Hybridization and Speciation in Common and Black-Tufted Marmosets

(*Callithrix jacchus* and *C. penicillata*)

by

Joanna Malukiewicz

A Dissertation Presented in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

Approved November 2013 by the
Graduate Supervisory Committee:

Anne Stone, Chair
Leanne Nash
Carlos Ruiz-Miranda
Philip Hedrick
Michael Rosenberg

ARIZONA STATE UNIVERSITY

December 2013

ABSTRACT

As an evolutionary force, hybridization outcomes include introgression, admixture, speciation, and reproductive isolation. While hybridization has been studied in several primates, the marmoset genus *Callithrix* is an important, but little studied example of Neotropical hybridization. Varying degrees of reproductive isolation exist between *Callithrix* species, and hybridization occurs at species borders or regions containing introduced and native species. Interbreeding between *Callithrix* species carries important implications for biodiversity and genetic integrity within the genus. However, species origins and levels of genetic admixture in marmoset hybrid zones are generally unknown, and few population genetic studies of individual *Callithrix* species exist.

Using the mitochondrial control region and 44 microsatellite markers, this work explored the genetic diversity and species origins of two *C. penicillata* and *C. jacchus* hybrid zones, as well as genetic diversity and divergence in the parental species. Both marker types showed that *C. penicillata* is more genetically diverse than *C. jacchus.* Based on mtDNA, *C. jacchus* seems to have experienced a past population expansion and *C. penicillata* evolved under constant population size. The data revealed the existence of a previously undocumented natural hybrid zone along the São Francisco River in NE Brazil and confirmed species origins of an anthropogenic zone in Rio de Janeiro state. The data also showed much lower levels of admixture and genetic diversity within the natural hybrid zone than in the anthropogenic zone. Further, the data suggested that the São Francisco River is an important geographic barrier to gene flow in the natural hybrid zone. On the other hand, admixture patterns within the anthropogenic hybrid zone suggested collapse of reproductive barriers, and the formation of a hybrid marmoset

swarm. Thus, this work suggested different evolutionary dynamics in anthropogenic vs. natural animal hybrid zones.

Restriction Associated DNA sequencing (RADseq) identified a large number of single nucleotide polymorphisms within *C. jacchus* and *C. penicillata* genomes. These preliminary data were used to measure intraspecific genomic diversity and interspecific divergence. In the future, RADseq will be used to study genus-wide diversity of *Callithrix* species, examine past and present marmoset demographic history, and applied to the evolutionary study of marmoset hybridization.

DEDICATION

I dedicate my doctoral work to my mother Boguslawa Malukiewicz for sparking the fire within and to my husband Michael James Walter for helping to keep the fire lit. Thank you for believing in me through all the twists and turns of this journey. I love you both.

ACKNOWLEDGMENTS

I give many thanks to my doctoral advisor Dr. Anne Stone for her guidance and amazing support though my graduate journey. Thank you for taking me under your wing, giving me your confidence, giving me the freedom to develop my research, allowing me to make my own mistakes, and helping me learn from them. Your door was always open (except for Starbucks trips!) to help me with bumps along the way. You have been and continue to be an amazing role model, I could not have asked for a better advisor, and I am truly indebted to you for what I have been able to accomplish.

This project would not have been possible without the guidance and support of my Brazilian academic advisor Dr. Carlos Ruiz-Miranda. Your work planted the seed for my own research. You provided all the logistical support I needed with collection permits, at the field sites, and helped me find a home in Brazil.

To my project collaborator (and unofficial Brazilian academic advisor) Dr. Adriana Grativol, you helped me find a genetics home in Brazil and helped me in numerous professional and personal ways. Thank you for helping me find the space and equipment I needed for the molecular components of this research, and for helping me navigate the maze of international export/import permits. Your door was always open to me and helped me through so many bumps in the road in trying to adjust to life and research as a geneticist in Brazil.

To my committee member Dr. Leanne Nash, thank you for constantly reminding me to keep the broader perspective in mind and for all the great research suggestions and questions. All our discussions have helped me grow as a primatologist, researcher, and person. Thank you for sharing so much of your experience and knowledge with me.

To my committee members Drs. Michael Rosenberg and Phil Hedrick, thank your for all feedback, advice, and suggestions for the development of this project. You always pointed out when I was missing the obvious, and helped me fill open holes in my research design, which helped to avert disaster in many cases.

Drs. Vanner Boere, Ita de Oliveira e Silva, Adélia Borstelmann de Oliveira, and Luiz Pereira Machado gave immense support regarding permits, transportation, captures, and other logistics. Thank you to Dr. Jeffrey French and the New England Regional Primate Center in providing valuable captive *Callithrix* samples in support of this work. Thank you to Andreia Martins, Luiz Paulo Ferraz, and other associates of the Golden Lion Tamarin Association for assistance in Rio de Janeiro state. Thank you to Lisieux Fuzessy, Edilberto Martinez, and Yuri Marinho for help in field collections. I am especially indebted to Kaity Buss and Dani Johnson for help with processing of laboratory samples. Hermano Gomes-Nunes thank you for your logistical help in the field and your friendship. Being “stuck” in the field with you was one of the highlights of my doctoral field work. Thank you to Andreia Magro Moraes for your help in getting settled in Brazil, for all the kindness you have shown me and for being a great friend to me. Thank you to Scott Bingham for the great service at the SOLS DNA Laboratory. Ramona Flatz’s suggestions on microsatellites have been indispensible for that component of my doctoral research. Thank you to all those that helped with my work but I do not have room to mention. The National Science Foundation Anthropological DDIG, Fulbright Fellowship to Brazil, SOLS FIGG grant, ASU GPSA Jump Start and Research Grants, ASU Chapter of Sigma Xi, and an International Primate Society Research Grant provided financial support for this work.

TABLE OF CONTENTS

Page

APPENDIX

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Hybrid zones are heralded as “natural evolutionary laboratories” and marmosets are distinctive primates offering unprecedented opportunities to study the evolutionary effects of natural and anthropogenic hybridization (mating between members of different populations). *Callithrix* marmosets are a recently diverged genus endemic to the degraded biomes of central and eastern Brazil (Ryland et al., 1993, 2009). While the six *Callithrix* species are historically allopatric (Ryland et al., 1993, 2009), common and black-tufted marmosets have been introduced to areas outside of their borders through Brazil’s illegal pet trade (e.g. Ruiz-Miranda et al., 2006). As a result, introduced common and black-tufted marmosets are now found in artificial sympatry with each other and other congeners. While the entire genus is specialized for gummivory, these two species possess the most extreme exudativore adaptations within *Callithrix*, which allows them to exploit disturbed habitats (Ferrari, 1993). Thus, the two species may be a demographic threat to congeners as they are better adapted to access nutritional resources within Brazil’s disturbed biomes. These species may also be a genetic threat as genus-wide hybridization occurs regularly between native and introduced marmosets. Besides anthropogenic hybridization, natural hybridization occurs between marmoset species at geographical contact zones (Rylands et al., 1988; Mendes, 1997a&b; Passamani et al., 1997). Thus, we use *Callithrix* as a model to address the following question: **What is the role of hybridization and genetic introgression in shaping animal evolutionary population histories?**

Marmosets are compelling models for the study of hybrid evolution because phylogenetic, phenotypic, and behavioral evidence suggests an important role for hybridization throughout *Callithrix* population history. Based on the maximum likelihood phylogeny of the *Callithrix* mtDNA control region produced by Tagliaro et al. (1997), Arnold and Meyer (2006) hypothesize that black-tufted and common marmosets are the parental species of the hybrid *C. kuhlii* lineage. C. *kuhlii* haplotypes are paraphyletic in the Tagliaro et al. (1997) phylogeny between the two purported parental species. Haplotypes from these three species also tend to cluster together in other autosomal and mtDNA phylogenies; branches leading to these haplotypes tend to be short, not fully resolved, nor strongly supported (Canavez et al., 1999; Tagliaro et al., 2000). Interspecific crosses in captivity yield fertile hybrids with ancestry from two to three *Callithrix* species (Coimbra-Filho, 1970; Coimbra- Filho et al., 1993; Coimbra-Filho and Mittermeir, 1973) suggesting incomplete reproductive isolation within the genus. This observation along with *Callithrix* phylogenetic patterns tells of a relatively young age for this genus (e.g. Perelmen et al., 2011), and one that may be exhibiting the effects of hybridization evolution as described by Mallet (2005). An alternative explanation for the phylogenetic signals observed between *Callithrix* species is incomplete lineage sorting where certain ancestral polymorphisms fail to sort prior to divergence (Zinner et al., 2009). Further studies are needed to clarify whether hybridization, incomplete lineage sorting, or both have most strongly shaped marmoset population histories.

Hybridization is also an important force in shaping recent population histories of marmosets. A hybrid zone has been observed in the eastern part of the state of Bahia close to the city of Salvador in an area of black-tufted/common marmoset range overlap (Alonso et al., 1987). Additionally, common marmosets are expanding from their range along the São Francisco River's northern banks in northern Bahia into the range of black-tufted marmosets south of the river (Ryland et al., 1993). Hybridization is likely to be occurring in this region (personal communication, Dr. Borstelmann de Oliveira). A human-induced hybrid zone exists in the state of Rio de Janeiro where illegal human transport established exotic (i.e., non-native) populations of black-tufted and common marmosets (Ruiz-Miranda et al., 2006). Rio de Janeiro is hypothesized to be a hybrid zone between common and black-tufted marmosets due to the presence of individuals possessing phenotypes intermediate between the two parental phenotypes (Ruiz-Miranda et al., 2006). The hypotheses of marmoset hybridization in Rio de Janeiro and northern Bahia have not been tested genetically, and thus, molecular data from hybridizing marmosets within these areas will offer a glimpse into both modern and human-induced hybridization events.

CHAPTER 1

HYBRIDIZATION EFFECTS AND GENETIC DIVERSITY OF THE COMMON AND BLACK-TUFTED MARMOSET (*CALLITHRIX JACCHUS* AND *C. PENICILLATA*) MITOCHONDRIAL CONTROL REGION

Hybrid zones offer many opportunities to examine important evolutionary processes such as speciation, adaptation, and genetic introgression (Hewitt, 1988; Shurtliff, 2011), and here we define hybridization as successful mating between members of populations possessing distinct heritable traits (modified from Arnold, 1997). This phenomenon has been documented in several primate taxa, including but not limited to: (1) baboons (e.g., Nagel, 1973; Samuels and Altmann, 1986; Phillips-Conroy and Jolly, 1981; Zinner et al., 2009; Keller et al., 2010); (2) macaques (e.g., Bynum et al., 1997; Kanthaswamy et al., 2008; Stevison and Kohn, 2009); and (3) howler monkeys (e.g., Aguiar et al., 2007; Cortes-Ortiz et al., 2007; Bicca-Marques et al., 2008; Kelaita and Cortes-Ortiz, 2013). While hybridization in wild primates is shaped by both anthropogenic and natural elements (e.g., Phillips-Conroy et al., 1992; Detwiler et al., 2005; Bonhomme et al., 2009), the role of each factor in driving hybridization in primates and other animals is still open to debate (Mallet, 2005). Further, these two types of hybridization may have different effects on biodiversity and may be difficult to differentiate from one another (Allendorf et al., 2001), which carries important conservation implications for primate taxa. Thus, there is a need to understand the frequency and signatures of hybridization under conditions that differentiate between anthropogenic and natural contexts as clearly as possible within the framework of primate evolution.

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 the Callitrichidae family (Rylands et al., 2000; Rylands and Mittermeier, 2009; Rylands et al, 2009), known to possess rare primate characteristics including cooperative breeding and twinning (Digby et al., 1997), as well as social modulation of female reproduction (e.g., Smith et al., 1997). The six *Callithrix* species (*C. penicillata, C. jacchus, C. aurita, C. flaviceps, C. geoffroyi* and *C. kuhlii*) have distinct geographic distributions throughout central-eastern Brazil and along the Brazilian coast (Rylands et al., 1993 and 2009). 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 *C. penicillata* and *C. jacchus* have occurred extensively within the state of Minas Gerais (personal observation, Ita de Oliveira e Silva and Vanner Boere). In Rio de Janeiro state, exotic *C. jacchus* and *C. penicillata* exist in the city proper (Hershkovitz, 1975; Rylands et al., 1993), as well as in coastal regions east of the city (Affonso et al., 2000; Ruiz-Miranda et al., 2006), where exotic marmosets were probably introduced in the mid-1980s as illegal pets (Ruiz-Miranda et al., 2000; Affonso et al., 2004; Ruiz-Miranda et al., 2006). *Callithrix* species are characterized by unique ear tuft shape and color as well as facial mask shape and pigmentation, and admixed individuals are usually identified through intermediate phenotypic combinations of these parental traits (Hershkovitz, 1977). The observation of marmosets possessing such admixed parental species phenotypes within the above areas of human-induced marmoset sympatry suggests the occurrence of hybridization in these regions.

