zones within Brazil. Solid gray and blue represent *C. jacchus* and *C. penicillata* ranges, respectively, based on 2012 IUCN Red List Spatial Data (http://www.iucnredlist.org/technical-documents/spatial-data). Thatched blue indicates an extension of the *C. penicillata* range based on Rylands et al. (1993, 2009). Degrees of longitude and latitude are, respectively, represented by the *x*- and the *y*-axes. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

As a relatively little-studied example of hybridizing New World primates, eastern Brazilian marmosets (genus *Callithrix*) represent a unique opportunity to study primate interbreeding within a clear anthropogenic context. Marmosets are part of the Callitrichidae family (Rylands and Mittermeier, 2009; Rylands et al., 2009), known to possess rare primate characteristics including cooperative breeding (Digby et al., 2007) and socially modulated female reproduction (Smith et al., 1997). All *Callithrix* species have distinct geographic distributions throughout central-eastern Brazil and along the Brazilian coast (Rylands et al., 1993). However, sympatry exists between exotic populations of *Callithrix* species introduced by anthropogenic factors into areas far outside of their natural geographic ranges. For example, human introductions of *Callithrix penicillata* and *Callithrix jacchus* have resulted in marmoset hybridization within the states of Minas Gerais (MG; personal observation, IOS and VB) and Rio de Janeiro (RJ; Hershkovitz, 1975; Rylands et al., 1993; Ruiz-Miranda et al., 2006).

Besides areas of artificial sympatry, intertaxa contact also occurs at geographical distribution boundaries of different *Callithrix* species (e.g., Rylands et al., 1993; Mendes, 1997; Passamani et al., 1997). Originally, Coimbra-Filho and Mittermeier (1973) noted that no known cases of marmoset hybridization existed in such areas of species contact. Hershkovitz (1975, 1977), however, did show some evidence of natural intergradation between *Callithrix* taxa based on admixed marmoset museum skins. The first documented field study of a marmoset hybrid zone came in the late 1980s when Alonso et al. (1987) examined a *C. jacchus* × *C. penicillata* hybrid zone outside of Salvador, Bahia state (BA). Since then, the number of hybridization reports between various *Callithrix* species has been steadily increasing (e.g., Mendes, 1997; Passamani et al., 1997; Ruiz-Miranda et al., 2000; personal observations, IOS, JM and VB). Further, hybridization is thought to occur in about 10% of mammalian species, usually among the youngest ones between 1 and 2 million years old (Mallet, 2005). *Callithrix* arose about 2.5 million years ago (MYA; Perelman et al., 2011; Schneider et al., 2012), and *C. jacchus* and *C. penicillata* were most likely the last to diverge (as sister species) within the genus less than 1 MYA (Perelman et al., 2011). Thus, the young age of the *Callithrix* genus and recent reports of interspecific breeding suggest a potential role for hybridization in marmoset evolutionary history.

However, few studies about the evolutionary biology and population genetics of the *Callithrix* genus are available. Many of these studies have used mitochondrial DNA (mtDNA) as a rapidly evolving marker employed in studying evolution of recently diverged taxa (Brown et al., 1979). Faulkes et al. (2003), whose work focused on *C. jacchus*, conducted one of the few population genetics studies available for any *Callithrix* species. Work considering the evolutionary history of *Callithrix* has examined the phylogenetics of the mtDNA control region (CR) as well as a few nuclear loci (e.g., Tagliaro et al., 1997, 2000; Schneider et al., 2012) without full resolution of species-level evolutionary relationships. The multilocus nuclear approach of Perelman et al. (2011) perhaps has given the most robust phylogeny of the *Callithrix* genus so far, but branching order between some *Callithrix* species remains unclear. Thus, large gaps in our understanding of the genetic diversity as well as recent and past population history of the *Callithrix* genus remain, particularly with regards to the impact of recent hybridization on the genus.

In this study, we assess the existence of a *C. jacchus* and *C. penicillata* hybrid zone at a natural species border in NE Brazil (between the cities of Petrolina, Pernambuco (PE) state and Juazeiro, BA) and in an area of artificial introduction in SE Brazil (cities of Rio Bonito, RJ and Silva Jardim, RJ). Additionally, we report on the genetic diversity and demographic history of pure common and black-tufted marmosets to understand better the evolutionary history of these two species. We use the mtDNA CR to build on previous work about the evolutionary biology of *Callithrix* and address the following questions: (i) Are *C. jacchus* and *C. penicillata* source species for the two putative hybrid zones mentioned above? (ii) What do the genetic patterns of the mtDNA CR let us infer about past demographic history of *C. jacchus* and *C. penicillata*? (iii) What are the patterns of mtDNA CR genetic diversity and differentiation inside and outside of putative *C. jacchus* × *C. penicillata* hybrid zones?

MATERIALS AND METHODS

Sample populations and hybrid zones

Between 2010 and 2011, biological samples were obtained from captive and wild populations of pure and likely hybrid *Callithrix* populations (detailed in Table 1). General locations of wild caught marmosets are shown in Figure 1, and latitude/longitude coordinates of the collection site for each individual are given in Supporting Information Table S1. For both captive and wild pure

TABLE 1. Summary of sampled individuals from captive and wild pure populations and wild hybrid zones

Populations	Type	Source	Year collected	Biological samples	Individuals sampled
C. jacchus	Captive	CRC,[a] Omaha, NE, USA	2011	B,S	2 (2)
	Wild	IBAMA CETAS,[b] Recife, PE, Brazil	2011	S	24 (20)
	Captive	NEPRC,[c] Southborough, MA, USA	2010	B,S	10 (10)
	Wild	Parque Dois Irmãos & Tapacurá Reserve, PE, Brazil[d]	2005	S	43 (1)
C. penicillata	Captive	CRC,[a] Omaha, NE, USA	2011	B,S	8 (7)
	Wild	Muriaé, MG; Brasilía, DF; Goiânia, GO, Brazil	2011	S	28 (25)
	Captive	IBAMA CETAS,[b] Recife, PE, Brazil	2011	S	3 (3)
	Wild	IBAMA CETAS,[b] Goiânia, GO, Brazil	2011	S	5 (5)
C. jacchus × *C. penicillata* hybrids	Wild	Silva Jardim and Rio Bonito Municipalities, RJ, Brazil	2011	S	46 (45)
C. jacchus × *C. penicillata* hybrids	Wild	Petrolina, PE and Juazeiro, BA, Brazil	2011	S	40 (38)
	Captive	CEMAFAUNA, Petrolina, PE, Brazil[e]	2011	S	3 (3)
C. kuhlii	Captive	CRC,[a] Omaha, NE, USA	2011	B,S	4 (3)
C. geoffroyi	Captive	CRC,[a] Omaha, NE, USA	2011	B,S	8 (4)
		Institute, Camden, NJ, USA	2010	N/A	1(1)

B, blood; S, skin.
Numbers in parentheses indicate number of samples used in statistical analysis.
[a] Callitrichid Research Center, University of Nebraska at Omaha.
[b] Wild Animal Triage Center, Brazilian Institute of the Environment and Natural Resources.
[c] New England Primate Research Center.
[d] Collected by Dr. Maria Adélia Borstelmann de Oliveira, most samples were too degraded to amplify the mtDNA control region.
[e] Center for Management of Fauna of the Caatinga.

individuals, the following samples were obtained: 79 *C. jacchus*, 44 *C. penicillata*, 4 *Callithrix kuhlii*, and 8 *Callithrix geoffroyi*. An additional *C. geoffroyi* DNA sample was obtained from the Corielle Institute (Camden, NJ).

Figure 1 shows general locations of hybrid and nonhybrid zone capture sites. The figure is largely based on 2012 IUCN Red List Spatial Data (http://www.iucnredlist.org/technical-documents/spatial-data), which shows that *C. jacchus* natively occurs in NE Brazil, and *C. penicillata* occurs in east central Brazil. The figure also extends part of the range of *C. penicillata* based on Rylands et al. (1993, 2009), who consider the natural distribution of *C. penicillata* to be wider than that of the IUCN data. Rylands et al. (1993, 2009) include the putative natural hybrid zone between Petrolina, PE and Juazeiro, BA (the "PJ hybrid zone") to lie at the natural species border between *C. jacchus* and *C. penicillata*. The Muriaé, MG capture location shown in Figure 1 lies in the area that Rylands et al. (1993, 2009) consider part of *C. aurita's* natural species distribution. However, these authors state that *C. penicillata* has recently been expanding its range into that of other marmoset species, and the Muriaé, MG site may be an example of such a *C. penicillata* range expansion.

The PJ hybrid zone (Figs. 1 and 2) occurs along the São Francisco River in the Caatinga biome (Leal et al., 2005). We collected samples from 40 wild caught marmosets along an approximate 50 km transect following the course of the São Francisco River, and three captive marmosets within the PJ zone. Along the São Francisco River, marmoset populations occur in fragmented forest patches, and populations may be able to get from one bank to another via fluvial islands continually formed and altered by the river (personal observation, LMP). Most collection was carried out on the *C. jacchus* side of the PJ zone, with six sites sampled north of the river and three sites sampled south of the river, due to more limited access to private farms on the *C. penicillata* side of the zone.

Forty-six samples were collected from exotic marmoset populations that probably originated as illegally introduced pets within the putative artificial hybrid zone of the São João watershed in the municipalities of Rio Bonito and Silva Jardim, Rio de Janeiro state (Figs. 1 and 3). We refer to this area as the "RJ hybrid zone." The RJ zone occurs within the Atlantic Forest Biome (Ribeiro et al., 2009), and it is characterized by highly disturbed/fragmented forest patches. Sampling sites of the RJ hybrid zone were located along an approximately 30 km long transect, and separated by a major highway, BR-101, with four sites each located on the north and south sides (Fig. 3).