Besides areas of artificial sympatry, intertaxa contact also occurs at geographical distribution boundaries of different *Callithrix* species: (1) *C. jacchus* and *C. penicillata* in Petrolina, Pernambuco state (PE), and Juazeiro, Bahia state (BA), (personal observation by Joanna Malukiewicz); (2) *C. penicillata* and *C. aurita* at Rio Doce State Park, Minas Gerais state (MG) and Almenara, MG (Rylands et al., 1993); (3) *C. penicillata* and *C. geoffroyi*, at Santa Barbara, MG (Rylands et al., 1993) and Antonio Dias, MG (Passamani et al., 1997); and (4) *C. geoffroyi* and *C. flaviceps* in the state of Espírito Santo (Mendes, 1997a; Rylands et al., 1993). 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 did not come until the late 1980s when Alonso et al. (1987) examined a *C. jacchus* x *C. penicillata* hybrid zone outside of Salvador, BA. Since the Alonso et al. (1987) study, the number of hybridization reports between various *Callithrix* species has been steadily increasing (Rylands et al., 1988; Mendes, 1997a&b; Passamani et al., 1997; Ruiz-Miranda et al., 2000; Affonso et al., 2004; Ruiz-Miranda et al., 2006; Pereira, 2010; Nogueira et al., 2011; personal observation, Joanna Malukiewicz).

Hybridization is thought to occur in about 10% of mammalian species, usually among the youngest ones that are between 1 and 2 million years old (Mallet, 2005). The evolutionary effects of hybridization vary from population to population, and outcomes include speciation, hybrid swarming, neutral introgression, and adaptation (Seehausen, 2004; Mallet, 2005; Shurtliff, 2011). *Callithrix* is thought to have arisen 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 suggests a potential role for hybridization in their recent evolutionary history.

However, few studies about the evolutionary biology and population genetics of the *Callithrix* genus are available. Many of these studies have utilized mitochondrial DNA (mtDNA) as a rapidly evolving marker employed in studying evolution of recently diverged taxa (Brown et al., 1979). Faulkes et al. (2003) conducted one of the few population genetics studies of *C. jacchus*, where the authors reported highly significant genetic structuring among studied social groups and populations at the mitochondrial control region (mtDNA CR). Work considering the evolutionary history of *Callithrix* has examined the phylogenetics of the mtDNA CR as well as a few nuclear loci (e.g.: Canavez et al., 1999; Sena et al., 2002; Schneider et al., 2012; Tagliaro et al., 1997, Tagliaro et al., 2000) without full resolution of species-level evolutionary relationships. The multi-locus 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. Quantitative assessment of hybridization is important both for understanding evolutionary history and speciation, as well as for developing conservation strategies regarding hybridizing taxa.

In this study, we genetically assess the existence of a *C. jacchus* and *C. penicillata* hybrid zone at a natural species border (between the cities of Petrolina, PE and Juazeiro, BA) and in an area of artificial introduction (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 x 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 collection site for each individual are given in Table S1. For both captive and wild pure individuals, total samples obtained were: 81 *C. jacchus*, 45 *C. penicillata,* four *C. kuhlii,* and eight *C. geoffroyi*. An additional *C. geoffroyi* DNA sample was obtained from the Corielle Institute (Camden, NJ).

Figure 1 shows general locations of hybrid and non-hybrid zones 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

Table 1.

Summary of Sampled Individuals from Captive and Wild Pure Populations and Wild Hybrid Zones

Populations	**Type**	**Source**	**Year Collected**	**Biological Samples**[f]	**Individuals Sampled**
C. jacchus	Captive	CRC[a], Omaha, NE, US	2011	B, S, C, H	2 (2)
	Wild	IBAMA CETAS[b], Recife, PE, Brazil	2011	S, C, H	27 (20)
	Captive	NEPRC[c], Southborough, MA, US	2010	B, S	10 (10)
	Wild	Parque Dois Irmãos & Tapacurá Reserve, PE, Brazil[d]	2005	S	42 (1)
C. penicillata	Captive	CRC[a], Omaha, NE, US	2011	B, S, C, H	8 (7)
	Wild	Muriaé, MG; Brasilía, DF; Goiânia, GO, Brazil	2011	S, C, H	29 (25)
	Captive	IBAMA CETAS[b], Recife, PE, Brazil	2011	S, C, H	3 (3)
	Wild	IBAMA CETAS[b], Goiânia,GO, Brazil	2011	S, C, H	5 (5)
C .jacchus x C. penicillata hybrids	Wild	Silva Jardim and Rio Bonito Municipalities, RJ, Brazil	2011	S, C, H	46 (45)
C .jacchus x C. penicillata hybrids	Wild	Petrolina, PE and Juazeiro, BA, Brazil	2011	S, C, H	42 (41)
	Captive	CEMAFAUNA, Petrolina, PE	2011	S,C,H	3 (3)
C. kuhlii	Captive	CRC[a], Omaha, NE, US	2011	B, S, C, H	4 (3)
C. geoffroyi	Captive	CRC[a], Omaha, NE, US	2011	B, S, C, H	8 (4)

Note: Numbers in parenthesis indicate 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 Borstelmanna de Oliveira, most samples were too degraded to amplify the mtDNA control region, [e]Center for Management of Fauna of the Caatinga[f]B=blood, S=skin, C=cheek swab, H=hair sample

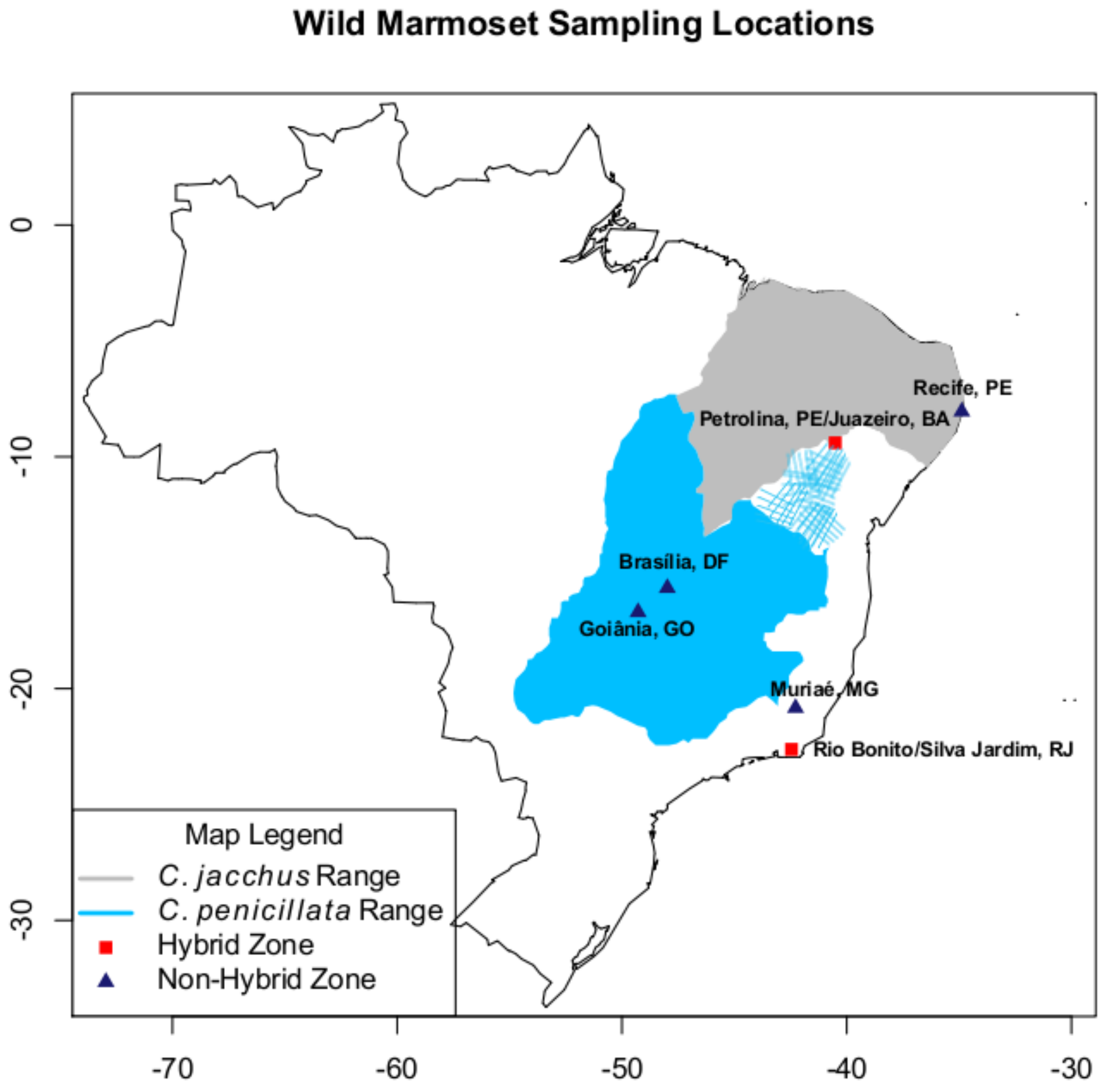

Figure 1. Overview of approximate natural ranges of *C. jacchus* and *C. penicillata* as well as the location of the study hybrid zones within Brazil.
Solid grey and blue represent *C. jacchus* and *C. penicillata* ranges, respectively, based on 2012 IUCN Red List Spatial Data (http://www.iucnredlist.org/technical-documents/spatial-data). Thatched blue indicates an extension of the *C. penicillata* range based on Rylands et al. (1993&2009). Also indicated is the sampling scheme of wild pure *C. jacchus* and *C. penicillata* and their hybrids. The x-axis represents degrees of longtitude and the y-axis represents degrees of latitude.

figure also extend 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, NE Brazil zone (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 occurs along the São Francisco River between the municipalities of Petrolina, PE and Juazeiro, BA, NE Brazil (Figs. 1 and 2), in the Caatinga biome (Leal et al., 2005). We collected samples from 42 wild caught marmosets, 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 islands continually formed and altered by the river (personal observation, Luiz Machado Pereira). 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 river.

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 parent

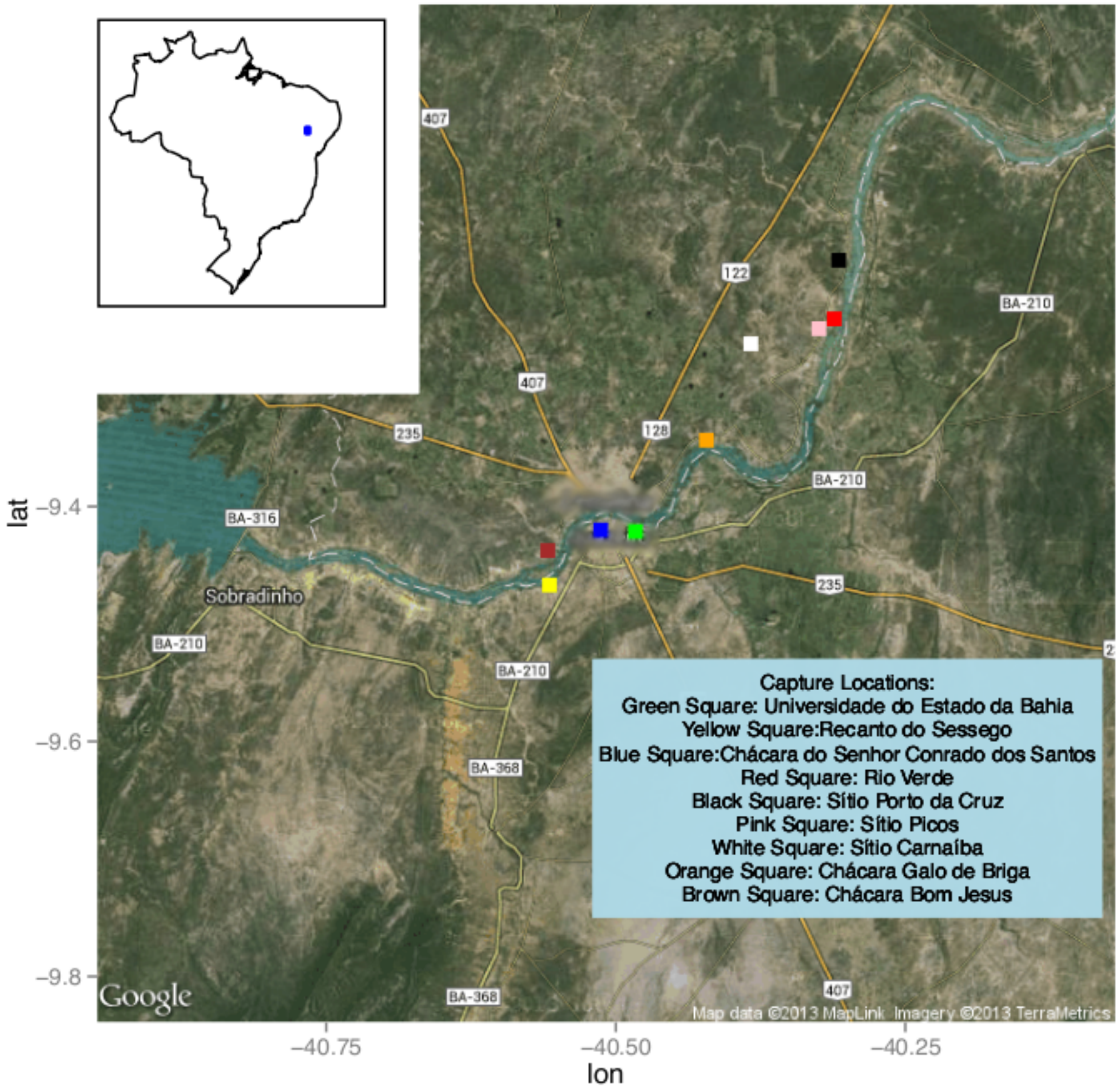

Figure 2. Close-up of the Petrolina-Juazeiro hybrid zone. The zone lies along an approximately 50 km transect along the São Francisco River. Six sites are found to the north of the river and three are found to the south of the river. The x-axis represents degrees of longtitude and the y-axis represents degrees of latitude.

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	Tufts 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% to 100% area around ear is covered by tuft hair		50% of area around ear is covered in tuft hair		Approximately 25% of area around ears covered by tuft hair
Head Coloration	Head region between ear tufts and around face is mostly grey 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

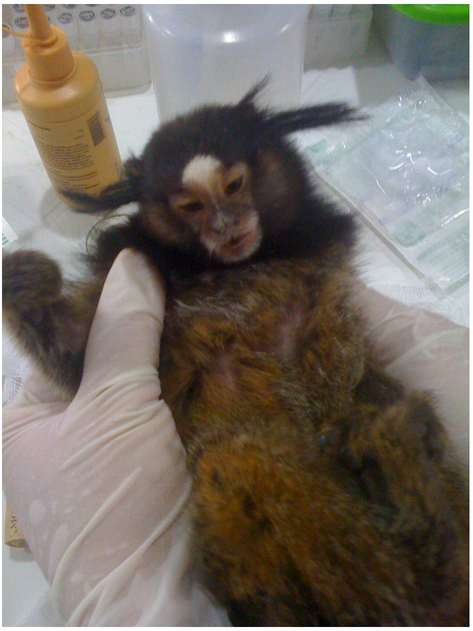

Figure 3a. *Figure 3b.*

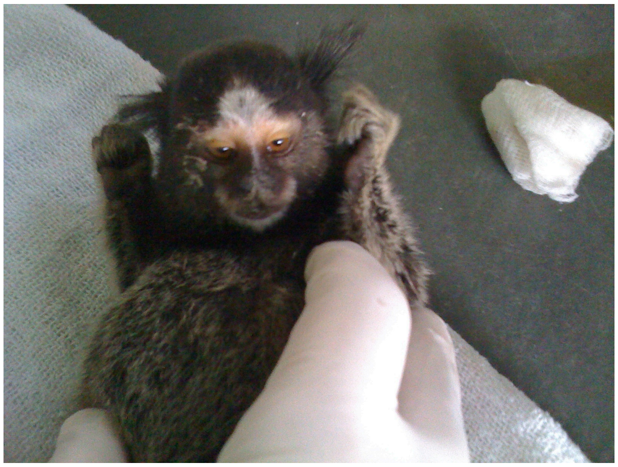

Figure 3c. *Figure 3d.*

Figure 3e.

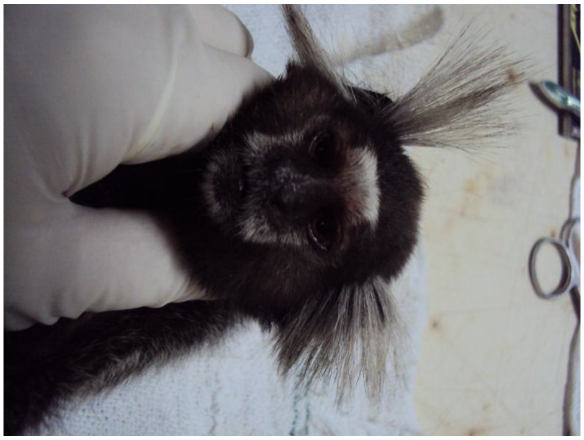

Figure 3f.

Figure 3a-e. Hybrid and pure phenotypes. 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 sparser and black ear tufts, (c) a C. *penicillata*-like hybrid that possess a mostly *C. penicillata* phenotype accented by greyish ear tufts, (d) a C. *jacchus*-like hybrid that possess a mostly *C. jacchus* phenotype accented by greyish ear tufts that also lack the full parental bushiness, and (e&f) show intermediate hybrids.

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 (described below). The scale of the hybrid index was as follows: zero to two indicates a pure *C. jacchus* phenotype, one half to two indicate 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 3 also shows examples of pure *C. jacchus,* pure *C. penicillata, C. jacchus*-like hybrid, *C. penicillata*-like hybrid, and intermediate hybrid phenotypes. Adult and non-adult animals were distinguished based on body mass following the descriptions of Hershkovitz (1979), Yamamoto (1993), and de Morais Jr (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).

Forty-six samples were collected from exotic marmosets 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 4). 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. 4). Pure and hybrid marmosets were classified as described above for the PJ hybrid zone.

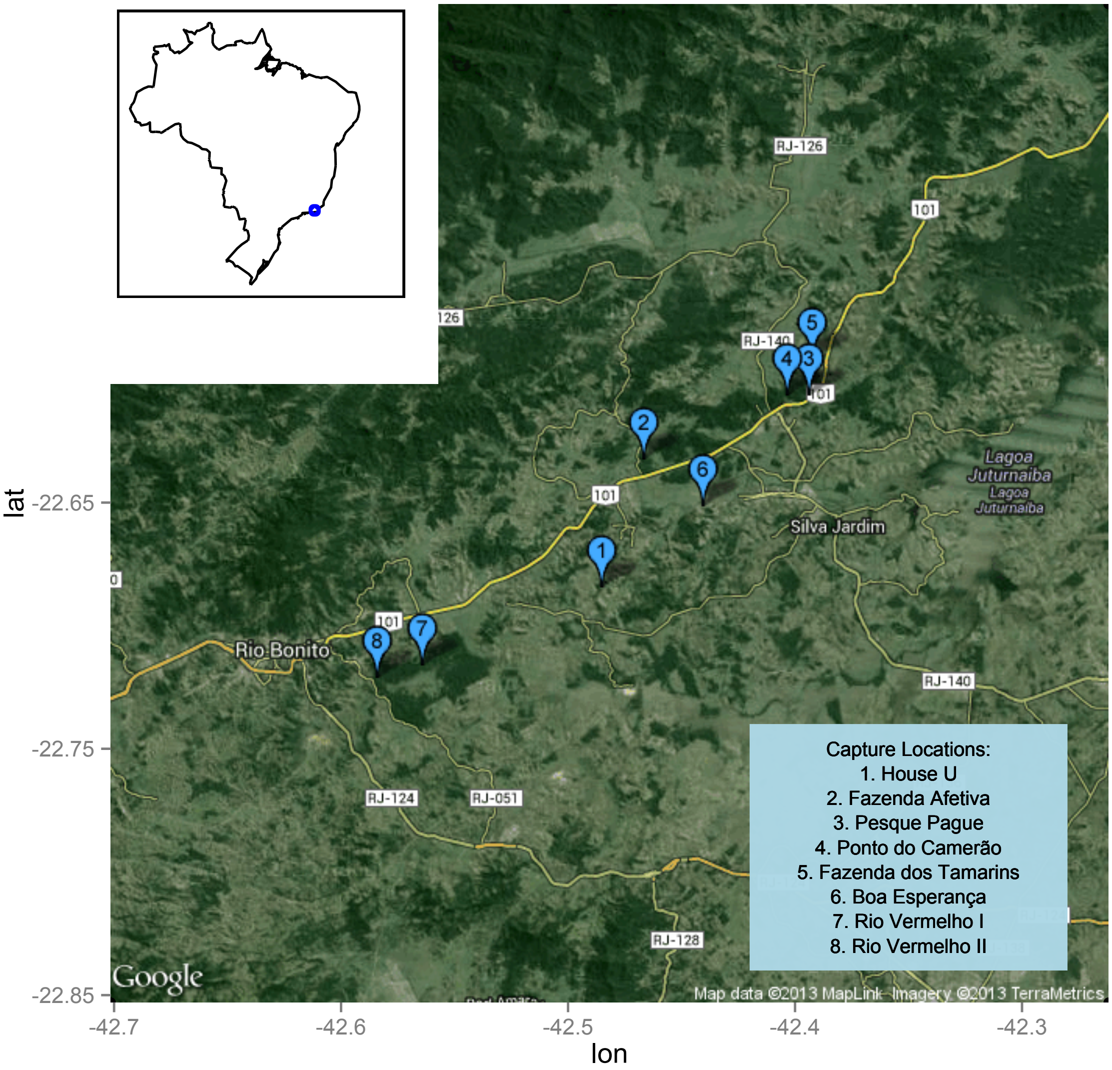

Figure 4. Close-up of Rio de Janeiro State hybrid zone. The zone lies along an approximately 30 km transect along highway BR-101. Four sites are found to the north of the highway and four are found to the south of the highway. The x-axis represents degrees of longtitude and the y-axis represents degrees of latitude.

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 and Use Committee (ASU IACUC, protocol #11-1150R, Appendix B). Captive animals were processed under protocols established at each primate center (see Table 1 and S1 for locations). Blood, cheek swabs 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 -80ºC. Buccal samples were collected with Omni swabs (Whatman), placed in cheek cell lysis buffer (50 mM Tris-HCl, pH 8.0, 50 mM EDTA, pH 8.0, 50 mM sucrose, 100 mM NaCl, 1% SDS), and refrigerated. Skin samples were obtained from the ear using a 0.5 cm skin punch and then frozen at -20ºC.

Collection of biological materials from wild marmosets was conducted following a protocol established by Vanner Boere and Carlos Ruiz-Miranda, 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 cheek swabs and skin punches. Field cheek swabs were placed into in the same cheek cell lysis buffer as used for captive samples, stored at room temperature for 1-4 weeks under field conditions, and then frozen at -20ºC in the laboratory. Skin samples were obtained from the ear using a 0.5 cm skin punch. These skin samples were stored from 1-8 weeks in 25% (w/v) DMSO dissolved in 6M NaCl (Goosens et al., 2003) under field conditions, and then frozen at -20ºC in the laboratory.

DNA from blood and epithelial samples was extracted using a standard proteinase K/phenol/choloform protocol (Sambrook and Russell, 2002). Cell lysis of cheek swab samples with proteinase K was carried out in the cheek cell lysis buffer mentioned above; for blood cells the following buffers were used: (1) 50 mM sucrose, 10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1% Triton X-100, (2) 75 mM NaCl, and 24 mM EDTA, pH 8.0. Lysis buffer for skin cells consisted of 10 mM Tris-HCl, pH 7.5, 10 mM EDTA, pH 8.0, 100 mM NaCl, and 0.5% SDS. Following phenol/cholorform extraction, DNA from all tissuse types was precipitated in ethanol and eluted in 50 uL of low TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, pH 8.0).