Hershkovitz (1977) considered pure *C. jacchus* to have ear tufts characterized by bushy hair colored white or white with black-tips and for pure *C. penicillata* to have dark-brown to black, fine, low sloping ear tufts. He describes intermediate hybrids to be those individuals that have ear tuft phenotypes that fall halfway between the two parental phenotypes. Based on the descriptions of Hershkovitz (1977) and personal observations a hybrid index was developed for the phenotypic classification of sampled individuals (Table 2). Hybrid index scores were based on individual photographs taken during sample collections (see subsequently), and scaled as follows: zero indicates a pure *C. jacchus* phenotype, one-half to two indicates a *C. jacchus*-like phenotype, two and a half to three and a half indicates an intermediate phenotype, four to five and a half indicates a *C. penicillata*-like phenotype, and six indicates a pure *C. penicillata* phenotype. Figure 4 shows examples of these phenotypes. Adult and nonadult animals were distinguished based on body mass following the descriptions of Hershkovitz (1977) and de Morais (2010). We only measured a hybrid index score for adult individuals, as younger animals do not yet have a fully developed adult phenotype (Hershkovitz, 1977).

Sample collection and laboratory procedures

Animals from both wild Brazilian and US captive populations (Table 1) were collected under the approval of the Arizona State University Institutional Animal Care

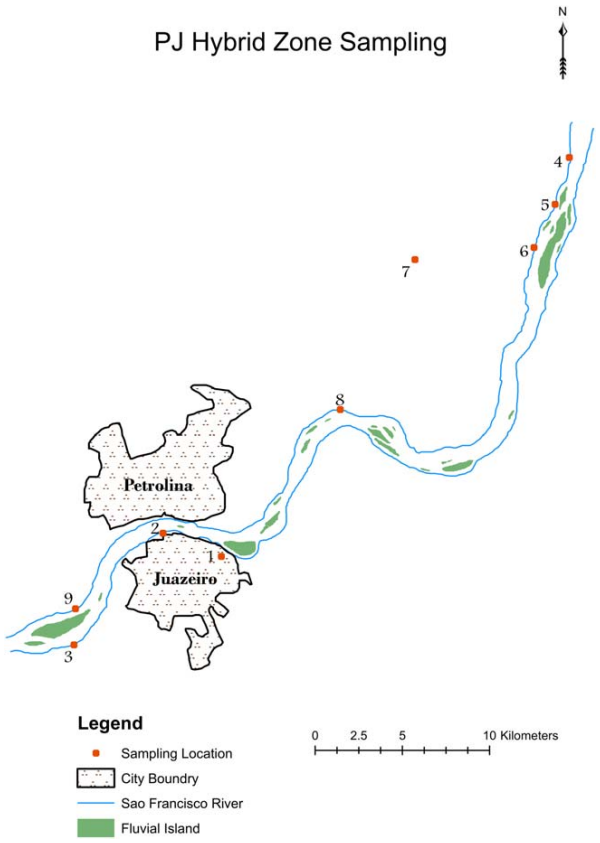

Fig. 2. Close-up of the Petrolina-Juazeiro hybrid zone. The zone lies along an approximately 50 km transect along the São Francisco River. Three sites are found to the south of the river: (1) Universidade do Estado da Bahia; (2) Chácara do Senhor Conrado dos Santos; and (3) Recanto do Sessego. Six sites are found to the north of the river: (4) Sítio Porto da Cruz; (5) Rio Verde; (6) Sítio Picos; (7) Sítio Carnaíba; (8) Chácara Galo da Briga; and (9) Chácara Bom Jesus. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

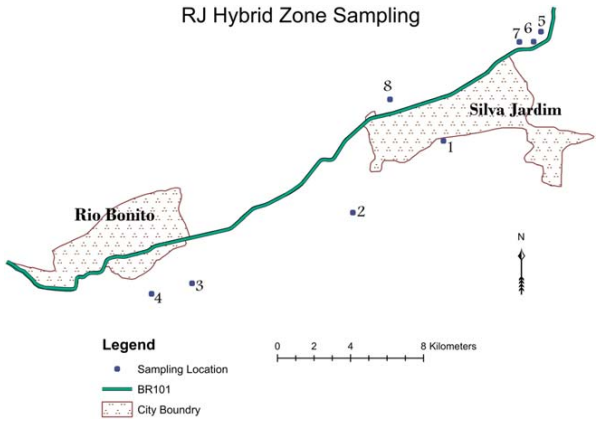

Fig. 3. Close-up of Rio de Janeiro State hybrid zone. The zone lies along an approximately 30 km transect along highway BR-101. Four sites are found to the south of the highway: (1) Boa Esperança; (2) House U; (3) Rio Vermelho I; and Rio Vermelho II. Four sites are found to the north of the highway: (5) Fazenda dos Tamarins; (6) Pesque Pague; (7) Ponto do Camarão; and (8) Fazenda Afetiva. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

and Use Committee (ASU IACUC, protocol #11-1150R). Captive animals were processed under protocols established at each primate center (see Table 1 and Supporting Information Table S1 for locations). Blood and skin punches were obtained from captive animals during routine physical examinations. For the captive samples, approximately 1–2 mL of whole blood were collected, preserved in EDTA, and then frozen at −80°C. Skin samples were obtained from the ear using a 0.5-cm skin punch and then frozen at −20°C.

Collection of biological materials from wild marmosets was conducted following a protocol established by VB and CRRM, and permission for capture of wild marmosets was obtained from the Brazilian National Council on the Development of Science and Technology (CNPq) and the Brazilian Ministry for the Environment and Natural Resources (IBAMA, protocol #28075-2). Wild animals were captured with auto-close, Tomahawk-style traps baited with bananas, and then traps were covered with cloth to calm the animals. At the RJ and PJ sites as well as the CETAS and CEMAFAUNA facilities, animals were transported to indoor laboratories for tissue collection, while collection at other locations was conducted about 500 m from the capture sites. Wild captured animals were monitored under veterinary care, and immobilized with injection of ketamine (approximately 20 mg/kg) into the intramuscular region of the inner thigh, photographed, weighed, and then biological samples were taken. Afterward, animals were returned to cages, given a banana, allowed to recover, and released at the original capture site on the same day they were captured. Biological tissues collected in the field consisted of skin punches. Skin samples were obtained from the ear using a 0.5-cm skin punch. These skin samples were stored from one to eight weeks in 25% (w/v) DMSO dissolved in 6 M NaCl (Goosens et al., 2003) under field conditions, and then frozen at −20°C in the laboratory.

DNA from blood and epithelial samples was extracted using a standard proteinase K/phenol/chloroform protocol (Sambrook and Russell, 2001). Buffers used for extraction, precipitation and elution of DNA from blood and skin tissue are listed in Supporting Information Table S2. DNA extraction of NEPRC and CRC samples (Table 1) was conducted at Arizona State University (ASU). DNA from all other samples was extracted at Northern State Fluminense University, Rio de Janeiro State, Brazil, and then exported to ASU (CITES permit #11BR007015/DF).

Initially, we attempted to amplify only the hypervariable regions of the mtDNA CR using the polymerase chain reaction with universal mtDNA primers of Kocher et al. (1989) and species-specific primers designed by JM. However, chromatograms from the sequences amplified with the above primers consistently resulted in multiple, overlapping nucleotide trace peaks, indicating possible amplification of nuclear inserts of mtDNA sequences (numts). Numts have been previously reported in marmosets (Mundy et al., 2000). To avoid numt amplification, we altered our strategy (described further in Supporting Information Methods) to instead amplify and sequence an approximately (depending on

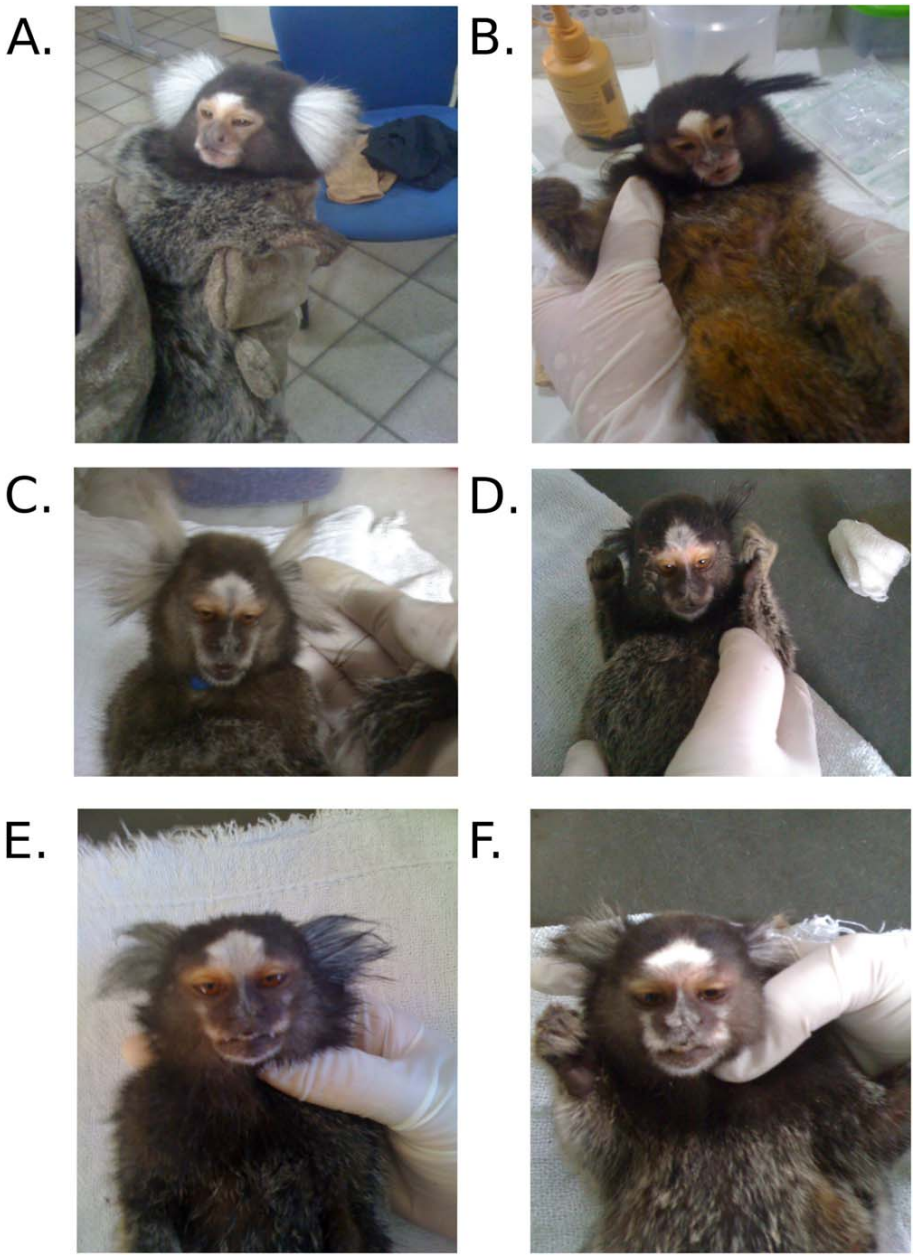

Fig. 4. (**A**–**F**) Phenotypes of (A) an individual with a pure *C. jacchus* phenotype, defined by full, white, bushy ear tufts; (B) an individual with a pure *C. penicillata* phenotype, defined by sparse, black ear tufts; (C) a *C. jacchus*-like hybrid that possesses a mostly *C. jacchus* phenotype accented by grayish ear tufts that lack the parental bushiness; (D) a *C. penicillata*-like hybrid that possesses a mostly *C. penicillata* phenotype accented by grayish ear tufts; and (E and F) show intermediate hybrids.

the species) 1,200 bp fragment that contained the majority of the marmoset mtDNA CR.