Initially, we attempted to amplify the hypervariable region I (HVI) of the mtDNA CR using the polymerase chain reaction (PCR) with universal mtDNA primer L15926 (Kocher et al., 1989) and a species-specific primer callithrix_HVI_R (5'-ATTCAATATCAGGCGCGATGATAG-3) designed by Joanna Malukiewicz, as well as mtDNA CR hypervariable region II (HVII) with another species-specific primer callithrix_HVII_F (5’-GTCTCTTAATCTACCAACCTCCGT G-3) designed by Joanna Malukiewicz and universal primer H00651 (Kocher et al., 1989). However, chromatograms from the sequences amplified with the above primers consistently resulted in multiple, overlapping nucleotide trace peaks, which may have indicated the amplification of nuclear inserts of mtDNA sequences (numts). Numts have been previously reported in marmosets (Moreira and Seuánez, 1999; Mundy et al., 2000).

To avoid further numt amplification, we followed the recommendations of Zhang and Hewitt (1996) and took the following steps: (1) new species-specific primers were designed by JM based on the *C. jacchus* mitochondrial genome (Genbank Accession #AB572419.1) and *Callithrix* D-loop alignment published by Tagliaro et al (1997); (2) primer pairs were tested *in silico* for unspecific replication against the marmoset nuclear genome (calJac3 build) in the USCS genome browser, and (3) since the length of most human numts is less than 500 bps (Bensasson et al., 2003), assuming that marmoset numts have similar lengths as those of humans, the marmoset CR was amplified at a length greater than 500 bp via PCR. Subsequently, most chromatograms of the amplified marmoset mtDNA CR produced single, non-overlapping nucleotide peaks and no extreme sequence variants were found.

With these precautions, AmpliTaq Gold (Life Technologies) was used to amplify 862 bp of the mtDNA control region using primer cal_D-loopR2 (5’-TGAGGTATGCGAGGAGTAAC-3’) in combination with either primer cal_dloopF1 (5’-CCCTAGTAGCTGACCTATTAAC-3’) or cal_dloopF2 (5’-GAAGTGATAGACGTCCTTGTAG-3’). PCR conditions consisted of an initial denaturation step at 95ºC for 5 min, followed by 35 cycles of denaturation at 95ºC for 45 sec, annealing at 53.9ºC or 51.4ºC for the F1/R2 pair and 54.5ºC for the F2/R2 pair for 30 sec, extension at 72ºC for 1:30 min, and a final extension of 72C at 4:30 min. Negative controls (reactions without DNA template) were included to check for contamination with extraneous DNA. PCR products were visualized on 1.5% 1X TBE agarose gel, cleaned up with shrimp alkaline phosphatase and exonuclease I, and sequenced on an ABI 3730 sequencer with the BigDye Cycle Sequencing Kit (Applied Biosystems). The above primers were also used as sequencing primers, in addition to HVIR (5’-ATTCAATATCAGGCGCGATGATAG -3’) and HVIIF (5’-GTCTCTTAATCTACCAACCTCCGTG -3’) internal primers. Trace files of resulting forward and reverse reads for each individual sequence were inspected by eye and merged into a single contig for each sampled individual using SEQMAN software from the DNAStar Lasergene Core 10 suite (DNASTAR, Madison, WI).

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 several sequences obtained from Genbank (Table 3). For phylogenetic analysis, the alignment was shortened from 862 bp to 805 bp (covering positions 86 to 880 of the original Tagliaro et al. (1997) mtDNA CR alignment) 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. For subsequent inter- and intrataxa analyses, subsets of this larger alignment were used, and the most appropriate nucleotide

Table 3.

Mico and *Callithrix* Genbank Sequences Used in this Study

Species	**Sequence Ids and Genbank Accession Numbers**
*Mico argentatus**	CAU120:U89005.1[1]
C. geoffroyi	CGE081: U88993.1,CGE083:U88994.1[1], CGE085: U88995.1[1], CGE087:U88996.1[1]
C. jacchus	CJAC:AB572419.1[3], NIS13:AY196762.1[2], NIS14:AY196763.1[2], NIS15:AY196764.1[2], NIS16:AY196765.1[2], NIS17:AY196757.1[2], NIS18:AY196756.1[2], NIS21:AY196755.1[2], NIS22:AY196758.1[2], NIS23:AY196759.1[2], NIS24:AY196760.1[2], NIS25:AY196761[2], REC19:AY196766.1[2], REC20: AY196767.1[2], TAP04:AY196771.1[2], TAP06: AY196772.1[2], TAP07: AY196775.1[2], TAP08: AY196774.1[2], TAP09: AY196773.1[2], TAP10:AY196768.1[2],
C. kuhlii	CKU122:U88991.1[1], CKU123:U88992.11[1], CKU094:U88841.1[1], CKU095:U88842.1[1], CKU096 :U88843.1[1]
C. penicillata	CPE089: JN541397.1[1]

[1] Published by Tagliaro et al. (1997)

[2] Published by Faulkes et al. (2003)

[3] Sequence published only in Genbank, Sequence ID given by current authors

**Mico argentatus* is referred to as *Callithrix argentatus* in Tagliaro et al. (1993), but was placed into another genus by Rylands et al. (2000). In the present study it is used as a phylogenetic outgroup.

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 5000 pseudoreplicate bootstrap runs to assess branch support. Under Bayesian phylogenetic interference, 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, as described below. MRBAYES 3.2 (Huelsbeck and Ronquist, 2001; Ronquist and Huelsbeck, 2003) was used for Bayesian tree construction using a HKY+I+G model, by carrying out six independent repetitions each set to a haploid ploidy level, running for 100,000,000 generations, with sampling from the posterior distribution every 500 generations. Of the six independent runs, two each were run according to the following branch length priors: (1) one pair with an exponential prior with a mean of 0.01, (2) another with an exponential prior with a mean of 0.1 (the default), (3) and the last with an exponential prior with a mean of 1. For each Bayesian run, four Metropolis coupled Markov Chain Monte Carlo chains were used at the default temperature setting. Runs were checked for convergence in TRACER 1.5 (Rambaut and Drummond, 2007) by discarding 10% of the initial sampled generations from each run. Convergence of each run was considered most likely when the standard deviation of split frequencies was below 0.01, no obvious increasing or decreasing trends appeared in the run convergence plots, the potential scale reduction factor was at 1.0, and the effective sample size values were above 100. A 50% majority consensus tree was constructed after a relative burn-in of 25% from each independent run and rooted with the *M. argentatus* sequence, and trees were visually compared among and between all three pairs of runs.

MRBAYES 3.2 was used to calculate harmonic mean (HM) and stepping-stone (SS) sampling log marginal likelihood estimates (6,000,000 generations and 200 steps between each generation, convergence was checked in TRACER) for model selection of the above Bayesian runs. The harmonic mean estimator can produce overestimated, biased values of the marginal likelihood; therefore other estimation methods such as SS as well as path sampling (PS- which MRBAYES does not calculate) are preferred to HM (Baele et al, 2012). HM and SS log marginal likelihood estimates were averaged for each pair of runs corresponding to the same branch length prior setting. Averaged marginal log likelihood estimates were then compared using Bayes Factors to select the best branch length prior model, using the criteria set by Baele et al. (2012) and Kass and Raftery (1995). Tree topology and posterior probability branch support were checked visually with FIGTREE 1.4 (http://tree.bio.ed.ac.uk/software/figtree/), and trees were annotated with the same program.

Finally, a network was constructed from sequences sampled from pure *C. jacchus* and *C. penicillata* individuals and *C. jacchus x 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 4.610 (Bandelt et al., 1999) software was used to create a median joining network, using the following settings: elipson 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.

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 ($\mathbf{\Phi}_{\mathrm{ST}}$) 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 record is scant (Schneider et al., 2012), fossil record 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). 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. The *C. jacchus* BSPs were run with the HKY+I model and *C. penicillata* BSPs were run with the HKY+I+G, both under a strict clock. Coalescent events were summarized into 10 groups (default setting). Each pair of species BSPs runs was inspected for convergence in TRACER. Additionally, two independent runs were conducted for each species and were repeated under a constant population size model, with the same substitution rate and settings as above. Marginal likelihoods for each BSP run were calculated using stepping stone sampling (SS) and path sampling (PS) (Baele et al., 2012a&b), and SS and PS were averaged for each pair of identical runs. Support for the BSP model was evaluated by comparing SS and PS scores with the Bayes Factor (Kass and Raftery, 1995; Suchard et al., 2001; Baele et al., 2012).

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 40 new haplotypes were obtained for *C. penicillata*. Including the Genbank and hybrid zone sequences, this gave 45 *C. jacchus* haplotypes (93 transitions, nine transversions, one indel), 25 *C. penicillata* haplotypes (120 transitions, 24 transversions, three indels), 15 in the PJ hybrid zone (81 transitions, 4 transversions, 2 indels), and three (57 transitions, five transversions, one indel) in the RJ zone. The average number of differences between common marmoset haplotypes was 13.38 and the average number of differences between black-tufted marmoset haplotypes was 37.68.

The Bayesian phylogenetic analyses resulted in topologically identical trees that show an overall pattern of complex species-level polyphyly (Figure 5). Terminal branches in the phylogenies were collapsed into larger clades for ease of tree viewing. 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 basal most clade in the phylogeny. The next *C. penicillata* clade was sister to one of the *C. kuhlii* clades. The other *C. kuhlii* clade was sister to the two *C. aurita* haplotypes. The remaining two *C. penicillata* clades were most closely related to a large *C. jacchus* clade. 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.

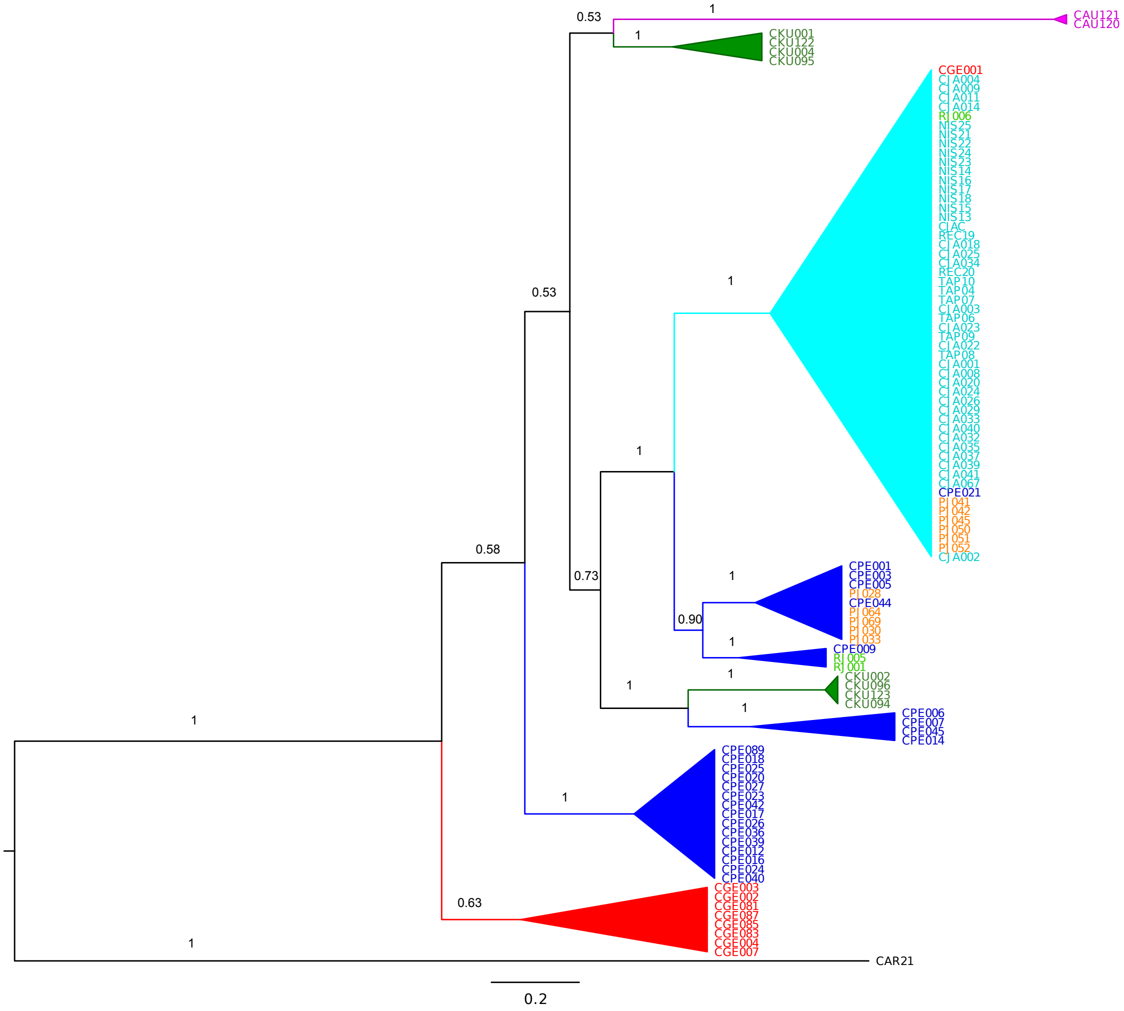
1
0.53
CAU121
CAU120
1
CKU001
CKU122
CKU004
CKU095
CGE001
CJA004
CJA009
CJA011
CJA014
RJ006
NIS25
NIS21
NIS22
NIS24
NIS23
NIS14
NIS16
NIS17
NIS18
NIS15
NIS13
CJAC
REC19
CJA018
CJA025
CJA034
REC20
TAP10
TAP04
TAP07
CJA003
TAP06
CJA023
TAP09
CJA022
TAP08
CJA001
CJA008
CJA020
CJA024
CJA026
CJA029
CJA033
CJA040
CJA032
CJA035
CJA037
CJA039
CJA041
CJA067
CPE021
PJ041
PJ042
PJ045
PJ050
PJ051
PJ052
CJA002
1
0.53
1
0.58
0.73
1
CPE001
CPE003
CPE005
PJ028
CPE044
PJ064
PJ069
PJ030
PJ033
0.90
1
CPE009
RJ005
RJ001
1
CKU002
CKU096
CKU123
CKU094
1
1
CPE006
CPE007
CPE045
CPE014
1
CPE089
CPE018
CPE025
CPE020
CPE027
CPE023
CPE042
CPE017
CPE026
CPE036
CPE039
CPE012
CPE016
CPE024
CPE040
1
0.63
CGE003
CGE002
CGE081
CGE087
CGE085
CGE083
CGE004
CGE007
1
CAR21
0.2

Figure 5a.

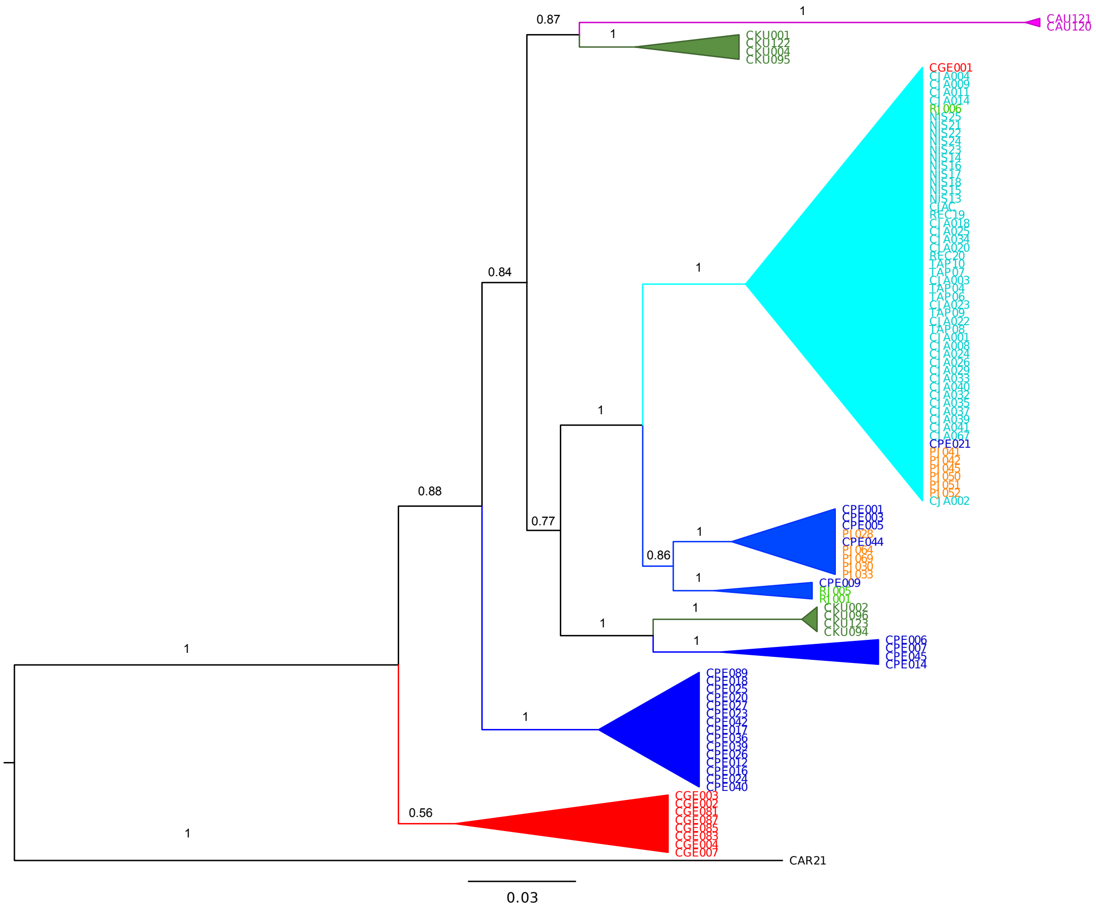

Figure 5b.

Figure 5a&b. Bayesian tree topologies for the *Callithrix* mtDNA control region. Outgroup rooted with *Mico argentatus* (CAR21). (a) Topology of tree modeled with a branch length prior set to the default unconstrained exponential branch length model with mean 0.1 and chosen by SS Bayes factor analysis. (b) Topology of tree modeled with a branch length prior set to an unconstrained exponential branch length model with mean of 0.01 and chosen by HS Bayes factor analysis. For a&b, branch posterior probabilities are indicated above branches. Clades were collapsed to more easily view the tree. Tip labels represent haplotypes found either outside of hybrid zones (with species haplotype classifications indicated by red for *C. geoffroyi,* purple for *C. penicillata*, light blue for *C. jacchus,* hunter green for *C. kuhlii,* and pink for *C. aurita*) or otherwise haplotypes found within hybrid zones (colored light green for the RJ zone and orange for the PJ zone). Clade colors represent majority species classification for non-hybrid zone haplotypes forming a given clade.

For the Bayesian trees described above, 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 and thus averaged together. However, branch support and branch length values differed between the two models (Figure 5). The ML tree did not provide strong branch support (above 75), thus it is not shown here.

The MJ network analysis only concentrated on the relationship between *C. jacchus* and *C. penicillata,* and shows relationships between haplotypes of the two species and their hybrids in finer detail (Figure 6). The circles presenting each haplotype are proportional to the frequency of a given haplotype within the *C. jacchus*, *C. penicillata*, and hybrid haplotype data set. 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 distributions of mtDNA D-loop haplotypes within hybrid zones. The geographical distributions of PJ zone haplotypes and phenotypes are shown in Figure 7a and 8a, respectively. Haplotypes and phenotypes of individuals PJ035-PJ037 were excluded from these figures because these individuals were not

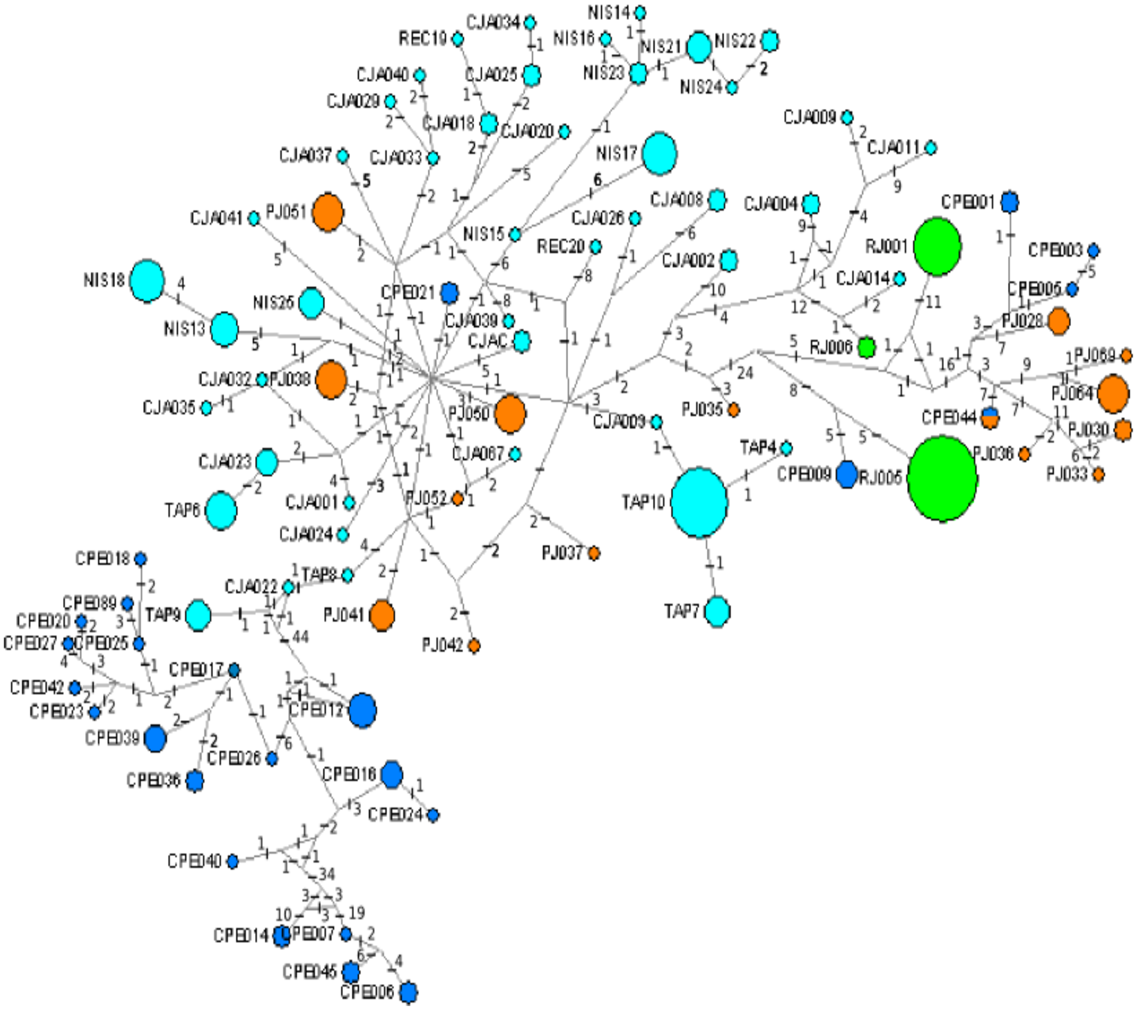

Figure 6. Median-joining network of mtDNA control region haplotypes for *C. jacchus, C. penicillata,* and their hybrids. Individual haplotypes are represented as colored pies, with the size of the pie scaled by the number of individuals possessing a given haplotype. The network is not drawn to scale for genetic distance between haplotypes. Numbers next to tick marks between two nodes represent the number of mutations between those nodes. Pie color indicates haplotypes found either outside of hybrid zones (with species haplotype classifications indicated by purple for *C. penicillata* and light blue for *C. jacchus*) or otherwise haplotypes found within hybrid zones (colored light green for the RJ zone and orange for the PJ zonec). Haplotype CPE044 is a haplotype was also found within the PJ hybrid zone as haplotype PJ032.