Data analysis

An alignment of the *Callithrix* mtDNA D-loop was made with the MUSCLE algorithm (Edgar, 2004) within MEGA 5.05 (Tamura et al., 2011) using the described samples (Table 1) as well as sequences obtained from Genbank (Supporting Information Table S3). For phylogenetic analysis, the alignment was shortened to 805 bp (positions 86–880 of the original marmoset mtDNA CR alignment Tagliaro et al., 1997) to accommodate the length of all obtained Genbank sequences. Our mtDNA D-loop alignment consisted of hypervariable region I, the conserved domain, and part of hypervariable region II, none of which code for genes. Sequences of *Callithrix* mtDNA CR haplotypes resulting from the samples listed in Table 1 and Supporting Information Table S1 and used in our alignment are available in Genbank (www.

TABLE 2. Phenotypic characteristics used for hybrid scoring

Score	Pure *C. jacchus*	*C. jacchus*-like	Intermediate	*C. penicillata*-like	Pure *C. penicillata*
	0	0.5	1	1.5	2
Ear tuft color	Tuft hairs completely white or white with black tips		Mixture of dark brown to black and white tufts hairs		Tuft hairs dark brown to black
Ear tuft volume	85–100% area around ear is covered by tuft hair		50% of area around ear is covered by tuft hair		Approximately 25% of area around ears covered by tuft hair
Head coloration	Head region between ear tufts and around face is mostly gray but sometimes interspersed with some black or beige		Intermediate		Head region around ear tufts is colored dark brown to black and interspersed with some beige, cheek region shows opposite pattern

ncbi.nlm.nih.gov/genbank) under accession numbers KJ020024-KJ020094. For subsequent inter- and intrataxa analyses, subsets of our larger alignment were used, and the most appropriate nucleotide substitution model was found for each subset with jModelTest 2.1 (Guindon and Gascuel, 2003; Darriba et al., 2012) using the Bayesian Information Criterion (Posada and Buckley, 2004). Maximum likelihood (ML) and Bayesian phylogenetic trees were based on an alignment of the full data set of captive, wild, and Genbank sequences, with identical sequences removed. *Mico argentatus* (CAR21) was used as an outgroup.

The ML tree was constructed in MEGA under the Tamura-Nei + I + G evolutionary model using Nearest Neighbor Interchange with 5,000 pseudoreplicate bootstrap runs to assess branch support. Under Bayesian phylogenetic inference, resultant trees can be sensitive to the branch length prior used (Ekman and Blaalid, 2011). Thus, we tested for such sensitivity in our data set by analyzing it under three different branch length priors (Supporting Information Table S4). MRBAYES 3.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) was used for Bayesian tree construction using a HKY+I+G model, by carrying out six independent repetitions (two repetitions per branch length prior), whose settings and convergence checks are further described in Supporting Information Methods. Bayesian tree topology and annotation were finalized with FIGTREE 1.4 (http://tree.bio.ed.ac.uk/software/figtree/).

Finally, a network was constructed from sequences sampled from pure *C. jacchus* and *C. penicillata* individuals and *C. jacchus* × *C. penicillata* hybrid zones to investigate population-level genealogies, as network methods can accommodate for low divergence, extant ancestral nodes, and multifurcations between sequences (Posada and Crandall, 2001). NETWORK 4.6.1.0 (Bandelt et al., 1999) software was used to create a median joining (MJ) network, using the following settings: elipson set to zero, including the frequency of each unique haplotype, using a 3:1 transversions-to-transitions ratio, and a 5:20:10 ratio for hypervariable sites/rare events such as indels/remaining sites. These settings follow the NETWORK user guide's suggestion to give greater weight to rare mutation events such as transversions than to more frequent mutations such as transitions.

DNASP 5.10.1 (Librado and Rozas, 2009) was used to identify unique haplotypes within the full dataset. Genetic variation was examined in terms of haplotype diversity (h), nucleotide diversity (π), theta based on the number of segregating sites (θs), and number of polymorphic sites for *C. jacchus* and *C. penicillata*, as well as the PJ and RJ hybrid zones using ARLEQUIN 3.5.1.2 (Excoffier and Lischer, 2010) set to the Tamura-Nei + G model of substitution. Genetic structure was investigated for the following pairings: *C. jacchus* and *C. penicillata*, north and south São Francisco River subpopulations in the PJ zone, and north and south subpopulations separated by highway BR-101 in the RJ zone. An analysis of molecular variance (AMOVA) was run in ARLEQUIN with the Tamura-Nei + G model to generate variance components and a fixation index (φ_{ST}) between the two subpopulations within each respective population. Significance of the analysis was assessed using resampling with the default setting of 16,000 permutations.

Changes in the demographic histories of *C. jacchus* and *C. penicillata* were inferred using Tajima's D (Tajima, 1989) and Fu's F (Fu, 1997) tests for neutrality in ARLEQUIN. Confidence intervals for the statistics were calculated with 16,000 resampling permutations. ARLEQUIN was also used to calculate population mismatch distributions for the two species to test the null hypothesis of sudden population expansion, with confidence intervals calculated same as above. The time (t) of expansion can be estimated through its relationship with the τ parameter in the equation $\tau = 2ut$ (where u is the mutation rate over the entire locus; Rogers and Harpending, 1992).

A Bayesian Skyline Plot (BSP; Drummond et al., 2005) in BEAST 1.7.5 (Drummond et al., 2012) was also used to explore past demographic changes in both species. As the marmoset fossil record is scant (Schneider et al., 2012), fossil calibration points could not be employed in the analysis. Consequently, a mean human substitution rate of 9.883×10^{-8} substitutions per nucleotide per year was used over the entire mtDNA CR (Soares et al., 2009), following the strategy of Yang et al. (2012). Two separate BSPs were made for each species, respectively, with a total chain length of 2.5×10^8 generations, logging every 1,000th generation, using only sequences from pure individuals. Additional runs were conducted for each species under a constant population size model, and support for each model was evaluated by stepping-stone sampling (SS) and path sampling (PS; Baele et al., 2012, 2013), See Supporting Information Methods for further details on BSP analysis.

RESULTS

Callithrix phylogenetics and network analyses

From pure captive and wild populations, 25 new, previously unreported, mtDNA CR haplotypes were obtained for *C. jacchus* and 24 new haplotypes were obtained for *C. penicillata*. Including the Genbank and hybrid zone sequences, this gave 45 *C. jacchus* haplotypes (93 transitions, 9 transversions, 1 indel), 25 *C. penicillata* haplotypes (120 transitions, 24 transversions, 3 indels), 15 haplotypes in the PJ hybrid zone (81 transitions, 4 transversions, 2 indels), and 3 haplotypes (57 transitions, 5 transversions, 1 indel) in the RJ zone. The average number of differences between common marmoset haplotypes was 13.38 bp and the average number of differences between black-tufted marmoset haplotypes was 37.68 bp.

The Bayesian phylogenetic analyses resulted in a topology that shows an overall pattern of complex species-level polyphyly (Fig. 5). The polyphyletic pattern is particularly characteristic of *C. penicillata* and *C. kuhlii* haplotypes. *C. penicillata* grouped into four distinct polyphyletic clades and *C. kuhlii* grouped into two polyphyletic clades. One of the *C. penicillata* clades was basal to all other species-level clades, except to a single *C. geoffroyi* clade. The *C. geoffroyi* clade was the basalmost clade in the phylogeny. The *C. jacchus* clade contained all haplotypes classified under this species as well as a single *C. geoffroyi* and *C. penicillata* haplotype. PJ zone haplotypes were essentially split between a single *C. penicillata* clade and the *C. jacchus* clade. Two of the three RJ zone haplotypes grouped within the same *C. penicillata* clade, and the third grouped within the *C. jacchus* clade.

For the Bayesian trees, SS Bayes Factor model selection chose the model set to the default unconstrained exponential branch length model with mean 0.1 and HS Bayes Factor model selection chose the model set to an unconstrained exponential branch length model with mean of 0.01 (data not shown). Topology was identical for the two independent runs done per model; branch support values were similar for each pair of these runs

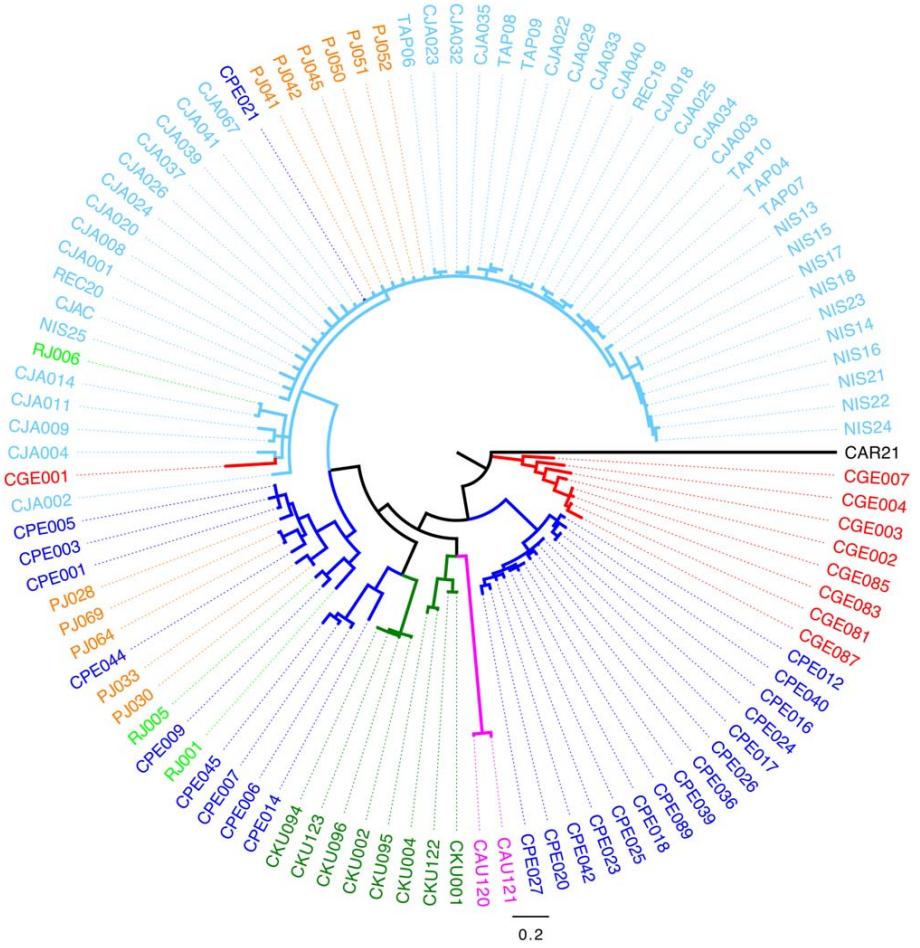

Fig. 5. Bayesian tree topology for the *Callithrix* mtDNA CR as chosen by SS Bayes Factor analysis for the default MRBAYES unconstrained exponential branch length model. Tree is rooted with the *Micoargentatus* (CAR21) outgroup. Branch posterior probabilities of at least 90% are marked with an asterisk above branches. Tip labels represent species haplotypes (*C. geoffroyi* is red, *C. penicillata* is dark blue, *C. jacchus* is light blue, *C. kuhlii* is hunter green, and pink for *C. aurita*) and hybrid zone haplotypes (RJ zone is light green and PJ zone is orange). Clade colors represent the majority of species haplotypes forming each clade. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

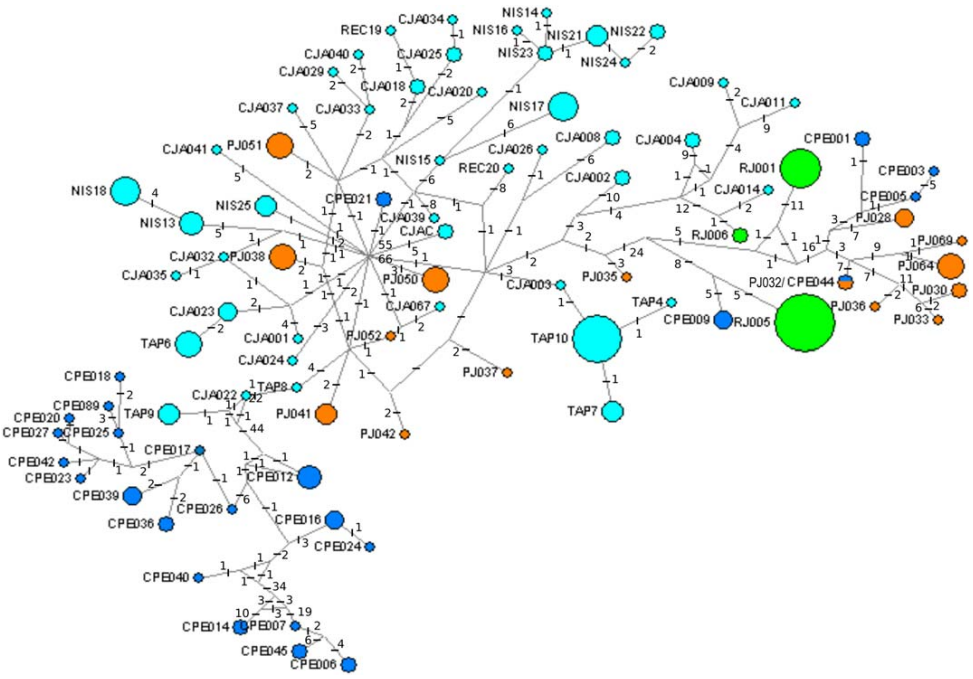

Fig. 6. Median-joining network of *C. jacchus, C. penicillata*, and hybrid mtDNA CR haplotypes. Individual haplotypes are represented as colored pies, scaled by the number of individuals possessing each haplotype. Numbers next to tick marks between two nodes represent the internode number of mutations. Pie colors indicate species haplotypes (*C. penicillata* is dark blue and *C. jacchus* is light blue) and hybrid zone haplotypes (RJ zone is light green and PJ zone is orange). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

and thus averaged together. However, branch support and branch length values differed between the two models, and in Figure 5 we only show the tree chosen by SS Bayes Factor model selection as it is preferred over the HS method. The ML tree did not provide strong branch support (above 75), thus it is not shown here.

The MJ network analysis was focused on the relationship between *C. jacchus* and *C. penicillata*, and shows relationships between haplotypes of the two species and their hybrids in finer detail (Fig. 6). The circles presenting each haplotype are proportional to the frequency of a given haplotype within the *C. jacchus, C. penicillata*, and hybrid haplotype dataset. In the network, *C. jacchus* forms a large, single, star-like group with a single pure *C. penicillata* haplotype embedded within it. The remaining pure *C. penicillata* haplotypes form two offshoots extending from the main *C. jacchus* group, both bearing clearer branching structure than that of *C. jacchus* haplotypes. PJ and RJ zone haplotypes group within the network analysis similarly to that already described in phylogenetic analysis.

Hybrid phenotype and mtDNA haplotypes distributions within hybrid zones

The geographical distributions of PJ zone haplotypes and phenotypes are shown in Figures 7a and 8a, respectively. Haplotypes and phenotypes of individuals PJ035-PJ037 were excluded from these figures because these individuals were not sampled in the wild. All individuals sampled on the south side of the PJ hybrid zone possessed a *C. penicillata* haplotype. Table 3 shows the percentage of photographed adult marmosets sampled within the PJ zone that fall within each hybrid phenotype category. Geographically, marmosets with *C. penicillata*-like and pure *C. penicillata* phenotypes were confined to the south side of the São Francisco River. *C. jacchus*-like and pure *C. jacchus* phenotypes were found to the north of the river. No intermediate hybrid phenotypes were found within the PJ hybrid zone. Interestingly, animals caught at the Chácara Bom Jesus site (see Fig. 2) on the *C. jacchus* side of the PJ zone possess haplotypes (PJ064 and PJ069) that group within a *C. penicillata* clade in both the phylogenetic and network analyses. The phenotypes of the Bom Jesus animals were that of pure *C. jacchus* and *C. jacchus*-like, but their *C. penicillata* haplotypes were closest in phylogenetic and geographic proximity to *C. penicillata* haplotypes found across on the southern riverbank. There is also a large fluvial island, *Ilha do Massangano*, found between the two banks around this spot. The rest of the individuals sampled on the northern bank possessed *C. jacchus* mtDNA haplotypes and pure *C. jacchus* and *C. jacchus*-like phenotypes.

Geographical distributions of the RJ zone haplotypes and phenotypic categories are shown in Figures 7b and 8b, respectively. For the RJ hybrid zone, the majority of individuals have an intermediate to *C. penicillata*-like phenotype (Table 3). Individuals with *C. penicillata*-like, pure *C. penicillata* and intermediate phenotypes were mostly found to the north of highway BR-101, and *C. jacchus*-like hybrids were found mostly to the south of highway BR-101. Haplotypes RJ001 and RJ005 grouped within a *C. penicillata* clade and haplotype RJ006 within the *C. jacchus* clade. The RJ001 haplotype is found mostly in marmosets with *C. jacchus*-like, intermediate,

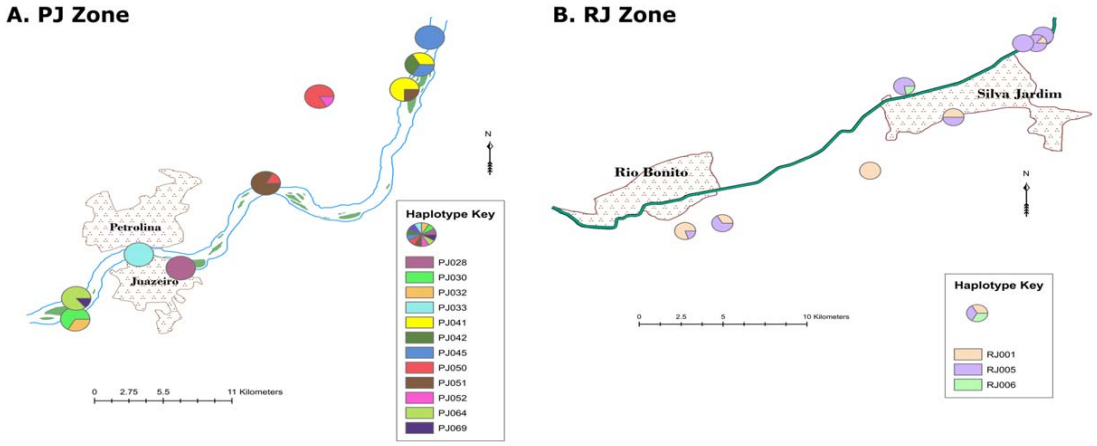

Fig. 7. Geographic distribution of mtDNA haplotypes in (**A**) the PJ hybrid zone and (**B**) the RJ hybrid zone. "Cpe" and "Cja" labels next to haplotype names indicate phylogenetic designation of each haplotype, *C. penicillata* and *C. jacchus*, respectively. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

and *C. penicillata*-like phenotypes. The RJ006 haplotype is found in a *C. jacchus*-like and intermediate individual, and the RJ005 haplotype is found mostly in individuals with an intermediate to pure *C. penicillata* phenotype.