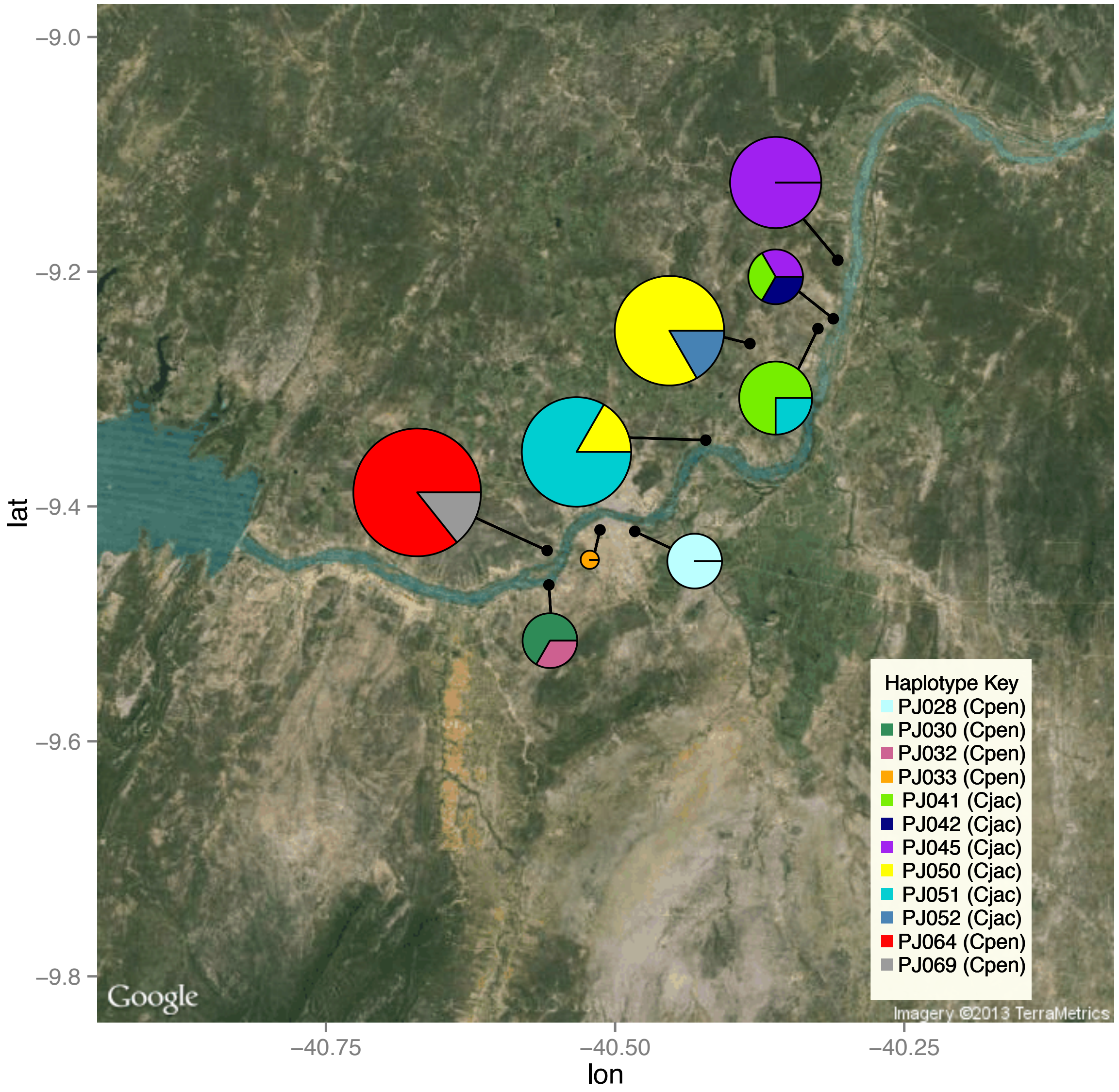
−9.0
−9.2
−9.4
−9.6
−9.8
lat
−40.75
−40.50
−40.25
lon
Haplotype Key
PJ028 (Cpen)
PJ030 (Cpen)
PJ032 (Cpen)
PJ033 (Cpen)
PJ041 (Cjac)
PJ042 (Cjac)
PJ045 (Cjac)
PJ050 (Cjac)
PJ051 (Cjac)
PJ052 (Cjac)
PJ064 (Cpen)
PJ069 (Cpen)
Google
Imagery ©2013 TerraMetrics

Figure 7a.

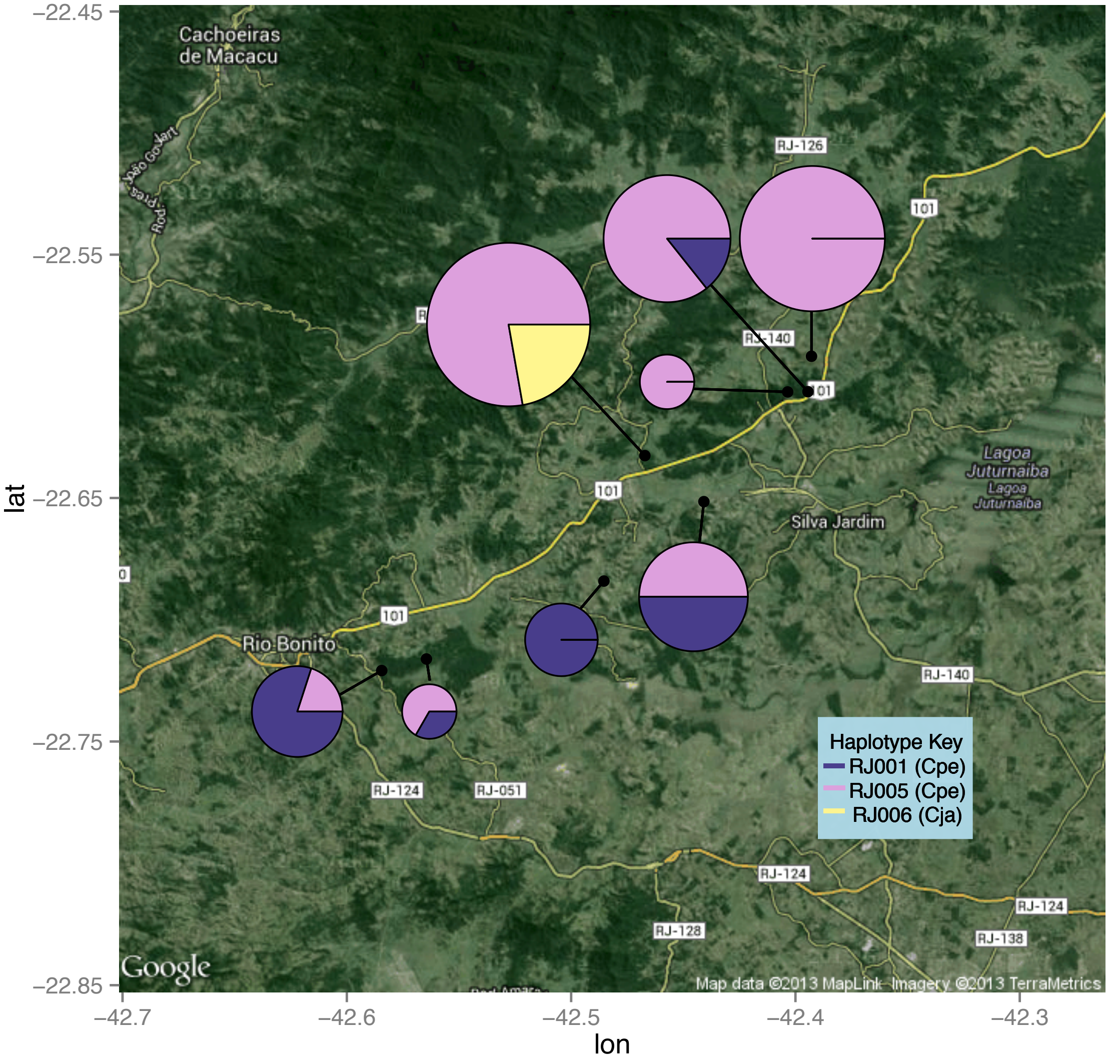

Figure 7b.

Figure 7a&b. 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.

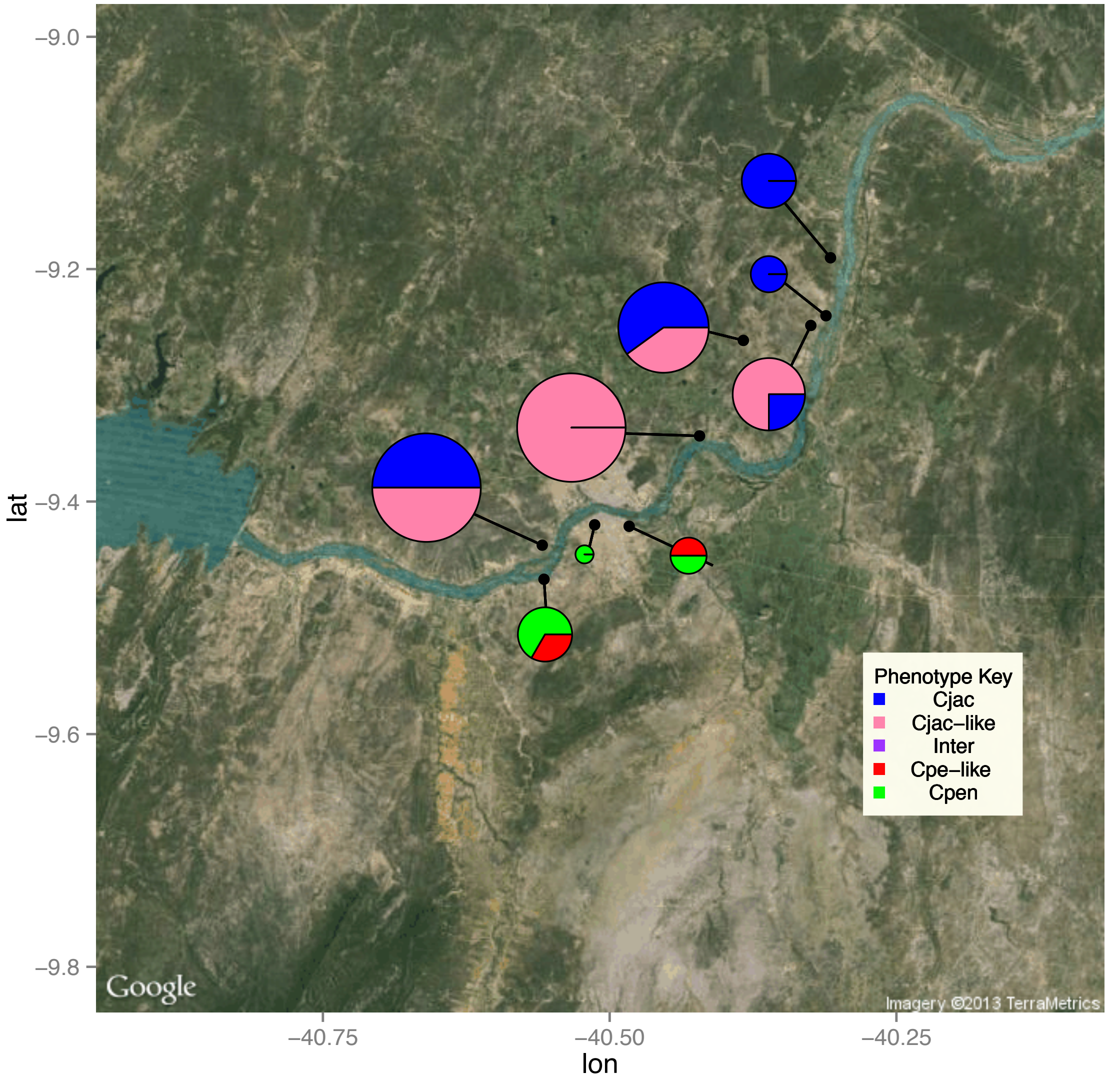
−9.0
−9.2
−9.4
−9.6
−9.8
lat
−40.75
−40.50
−40.25
lon
Phenotype Key
Cjac
Cjac−like
Inter
Cpe−like
Cpen
Google
Imagery ©2013 TerraMetrics

Figure 8a.