Genetic diversity and structure of pure and hybrid *C. jacchus* and *C. penicillata*

Genetic diversity data for pure *C. jacchus* and *C. penicillata* as well as the hybrid zone populations are summarized in Table 4. Pure *C. jacchus* and *C. penicillata* show similar levels of haplotype diversity. However, nucleotide diversity and theta estimates indicate that the mtDNA CR is more variable on both a per site basis and per haplotype basis, respectively, in *C. penicillata* than in *C. jacchus*. For the hybrid zones, the RJ zone showed lower levels of variation than the PJ zone. In particular, there is a much higher level of nucleotide diversity seen in the latter than the former. If we break down the PJ zone haplotypes according to their parental species origin, nucleotide diversity for the *C. jacchus* haplotypes is 0.007 and for *C. penicillata* haplotypes it is 0.023. Haplotype diversity for PJ zone *C. jacchus* and *C. penicillata* haplotypes, respectively, is 0.815 and 0.791. We did not carry out these analyses for the RJ zone due to the low number of haplotypes found within the zone.

AMOVA shows a significant species-level differentiation between *C. jacchus* and *C. penicillata* (φ_{ST} = 0.664, P = 0.000). When the population in the PJ hybrid zone is split into subpopulations north and south of the São Francisco River, those subpopulations show a level of differentiation similar to that seen for the pure species (φ_{ST} = 0.697, P = 0.000). On the other hand within the RJ hybrid zone, genetic structure between subpopulations to the north and south of BR-101 is not as strong (φ_{ST} = 0.208, P = 0.001) and 79.2% of genetic variation is found within subpopulations when the zone is divided by BR-101.

Demographic history of *C. jacchus* and *C. penicillata*

Table 5 shows results of neutrality tests and mismatch distribution calculations for *C. jacchus* and *C. penicillata*. Neither Tajima's D nor Fu's F_s showed evidence in favor of demographic expansion in *C. penicillata*, and its bimodal mismatch distribution is characteristic of a stable population (Fig. 9a). Only the raggedness index statistic did not reject the null hypothesis of sudden population expansion in this species. The BEAST BSP for *C. penicillata* (Fig. 10a) shows constant population size in the species, with a population decline toward the very recent past. Bayes Factor model selection based on SS estimates showed evidence in favor of the BSP demographic model and PS estimates were in favor of a constant population size demographic model for *C. penicillata* (data not shown).

A population expansion for *C. jacchus* is supported through a negative value for Fu's F_s neutrality test and a unimodal mismatch distribution (Fig. 9b). Using a human CR mutation rate of 9.883×10^{-8} mutations per nucleotide per year (Soares et al., 2009), since mutation rate estimates for marmosets are currently unavailable, and a marmoset generation time of 1.5 years (Tardif et al., 2003), time of population growth under the sudden population expansion model of *C. jacchus* is estimated to be 22,580 years ago. The BEAST BSP for *C. jacchus* (Fig. 10b) also supports a population expansion in common marmosets approximately 60,000 years ago, but a decline about 10,000 years ago. Bayes Factor analysis of SS and PS marginal likelihood estimates both supported the BSP model instead of a constant population size model to describe the demographic history of *C. jacchus* (data not shown). Tajima's D value for *C. jacchus* is nonsignificant, but negative in favor of expansion, while the raggedness index rejected the null sudden expansion hypothesis.

DISCUSSION

Speciation and evolutionary relationships within the *Callithrix* genus

Our phylogenetic results of the marmoset mtDNA CR, along with those of several other studies (e.g., Tagliaro et al., 1997, 2000; Schneider et al., 2012) show *C. penicillata* and *C. kuhlii* as polyphyletic. This pattern is suggestive of either hybridization of these species with one another or other marmosets or incomplete lineage

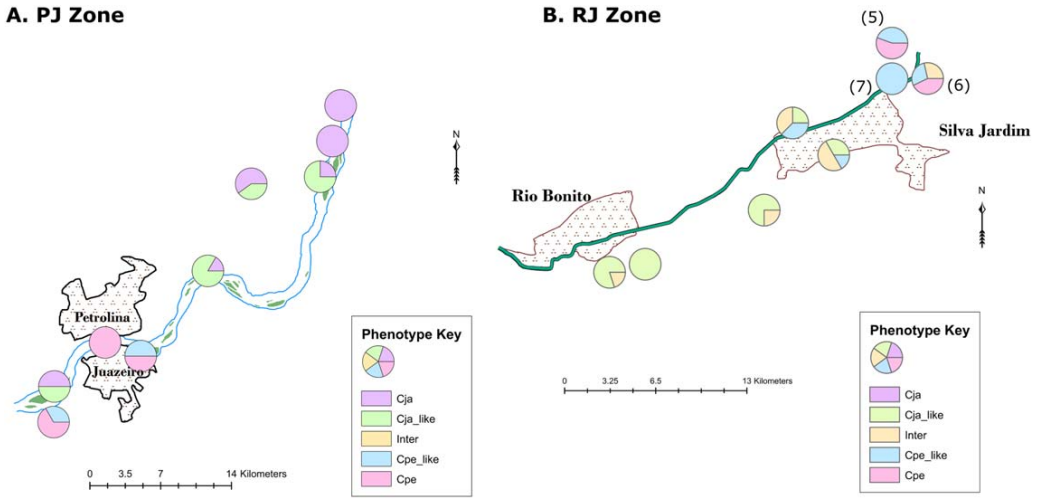

Fig. 8. Geographic distribution of phenotypic categories based on hybrid index scores in (**A**) the PJ hybrid zone and (**B**) the RJ hybrid zone (sites 5–7 from Fig. 3 are labeled for clarity). Phenotype key labels indicate the following phenotypic categories: "Cjac" is pure *C. jacchus*, "Cjac-like" is *C. jacchus*-like, "Inter" is intermediate, "Cpe-like" is *C. penicillata*-like, and "Cpen" is *C. penicillata* pure. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE 3. Percentage of photographed adult individuals sampled within each hybrid zone that fall into each phenotype category based on hybrid index score

	C. jacchus pure (0)	*C. jacchus*-like (0.5–2)	Intermediate (2.5–3.5)	*C. penicillata*-like (4–5.5)	*C. penicillata* pure (6)
RJ hybrid zone	0.00%	29.55%	25.00%	29.55%	15.91%
PJ hybrid zone	37.50%	43.75%	0.00%	6.25%	12.50%

Numbers in parentheses indicate hybrid index scores.

TABLE 4. Population genetic variables and indices for C. jacchus, C. penicillata*, and* C. jacchus × C. penicillata *hybrids*

Population	Sequences	Haplotype number	Haplotype diversity (h)	Nucleotide diversity (π)	Theta (θs)	Polymorphic sites
C. jacchus	108	45	0.946	0.017	19.030	101
C. penicillata	41	25	0.970	0.084	30.620	134
RJ hybrids	45	3	0.497	0.174	13.950	62
PJ hybrids	41	15	0.915	1.710	19.400	84

sorting (Funk and Omland, 2003). A hybrid origin for *C. kuhlii* has been previously suggested as a result of interbreeding between *C. penicillata* and *C. geoffroyi* (Hershkovitz, 1977) or *C. penicillata* and *C. jacchus* (Arnold and Meyer, 2006). Yet, Rylands et al. (1993) point out that experimental work in marmoset hybridization by Coimbra-Filho et al. (1993) failed to produce a *C. penicillata* × *C. geoffroyi* hybrid with the *C. kuhlii* phenotype (see Coimbra-Filho et al., 2006 for examples), although this work was mostly based on early generation F1, F2, and backcross hybrids. Also in the wild, none of the individuals within a group of *C. penicillata* × *C. geoffroyi* hybrids found in the Serra do Espinhaço mountains in Minas Gerais were observed with a phenotype reminiscent of *C. kuhlii* (Coimbra-Filho et al., 2006), where later generation hybridization is likely. In our own work, none of the animals sampled in either the RJ or PJ hybrid zones showed the phenotype typical of *C. kuhlii*. Additionally, no *C. kuhlii* mtDNA D-loop haplotypes included in our phylogeny fell into a *C. jacchus* or *C. penicillata* clade. Thus, considering the above data, it is unlikely that *C. kuhlii* arose through hybridization but rather mtDNA D-loop lineages have not completely sorted within the *Callithrix* genus.

Because of our focus on one short portion of the marmoset mtDNA genome, our reported Bayesian phylogenies do not agree topologically with previously published *Callithrix* phylogenies. Contrary to our phylogeny, *C. aurita* is consistently the basal *Callithrix* species when included in a given nuclear or mitochondrial phylogeny (Tagliaro et al., 1997, 2000; Perelman et al., 2011; Schneider et al., 2012). Other research also finds that *C. geoffroyi* forms a well-supported monophyletic clade that diverges next after *C. aurita* within the *Callithrix* genus (Tagliaro et al., 1997). The remaining branching order in our analysis broadly matches that of Tagliaro et al. (1997, 2000), but with higher branch supports. Incomplete lineage sorting of mtDNA (see discussion below), length differences in mtDNA sequences and the different applied phylogenetic methods are likely the main factors underlining differences in results from our study and those of previously published work.

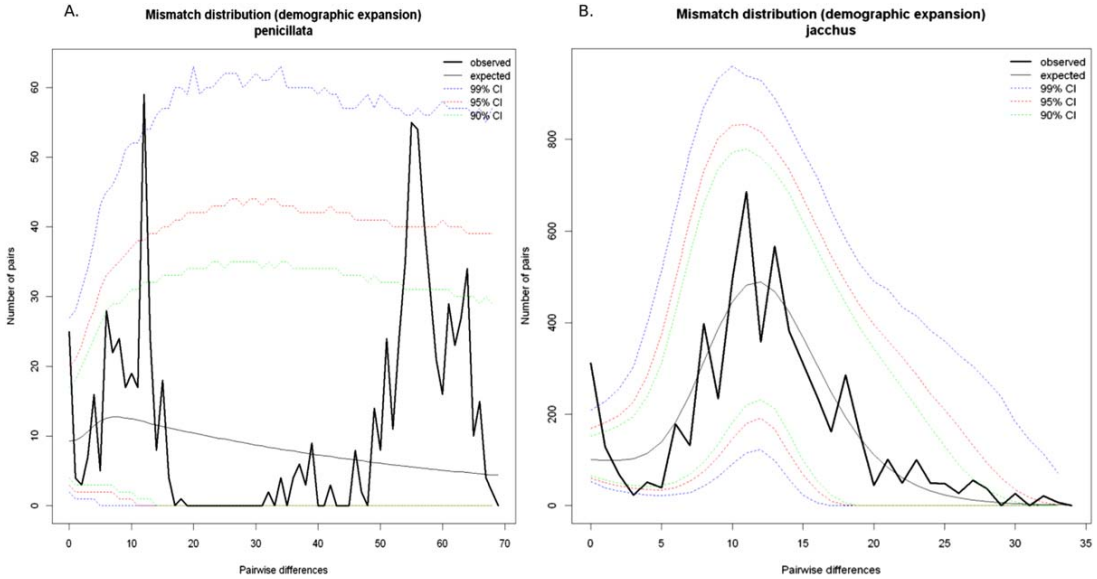

Fig. 9. MtDNA dloop mismatch distributions for (**A**) *C. penicillata* and (**B**) *C. jacchus*. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Despite mtDNA *Callithrix* phylogenies being characterized by polyphyly, mtDNA may be an important marker for tracking species relationships within this genus. The fixation time for mtDNA, as a uniparentally inherited, haploid marker with a small effective population size, is expected to be shorter than that of autosomal loci; indeed, phylogenies using the latter do tend to show relationships among *Callithrix* species as polytomies (e.g., Perelman et al., 2011; Schneider et al., 2012). One caveat in using mtDNA to track species relationships is that in female philopatric species, which covers most mammals, population structure and geography may strongly affect mtDNA phylogenies (Tosi et al., 2003). As a result, such mtDNA-based phylogenies may be incongruent to phylogenies of female philopatric species based on Y-chromosome and autosomal markers, which get spread between populations via male migration (Tosi et al., 2003). However, behavioral and genetic evidence suggests that female and male dispersal is the norm in several *Callithrix* species (Lazaro-Perea et al., 2000; Faulkes et al., 2003; Ferrari, 2009; Sousa et al., 2009; this study). Thus, mtDNA based phylogenies of *Callithrix* species can inform us of marmoset species evolutionary relationships where the comparatively long fixation time of autosomal markers may not yet allow for this and *Callithrix* Y-chromosome markers are still largely unavailable.

Population genetics and demographic history of *C. jacchus* and *C. penicillata*

Faulkes et al. (2003) reported on genetic structure in the mtDNA control region within and among *C. jacchus* populations, one of the few population genetic studies conducted on wild *Callithrix*. Their results showed high haplotypic diversity, but low genetic divergence in *C. jacchus*. Combining the dataset of Faulkes et al. (2003) with our own data, we observe a similar trend. In *C. penicillata*, we see high haplotypic diversity but greater variation among individual haplotypes. Our analyses also suggest separate demographic histories for these two species, with *C. jacchus* experiencing one past major population expansion and *C. penicillata* evolving at constant population size. Expanding populations are expected to have a higher frequency of rare variant sites and lower nucleotide diversity than a population at equilibrium. We would eventually expect an increase in frequency of some of those rare variants, and in turn an increase in the average number of differences between haplotypes and nucleotide diversity. This process is reflected in our MJ network through partially networked and partially star-like arrangements of *C. jacchus* haplotypes. We do note that that the BSP results should be interpreted with caution, as the analyses were calibrated with a human-based substitution rate. Because mitochondrial substitution rates vary among mammalian species (Galtier et al., 2009), our time estimates of BSP inferred *Callithrix* demographic changes might be a rough approximation at best.

TABLE 5. Neutrality tests and mismatch distribution analyses for C. jacchus *and* C. penicillata

Statistic	*C. jacchus*	*C. penicillata*
Tajima's D (*P*-value)	−1.188 (0.096)	0.885 (0.856)
Fu's F_s (*P*-value)	−9.250 (0.038)	2.271(0.820)
SSD (*P*-value)	0.006 (0.110)	0.024 (0.065)
r (*P*-value)	0.016 (0.007)	0.012 (0.184)
Θ_0	2.600	55.496
Θ_1	56.250	87.941
τ	10.688	4.418

SSD is sum of squared deviation and *r* is the raggedness index.

The studies of Perelman et al. (2011) and Schneider et al. (2012) suggest that *Callithrix* species diverged during the Pleistocene. It is hypothesized that climatic oscillations during the Pleistocene caused repeated contractions and expansions of forested areas (refugia) across South America and thus drove parapatric and allopatric speciation (Kinzey, 1982; Turchetto-Zolet

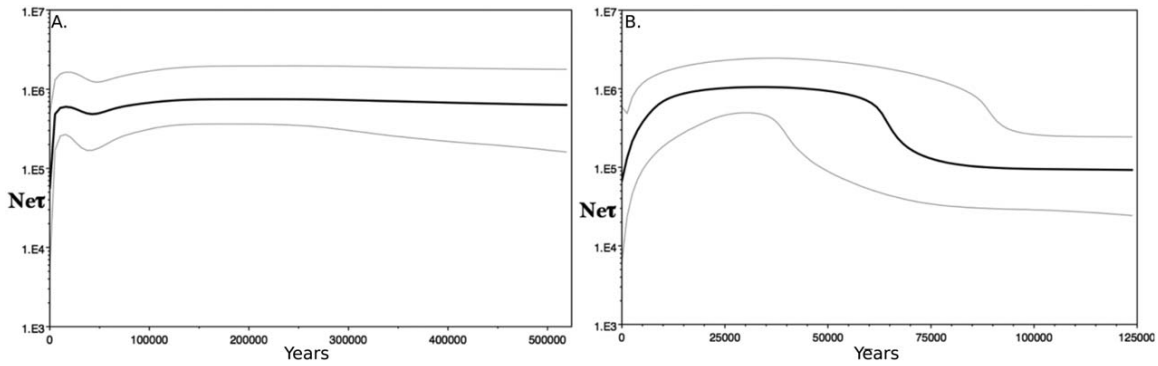

Fig. 10. Bayesian skyline plots for (**A**) *C. penicillata* and (**B**) *C. jacchus*. The black solid line shows the median estimate and the thin blue lines show the 95% highest posterior density limits. The *x*-axis shows time in years and the *y*-axis is the product of effective population size (N_e) and generation time (τ) measured in years.

et al., 2013). The historical separation of the geographical ranges of the *Callithrix* species (Rylands et al., 1993) certainly suggests the possibility of speciation modes in line with refugia theory. Further, the respective modern ranges of many *Callithrix* species are located in areas identified as historical forest refuges (e.g. Pernambuco refuge for *C. jacchus*, Bahia refuge for *C. kuhlii, C. penicillata*, and *C. geoffroyi*, Carnaval and Moritz, 2008). Rivers may have also played an important role in the diversification of terrestrial organisms in South America by acting as barriers to dispersal between different populations (Turchetto-Zolet et al., 2013). This may have certainly been the case for *Callithrix* species whose ranges are in part limited by rivers (Rylands et al., 1993), like *C. jacchus* and *C. penicillata* whose ranges are largely separated by the São Francisco River (but see the discussion below on fluvial islands and gene flow). Thus, it may have been a complex interplay between the paleoclimate of the Pleistocene and river barriers that played an important role in the divergence of our two focal species and other *Callithrix* species.

The Rio de Janeiro and Petrolina-Juazeiro hybrid zones

Our phylogenetic and network analyses confirmed the hypothesis of *C. jacchus* and *C. penicillata* being the parental species of the PJ and RJ hybrid zones. Occurring in an area of natural contact between our study species, the PJ hybrid zone shows much higher haplotype diversity, a larger number of haplotypes, and higher nucleotide diversity than found in the anthropogenic RJ hybrid zone. Further, the RJ zone showed much lower levels of genetic diversity than either parental species. Standing levels of genetic variation in the RJ zone come from only three mtDNA control region haplotypes, and there is probably very limited flow of new genetic variation (through additional marmoset introductions by humans) coming into the hybrid zone as it is far removed from the natural ranges of either parental species. Thus, we cannot rule out the lack of haplotype diversity in the RJ zone hybrids resulting from a founder effect. The PJ zone, with a location at the edges of parental species' distributions, more plausibly receives a continued influx of new genetic material from *C. jacchus* in the north and from *C. penicillata* in the south.

The RJ zone phenotype distributions identified here are similar to those found by de Morais (2010). de Morais (2010) argues that such a phenotypic pattern suggests multiple marmoset introductions into the RJ hybrid zone given that certain phenotypes are confined to specific regions of the zone. Our genetic data suggest multiple introductions into the RJ zone, with each of the three recovered mtDNA haplotypes being more common in certain areas than others. Haplotypes probably remained localized mainly within the original introduction areas since marmoset gene flow between forest patches within the RJ one is unlikely (see discussion subsequently). Additionally, highway BR-101 probably precludes gene flow between northern and southern portions of the RJ zone as suggested by the significant genetic structure between these two sides of the zone. While other female mtDNA lineages may have been introduced into the RJ zone, they may have been lost due to the effects of genetic drift on mtDNA, which has a small effective population size (Ballard and Whitlock, 2004).

Significant genetic structure is also evident between marmoset populations on the northern and southern sides of the PJ zone, which is divided by the São Francisco River. Genetic analyses of haplotypes from the PJ zone are essentially comparing a large number of sequences from two genetically differentiated species. This context helps explain the high level of nucleotide diversity found within the PJ hybrid zone when all haplotypes are considered together. Interestingly, we found evidence for the hypothesis that islands in the São Francisco River enable gene flow across the river since we found a group of *C. penicillata* haplotypes on both the *C. jacchus* and *C. penicillata* sides of the river at capture points between which lies a large island, *Ilha do Massangano*.