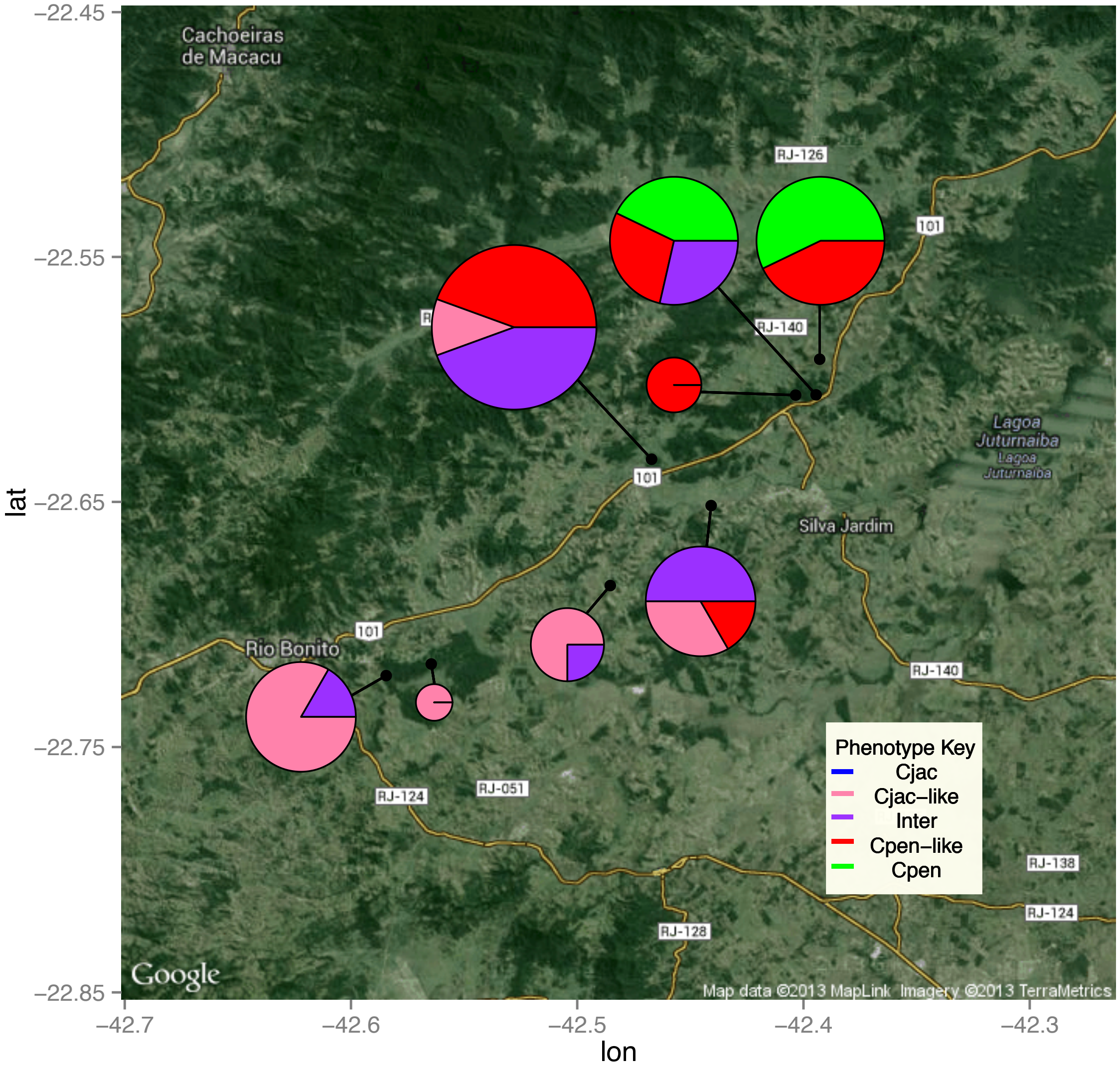

Figure 8.

Figure 8a&b. Geographic distribution of phenotypic categories based on hybrid index scores in (a) the PJ hybrid zone and (b) the RJ hybrid zone. Phenotype key labels indicate the following phenotypic categories: “Cjac” is pure *C. jacchus,* “Cjac” is *C. jacchus*-like, “Inter” is intermediate, “Cpe-like” is *C. penicillata*-like, and “Cpen” is *C. penicillata* pure.

sampled in the wild. All individuals sampled on the south side of the PJ hybrid zone possessed a *C. penicillata* haplotype. Table 4 shows the percentage of photographed adult marmosets sampled within the PJ zone that fall within each hybrid phenotype category. Table 5 shows a break down of phenotypically scored adults by their associated mtDNA haplotype. Geographically, marmosets with *C. penicillata*-like and pure *C. penicillata* phenotypes were confined to the south side of the São Francisco River, and all these individuals possessed *C. penicillata* mtDNA haplotypes. *Callithrix 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 (Figure 3) on the *C. jacchus* side of the PJ zone possess haplotypes that group within a *C. penicillata* clade in both the phylogenetic and network analyses. The phenotypes of these particular animals were that of pure *C. jacchus* and *C. jacchus*-like. 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. For the RJ hybrid zone, the percentages of sampled photographed adult individuals that fall within each hybrid phenotype category are also shown in Table 4, with the majority of individuals having a intermediate to *C. penicillata*-like phenotype. Table 5 also shows a break down of phenotypically scored adults by their associated mtDNA haplotype within the RJ hybrid zone. 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

Table 4.

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%

Table 5.

Breakdown of Number of Photographed Adult Individuals Sampled within each Hybrid Zone by Phenotype Category and Associated mtDNA Haplotype

	***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)**	**Total**
rj001 (cpe)	0	7	6	1	0	14
rj005 (cpe)	0	4	4	12	7	27
rj006 (cja)	0	1	1	0	0	2
pj028 (cpe)	0	0	0	1	1	2
pj030 (cpe)	0	0	0	1	1	2
pj032 (cpe)	0	0	0	0	1	1
pj033 (cpe)	0	0	0	0	1	1
pj041 (cja)	2	2	0	0	0	4
pj042 (cja)	1	0	0	0	0	1
pj045 (cja)	3	0	0	0	0	3
pj050 (cja)	2	3	0	0	0	5
pj051 (cja)	0	6	0	0	0	6
pj052 (cja)	1	0	0	0	0	1
pj064 (cpe)	3	2	0	0	0	5
pj069 (cpe)	0	1	0	0	0	1

Note: "Cpe" indicates a haplotype that was phylogenetically classified within a *C. penicillata* clade. "Cja" indicates a haplotype that was phylogenetically classified within a *C. jacchus* clade.

and haplotype RJ006 within the *C. jacchus* clade. The RJ001 haplotype is found mostly in marmosets with a *C. jacchus*-like, intermediate, and *C. penicillata*-like phenotype. The RJ006 was found in a *C. jacchus*-like and intermediate individual, and the RJ005 is found mostly in individuals with an intermediate to pure *C. penicillata* phenotype.

Genetic diversity and genetic 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 6. Pure *C. jacchus* and *C. penicillata* show similar levels of haplotype diversity. However, nucleotide diversity (π: *C. jacchus*-0.017, *C. penicillata*-0.084) and theta (θs: *C. jacchus*- 19.030, *C. penicillata*-30.620) 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 overall 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 (π: PJ zone-1.71, RJ zone- 0.174). If we further 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* ($\mathbf{\Phi}_{ST} = 0.664$, 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 ($\mathbf{\Phi}_{ST} = 0.697$,

Table 6.

Population genetic variables and indices for C. jacchus*,* C. penicillata*, and* C. jacchus *x* C. penicillata *hybrids*

Group	**Sequences**	**Haplotype Number**	**Haplotype Diversity (h)**	**Nucleotide Diversity (π)**	**Theta (θs)**	**Poly[a] 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

[a] Indicates polymorphic sites

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 ($\mathbf{\Phi}_{ST}$ = 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 7 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* (Tajima's D= 0.885, *P*-value=0.856; F_S =2.271, *P*-value=0.820), and its bimodal mismatch distribution is characteristic of a stable population (Figure 9a; SSD = 0.024, *P*-value = 0.064). Only the raggedness index statistic did not reject the null hypothesis of sudden population expansion (r=0.012, *P*-value= 0.184) in this species. The BEAST BSP for *C. penicillata* (Figure 10a) shows constant population size in the species. Bayes factor model selection based on SS estimates showed evidence in favor of the Bayesian Skyline Plot 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 (F_S = -9.250, P =0.038) and a unimodal mismatch distribution (SSD= 0.006, *P*-value=0.110, Figure 9b). Using a human CR mutation rate of 9.883×10^{-8} mutations per nucleotide per year (Soares et al., 2009), as mutation rates for marmosets

Table 7.

Neutrality tests and mismatch distribution analyses for C. jacchus*,* C. penicillata*, and* C. jacchus x C. penicillata *hybrids*

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

Note: SSD is sum of squared deviation, r is the raggedness index.

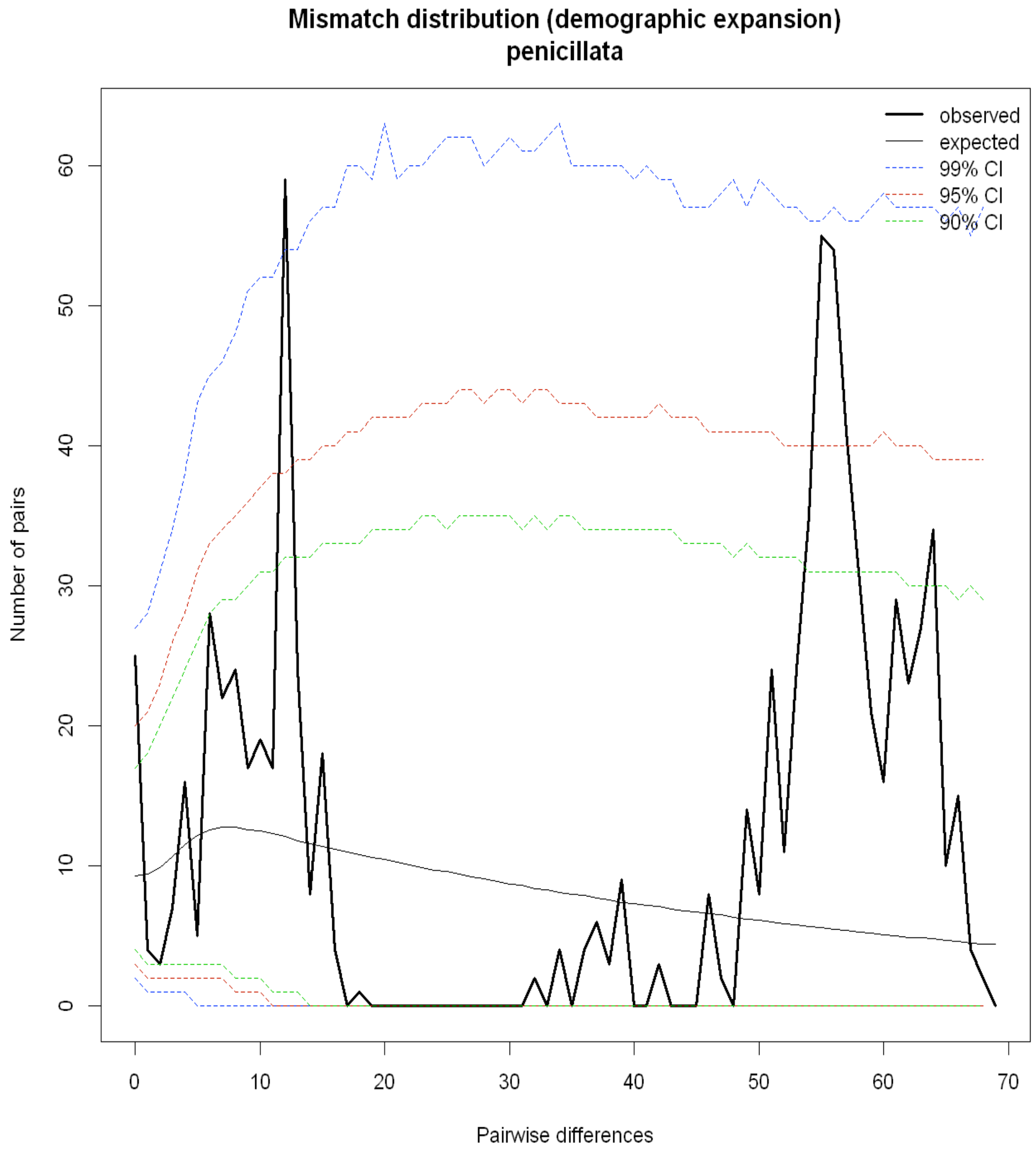
Mismatch distribution (demographic expansion)
penicillata
observed
expected
99% CI
95% CI
90% CI
Number of pairs
0
10
20
30
40
50
60
Pairwise differences
0
10
20
30
40
50
60
70

Figure 9a.