Alternately, the presence of the *C. penicillata* haplotypes found on either side of the São Francisco River may be explained by incomplete lineage sorting as this process may have affected other parts of our *Callithrix* mtDNA phylogeny. However, in spite of this possibility, our phylogeny showed only two cases of incongruence between an individual's phenotypic and haplotypic species classification. Rather, our phylogeny shows polyphyly of species-level clades that still maintain internal clade haplotypic and phenotypic congruence. Additionally, the *C. penicillata* mtDNA haplotypes observed on

riverbanks on either side of *Ilha do Massangano* fall into close phylogenetic proximity of each other within the same *C. penicillata* clade of our mtDNA CR phylogeny. Funk and Omland (2003) write "incomplete lineage sorting is not predicted to promote geographical proximity of interspecifically shared alleles that may be seen under local introgression." The geographic and phylogenetic proximity of *C. penicillata* haplotypes seen in marmoset groups on either side of *Ilha do Massangano* closely fit such a pattern pertaining to interspecific introgression. This observation along with the rare occurrence of haplotype/phenotype mismatches observed in our mtDNA CR *Callithrix* phylogeny suggests that the presence of *C. penicillata* haplotypes on *C. jacchus* side of the RJ zone is best explained by interspecific hybridization and introgression. Most likely, *C. penicillata* migrated north via large fluvial landmasses such as *Ilha do Massangano* and eventually encountered and mated with resident *C. jacchus* populations.

Implications for *Callithrix* biodiversity and genetic integrity

The Brazilian Atlantic Forest, the biome occupied by most *Callithrix* species (Rylands et al. (1993, 2009), currently faces severe habitat destruction. The *Callithrix* genus itself is already threatened with *C. aurita* as listed as vulnerable, *C. flaviceps* as endangered, and *C. kuhlii* as near-threatened (IUCN 2012. IUCN Red List of Threatened Species Version 2012. 2. <iucnredlist.org> Downloaded on May 01, 2013). Only about 11% of the original forest cover of the Brazilian Atlantic Forest remains as highly fragmented patches with 80% of fragments sized at <50 ha, and an average distance of 1,440 m between fragments (Ribeiro et al., 2009). Such fragmentation is certainly a threat to the *Callithrix* genus, as sizes of many Atlantic Forest fragments seem to be near the minimum of adequate marmoset group home range size (Rylands and de Faria, 1993). Forest patches with an average size of 50 ha characterize the RJ hybrid zone landscape, and distances between patches north of highway BR-101 are about 250 m (personal observation, CRRM). The capture sites of Fazenda dos Tamarins, Ponto do Camarão, and Pesque Pague are part of the same large forest fragment. The fragment is bordered on its western and southern sides by highway, and on its east and north side by large expanses of cleared land. Few marmosets sampled within this particular fragment had either a pure *C. jacchus* or *C. penicillata* appearance, suggesting the possibility of the formation of a hybrid swarm in this fragment. The hybrid swarm may be the result of many generations of hybridization within this forest fragment, barriers to gene flow into and out of the fragment, and no new genetic input from parental species into the fragment.

Unfortunately, the negative outlook for declining or fragmented species includes loss of genetic variation, alteration in levels of population differentiation, and changes in levels of inbreeding (Sherwin and Moritz, 2000). Further, lowered genetic variation between populations can result in the loss of adaptive responses to varied local conditions, reduction in adaptation opportunities, and ultimately curtail the viability of a population (Sherwin and Moritz, 2000). The addition of hybridization into this delicate mix may further threaten the biodiversity and genetic integrity of species within the *Callithrix* genus. *C. jacchus* and *C. penicillata* seem to be the most frequently introduced exotic species into the ranges of other native marmoset species. Such an influx of new genetic variation may initially be advantageous for a genetically depressed, endangered population. However, given the level of isolation and fragmentation of habitats where marmosets occur, hybridization between exotic and native marmoset species may lead to a situation of a hybrid swarm being contained within forest patches holding marmoset populations. Pure populations could be replaced by complex hybrids whose mosaic genomes have been shaped by different levels of admixture from two or more parental species. Hybridization has a multitude of positive and negative outcomes, but certainly, such a process would impact how "pure" each *Callithrix* species can remain, which would carry important implications for the conservation of species within this genus.

We observed evidence for low genetic variation and hybrid swarming within the RJ hybrid zone, whereas in the PJ zone, we observed levels of genetic variation comparable to that of pure, parental populations of *C. jacchus* and *C. penicillata*. Certainly, we cannot directly measure the effect of hybridization on marmoset fitness from our dataset. However, Reed and Frankham (2002) showed that when a measure such as genetic variation is used as a surrogate for fitness, it is positively and significantly correlated with population fitness. Thus, this leads us to suggest that the hybrid populations in the RJ hybrid zone are less fit than populations of parental marmoset species. On the other hand, marmosets within the PJ zone are probably comparable in fitness to parental *C. jacchus* and *C. penicillata* populations.

If we use the RJ and PJ hybrid zones as models to understand the dynamics of natural and artificial marmoset hybrid zones, our genetic data from these areas imply complex hybridization outcomes within other marmoset hybrid zones. As most marmoset species are located within the highly fragmented Brazilian Atlantic Forest, marmoset hybridization within this biome may carry potential implications of loss of genetic variation, biodiversity, and reduction in population fitness. However, we are just beginning to examine the genetics of hybridization within the *Callithrix* genus, and our study only examined one locus in two hybrid zones. *Callithrix* hybridization is a geographically widespread phenomenon, which occurs across the entire genus. Thus, to better understand the evolutionary consequences of hybridization on the *Callithrix* genus, it is important to consider marmoset hybridization within the context of other genetic loci, at other geographical locations, and between various species. Additionally, studies are needed to understand the effects of hybridization on marmoset fitness and adaptation.

ACKNOWLEDGMENTS

Thank you to two anonymous reviewers, Leanne Nash, Phil Hedrick, and Michael Rosenberg for comments on earlier drafts of this work. Adélia Borstelmann de Oliveira gave immense logistical support in obtaining samples. Hermano Gomes, Roberta Santos, Edilberto Martinez, Andreia Martins, and the AMLD META Team aided with fieldwork. Kaitlyn Buss and Danielle Johnson aided in molecular work. We thank Luiz Paulo Ferraz, and the AMLD and Rebio Poço das Antas staffs for additional support. We are also indebted to the Brasília Zoo

and Brasília Botanical Gardens, IBAMA CETAS, and private farms for access to their premises.

LITERATURE CITED

Allendorf F, Leary R, Spruell P, Wenburg J. 2001. The problems with hybrids: setting conservation guidelines. Trends Ecol Evol 16:613–622.

Alonso C, de Faria DS, Langguth A, Santee DF. 1987. Variação da pelagem na área de intergradação entre *Callithrix jacchus* e *Callithrix penicillata*. Rev Bras Biol 47:465–470.

Arnold ML. 1997. Natural hybridization and evolution. NY: Oxford University Press.

Arnold ML, Meyer A. 2006. Natural hybridization in primates: one evolutionary mechanism. Zoology 109:261–276.

Baele G, Lemey P, Bedford T, Rambaut A, Suchard MA, Alekseyenko AV. 2012. Improving the accuracy of demographic and molecular clock model comparison while accommodating phylogenetic uncertainty. Mol Biol Evol 29:2157–2167.

Baele G, Li WLS, Drummond AJ, Suchard MA, Lemey P. 2013. Accurate model selection of relaxed molecular clocks in Bayesian phylogenetics. Mol Biol Evol 30:239–243.

Ballard J, Whitlock M. 2004. The incomplete natural history of mitochondria. Mol Ecol 13:729–744.

Bandelt HJ, Forster P, Rohl A. 1999. Median-joining networks for inferring intraspecific phylogenies. Mol Biol Evol 16:37–48.

Bonhomme M, Cuartero S, Blancher A, Crouau-Roy B. 2009. Assessing natural introgression in 2 biomedical model species, the rhesus macaque (*Macaca mulatta*) and the long-tailed macaque (*Macaca fascicularis*). J Hered 100:158–169.

Brown WM, George M Jr, Wilson AC. 1979. Rapid evolution of animal mitochondrial DNA. Proc Natl Acad Sci USA 76:1967–1971.

Carnaval AC, Moritz C. 2008. Historical climate modeling predicts patterns of current biodiversity in the Brazilian Atlantic Forest. J Biogeogr 35:1187–1201.

Coimbra-Filho AF, Mittermeier RA. 1973. New data on the taxonomy of the Brazilian marmosets of the genus *Callithrix* Erxleben, 1777. Folia Primatol 20:241–264.

Coimbra-Filho AF, Mittermeier RA, Rylands AB, Mendes SL, Kierulff MCM, de S. Pinto LP. 2006. The taxonomic status of Wied's black-tufted-ear marmoset, *Callithrix kuhlii* (Callitrichidae, primates). Primate Conserv 21:1–24.

Coimbra-Filho AF, Pissinatti A, Rylands AB. 1993. Experimental multiple hybridism and natural hybrids among *Callithrix* species from eastern Brazil.In: Rylands AB, editor. Marmosets and tamarins: systematics, behaviour, and ecology. NY: Oxford University Press. p 95–120.

Darriba D, Taboada GL, Doallo R, Posada D. 2012. jModelTest 2: more models, new heuristics and parallel computing. Nat Methods 9:772.

de Morais Jr, MM. 2010. Os saguis (*Callithrix* spp., Erxleben, 1777) exóticos invasores na bacia do Rio São João, Rio de Janeiro: biologia populacional e padrão de distribuição em uma paisagem fragmentada [dissertation]. Campos, Rio de Janeiro, Brazil: Universidade Estadual do Norte Fluminense.

Detwiler KM, Burrell AS, Jolly CJ. 2005. Conservation implications in hybridization in African cercopithecine monkeys. Int J Primatol 26:661–684.

Digby LJ, Ferrari SF, Saltzman W. 2007. Callitrichines: the role of competition in cooperatively breeding species.In: Campbell CJ, Fuentes A, MacKinnon KC, et al., editors. Primates in perspective. NY: Oxford University Press. p 85–106.

Drummond AJ, Rambaut A, Shapiro B, Pybus OG. 2005. Bayesian coalescent inference of past population dynamics from molecular sequences. Mol Biol Evol 22:1185–1192.

Drummond AJ, MA, Xie D, Rambaut A. 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. Mol Biol Evol 29: 1969–1973.

Edgar RC. 2004. Muscle: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32: 1792–1797.

Ekman S, Blaalid R. 2011. The devil in the details: interactions between the branch-length prior and likelihood model affect node support and branch lengths in the phylogeny of the Psoraceae. Syst Biol 60:541–561.

Excoffier L, Lischer HEL. 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Mol Ecol Resour 10:564–567.

Faulkes CG, Arruda MF, Monteiro da Cruz MAO. 2003. Matrilineal genetic structure within and among populations of the cooperatively breeding common marmoset, *Callithrix jacchus*. Mol Ecol 12:1101–1108.

Ferrari SF. 2009. Social hierarchy and dispersal in free-ranging buffy-headed marmosets (*Callithrix flaviceps*).In: Ford SM, Porter LM, Davis LC, editors. The smallest anthropoids: the marmoset/*Callimico* radiation. New York: Springer. p 155–165.

Fu X. 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. Genetics 147:915–925.

Funk DJ, Omland KE. 2003. Species-level paraphyly and polyphyly: frequency, causes, and consequences, with insights from animal mitochondrial DNA. Annu Rev Ecol Evol Syst 34:397–423.

Goosens B, Anthony N, Jeffrey K, Johnson-Bawe M, Bruford MW. 2003. Collection, storage, and analysis of non-invasive genetic material in primate biology. In Setchell JM, Curtis DJ, editors, Field and laboratory methods in primatology. NY: Cambridge University Press. p 295–308.

Galtier N, Jobson RW, Nabholz B, Glemin S, Blier PU. 2009. Mitochondrial whims: metabolic rate, longevity, and the rate of molecular evolution. Biol Lett. 10.1098/rsbl.2008.0662.

Guindon S, Gascuel O. 2003. A simple, fast and accurate method to estimate large phylogenies by maximum-likelihood. Syst Biol 52:696–704.

Hershkovitz P. 1975. Taxonomy of Brazilian marmosets (*Callithrix*, Callitrichidae)—comments. Folia Primatol 24:137–172.

Hershkovitz P. 1977. Living new world monkeys (Platyrrhini): with an introduction to primates. Chicago: University of Chicago Press.

Huelsenbeck JP, Ronquist F. 2001. MRBAYES: Bayesian inference of phylogeny. Bioinformatics 17:754–755.

Kinzey WG. 1982. Distribution of primates and forest refuges.In: Prance GT, editor. Biological diversification in the tropics. NY: Columbia University Press. p 455–482.

Kelaita MA, Cortes-Ortiz L. 2013. Morphological variation of genetically confirmed *Alouatta pigra* × *A. palliata* hybrids from a natural hybrid zone in Tabasco, Mexico. Am J Phys Anthrop 150:223–234.

Kocher TD, Thomas WK, Meyer A, Edwards SV, Paabo S, Villablanca FX, Wilson AC. 1989. Dynamics of mitochondrial DNA evolution in animals—amplification and sequencing with conserved primers. Proc Natl Acad Sci USA 86:6196–6200.

Lazaro-Perea C, Castro CSS, Harrison R, Araujo A, Arruda MF, Snowdon CT. 2000. Behavioral and demographic changes following the loss of the breeding female in cooperatively breeding marmosets. Behav Ecol Sociobiol 48: 137–146.

Leal IR, Da Silva JMC, Tabarelli M, Lacher TE. 2005. Changing the course of biodiversity conservation in the Caatinga of northeastern Brazil. Conserv Biol 19:701–706.

Librado P, Rozas J. 2009. DNASP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25: 1451–1452.

Mallet J. 2005. Hybridization as an invasion of the genome. Trends Evol Ecol 20:229–237.

Mendes SL. 1997. Hybridization in free-ranging *Callithrix flaviceps* and the taxonomy of the Atlantic Forest marmosets. Neotrop Primates 5:6–8.

Mundy NI, Pissinatti A, Woodruff DS. 2000. Multiple nuclear insertions of mitochondrial cytochrome b sequences in callitrichine primates. Mol Biol Evol 17:1075–1080.

Passamani M, Agular L, Machado RB, Figueirdo E. 1997. Hybridization between *Callithrix geoffroyi* and *C. penicillata* in southeastern Minas Gerais, Brazil. Neotrop Primates 5:9–10.

Perelman P, Johnson WE, Roos C, Seuanez HN, Horvath JE, Moreira MAM, Kessing B, Pontius J, Roelke M, Rumpler Y, Schneider MP, Silva A, O'Brien SJ, Pecon-Slattery J. 2011. A molecular phylogeny of living primates. PLoS Genet 7: e1001342.

Phillips-Conroy JE, Jolly CJ. 1981. Sexual dimorphism in two subspecies of Ethiopian baboons (*Papio hamadryas*) and their hybrids. Am J Phys Anthrop 56:115–129.

Posada D, Buckley TR. 2004. Model selection and model averaging in phylogenetics: advantages of Akaike information criterion and Bayesian approaches over likelihood ratio tests. Syst Biol 53:793–808.

Posada D, Crandall KA. 2001. Intraspecific gene genealogies: trees grafting into networks. Trends Evol Ecol 16:37–45.

Reed DH, Frankham R. 2002. Correlation between fitness and genetic diversity. Conserv Biol 17: 230–237.

Ribeiro MC, Metzger JP, Martensen AC, Ponzoni FJ, Hirota MM. 2009. The Brazilian Atlantic forest: how much is left, and how is the remaining forest distributed? Implications for conservation. Biol Conserv 142:1141–1153.

Rogers AR, Harpending H. 1992. Population-growth makes waves in the distribution of pairwise genetic-differences. Mol Biol Evol 9:552–569.

Ronquist F, Huelsenbeck JP. 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574.

Ruiz-Miranda CR, Affonso AG, Martins A, Beck B. 2000. Distribuição do sagui (*Callithrix jacchus*) nas areas de ocorrência do mico-leao-dourado (*Leontopithecus rosalia*) no estado do Rio de Janeiro. Neotrop Primates 8:98–100.

Ruiz-Miranda CR, Affonso AG, de Morais MM, Verona CE, Martins A, Beck BB. 2006. Behavioral and ecological interactions between reintroduced golden lion tamarins (*Leontopithecus rosalia linnaeus*, 1766) and introduced marmosets (*Callithrix* spp, linnaeus, 1758) in Brazil's Atlantic coast forest fragments. Braz Arch Biol Technol 49:99–109.

Rylands AB, Coimbra-Filho AF, Mittermeier RA. 1993. Systematics, geographic distribution, and some notes on the conservation status of the Callithrichidae.In: Rylands AB, editor. Marmosets and tamarins: systematics, behaviour, and ecology. New York: Oxford University Press. p 9–77.

Rylands AB, de Faria DS. 1993. Habitats, feeding ecology, and home range size in the genus *Callithrix*. In: Rylands AB, editor. Marmosets and tamarins: systematics, behaviour, and ecology. New York: Oxford University Press. p 262–272.

Rylands AB, Mittermeier RA. 2009. The diversity of the New World primates (Platyrrhini): an annotated taxonomy.In: Garber PA, Estrada A, Bicca-Marques JC, et al., editors. South American primates: comparative perspectives in the study of behavior, ecology, and conservation. New York: Springer. p 23–54.

Rylands AB, Coimbra-Filho, AF, Mittermeier, RA. 2009. The Systematics and Distributions of the Marmosets (*Callithrix, Callibella, Cebuella*, and *Mico)* and *Callimico* (*Callimico*) (Callitrichidae, Primates).In: Ford SM, Porter LM, Davis LC, editors. The smallest anthropoids: the marmoset/*Callimico* radiation. New York: Springer. p 25–61.

Sambrook J, Russel DW. 2001. Molecular cloning, 3rd ed. Cold Spring Harbor: CSHL Press.

Schneider H, Bernardi JAR, da Cunha DB, Tagliaro CH, Vallinoto M, Ferrari SF, Sampaio I. 2012. A molecular analysis of the evolutionary relationships in the Callitrichinae, with emphasis on the position of the dwarf marmoset. Zool Scr 41:1–10.

Sherwin WB, Moritz C. 2000. Managing and monitoring genetic erosion.In: Young AG, Clarke GM, editors. Genetics, demography, and viability of fragmented populations. Cambridge: Cambridge University Press. p 9–34.

Shurtliff QR. 2013. Mammalian hybrid zones: a review. Mamm Rev 43:1–21.

Smith TE, Schaffner, CM, French, JA. 1997. Social and developmental influences on reproduction in female Wied's black tufted-ear marmosets (*Callithrix kuhli)*. Horm Behav 31:159–168.

Soares P, Ermini L, Thomson N, Mormina M, Rito T, Roehl A, Salas A, Oppenheimer S, Macaulay V, Richards MB. 2009. Correcting for purifying selection: an improved human mitochondrial molecular clock. Am J Hum Genet 84:740–759.

Sousa MBC, Albuquerque ACSR, Yamamoto ME, Araujo A, Arruda MF. 2009. Emigration as a reproductive strategy of the common marmoset (*Callithrix jacchus*). In: Ford SM, Porter LM, Davis LC, editors. The smallest anthropoids: the marmoset/*Callimico* radiation. New York: Springer. p 167–182.

Tagliaro CH, Schneider MPC, Schneider H, Sampaio IC, Stanhope MJ. 1997. Marmoset phylogenetics, conservation perspectives, and evolution of the mtDNA control region. Mol Biol Evol 14:674–684.

Tagliaro CH, Schneider MPC, Schneider H, Sampaio I, Stanhope M. 2000. Molecular studies of *Callithrix pygmaea* (primates, Platyrrhini) based on transferrin intronic and ND1 regions: implications for taxonomy and conservation. Genet Mol Biol 23:729–737.

Tajima F. 1989. Statistical-method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123:585–595.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739.

Tardif SD, Smucny DA, Abbott DH, Mansfield K, Schultz-Darken N, Yamamoto ME. 2003. Reproduction in captive common marmosets (*Callithrix jacchus*). Comp Med 53:364–368

Tosi AJ, Morales JC, Melnick DJ. 2003. Paternal, maternal, and biparental molecular markers provide unique windows onto the evolutionary history of macaque monkeys. Evolution 57:1419–1435.

Turchetto-Zolet AC, Pinheiro F, Salgueiro F, Palma-Silva C. 2013. Phylogeographical patterns shed light on evolutionary process in South America. Mol Ecol 22:1193–1213.

Yang M, Yang Y, Cui D, Fickenscher G, Zinner D, Roos C, Brameier M. 2012. Population genetic structure of Guizhou snub-nosed monkeys (*Rhinopithecus brelichi*) as inferred from mitochondrial control region sequences, and comparison with *R. roxellana* and *R. bieti*. Am J Phys Anthrop 147:1–10.