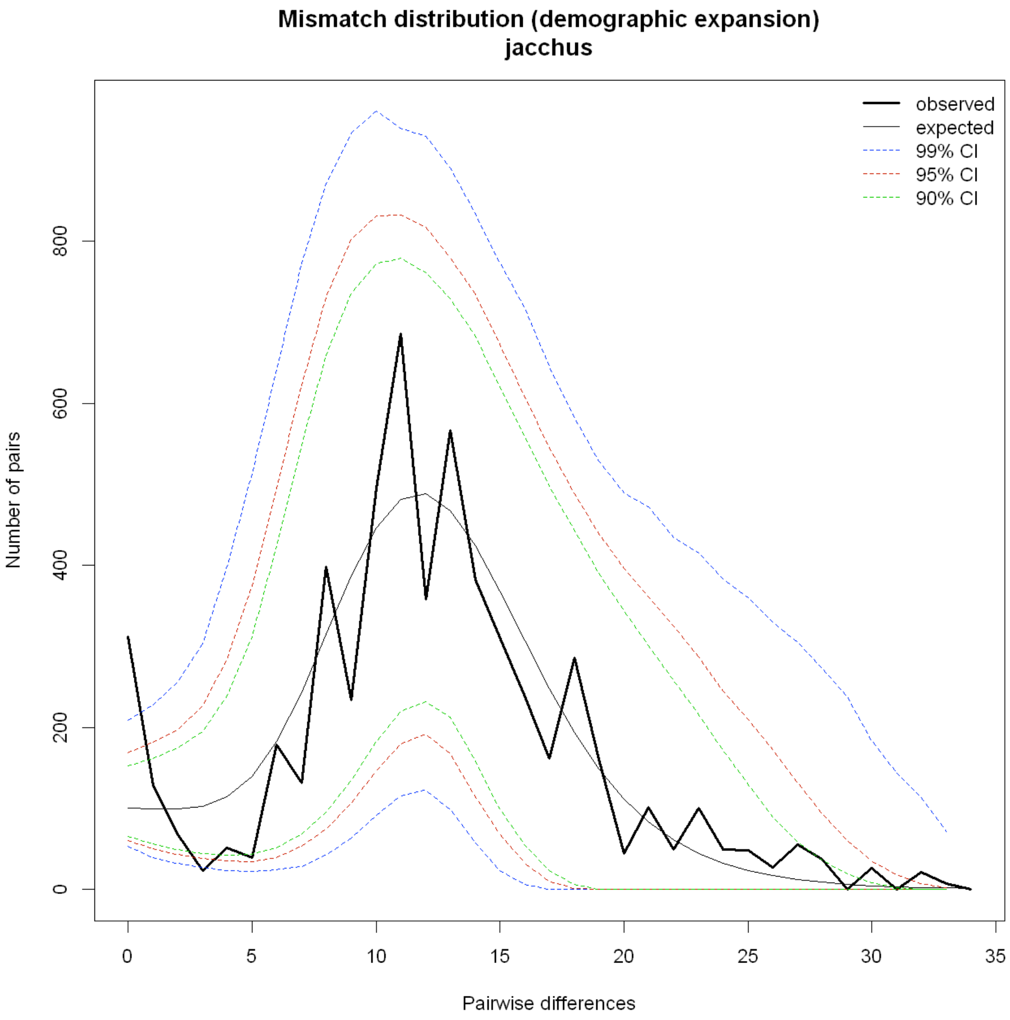

Figure 9b.

Figure 9. mtDNA D-loop mismatch distributions for (a) *C. penicillata* and (b) *C. jacchus.*

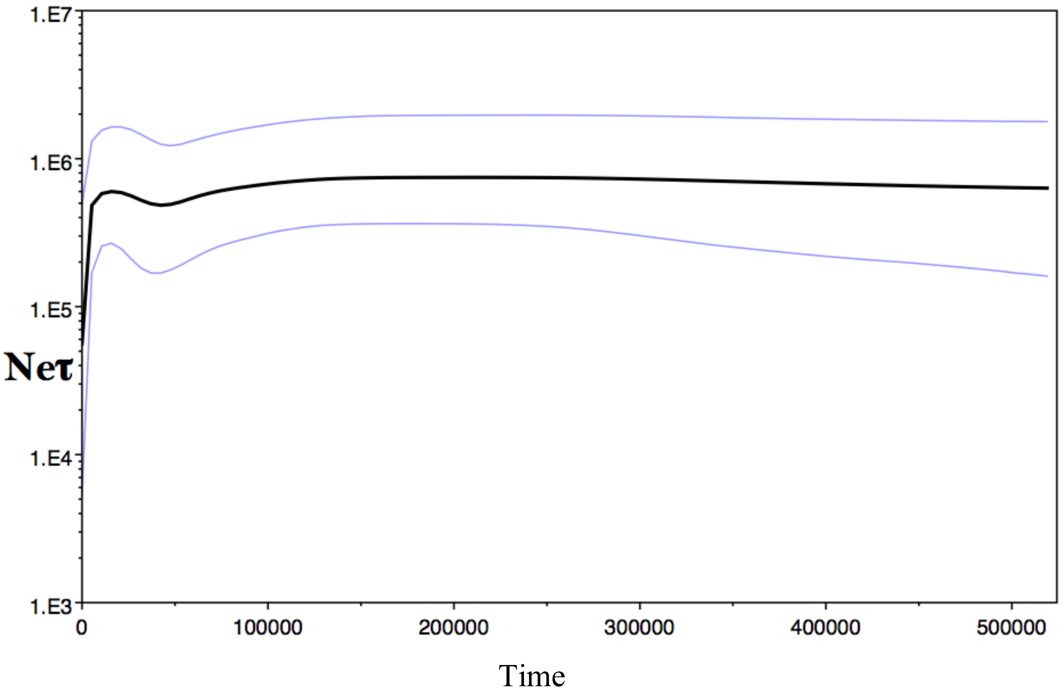
1.E7
1.E6
1.E5
1.E4
1.E3
Neτ
0
100000
200000
300000
400000
500000
Time

Figure 10a.

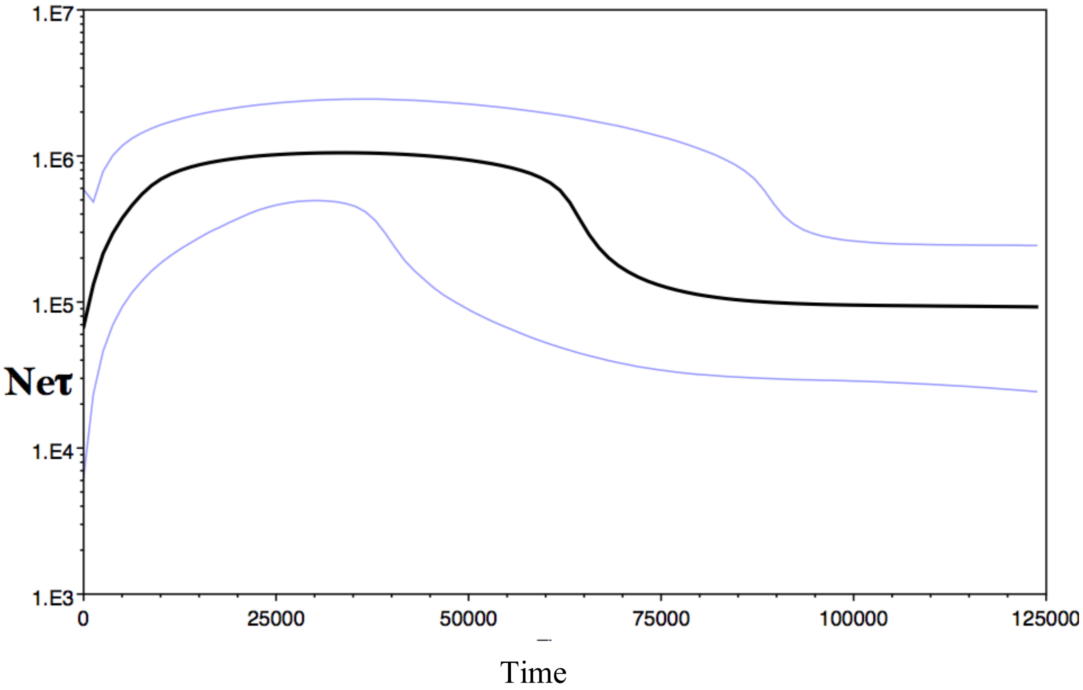

Figure 10b.

Figure 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 (blue) 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.

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* (Figure 10b) also supports a population expansion in common marmosets at approximately 60,000 years ago, but a decline at 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 non-significant, but negative in favor of expansion (Tajima's D=-1.188, *P*-value= 0.096), while the raggedness index is rejecting the null sudden expansion hypothesis.

Discussion

Speciation and evolutionary relationships within the *Callithrix* genus. The phylogenetic and network results of our study indicate the occurrence of common and black-tufted marmoset hybridization at a point of species contact between the Brazilian cities of Petrolina, PE and Juazeiro, BA. We also show genetic evidence for common and black-tufted marmoset hybridization in an artificial sympatry zone in Rio de Janeiro state. Marmosets sampled within these hybrid zones possessed a variety of ear-tuft and facial phenotypes that suggested either pure or admixed ancestry of *C. penicillata* and *C. jacchus*. Individuals with intermediate phenotypes possessed both *C. jacchus* and *C. penicillata* mtDNA CR haplotypes, and in some instances even individuals with pure or pure-like phenotypes of one species possessed haplotypes from the other species. Thus, our phylogenetic and network analyses corroborated the phenotypic data of *C. jacchus* and *C. penicillata* being the parental species of our sampled hybrid zones.

Our reported Bayesian phylogenies do not agree topologically with previously published *Callithrix* phylogenies. *C. aurita* is consistently the basal *Callithrix* species when included in a given nuclear or mitochondrial phylogeny (Sena et al., 2002; Perelman et al., 2011; van Roosmalen and van Roosmalen, 2003, Tagliaro et al., 1997, 2000; Schneider et al, 2012). However, our phylogeny did not agree with this basal position for *C. aurita.* Our results also do not agree with *C. geoffroyi* forming a well-supported monophyletic clade that diverges next within the *Callithrix* genus after *C. aurita* (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. The disagreement between ours and other studies may be due to incomplete lineage sorting at the mtDNA control region (see below), length differences in mtDNA sequence data, as well as the different phylogenetic methods used by us and other researchers.

However, our results along with several other studies (e.g.: Sena et al., 2002; van Roosmalen and van Roosmalen, 2003; Schneider et al., 2012; Tagliaro et al., 1997, 2000) show that *C. penicillata* and *C. kuhlii* both appear to be polyphyletic. This pattern is suggestive of either hybridization of these species with one another or other marmosets or incomplete lineage sorting (Funk and Omland, 2003). A hybrid origin for *C. kuhlii* has been previously suggested as a result of either hybridization between *C. penicillata* and *C. geoffroyi* (Hershkovitz, 1977) or *C. penicillata* and *C. jacchus* (Arnold and Meyer, 2006). Yet, Ryland et al. (1993) point out that experimental work in marmoset hybridization by Coimbra-Filho et al. (1993) failed to produce a *C. penicillata* x *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 x 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 more plausible than in the experimental setting. 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.

Population Genetics and Demographic History of *C. jacchus* and *C. Penicillata*. Faulkes et al. (2003) reported on genetic structure in the mtDNA control regions within and among *C. jacchus* populations, one of the few population genetics studies conducted on wild *Callithrix.* Their results showed high haplotypic diversity, but low genetic divergence in *C. jacchus*. Combining the data set 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 more low rare frequency 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 arrangement of *C. jacchus* haplotypes.

The studies of Perelman et al. (2011) and Schneider et al. (2012) place the origin of the *Callithrix* genus roughly at 2.5 MYA, around the Pliocene/Pleistocene boundary. *C. jacchus* and *C. penicillata* split from a common ancestor less than 1 MYA (Perelman et al., 2011), with the divergence of other *Callithrix* species occurring 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 (refugia theory: Kinzey, 1980; Turchetto-Zolet 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 American by acting as barriers to dispersal between different populations (riverine hypothesis, Turchetto-Zolet et al., 2013). This may have certainly been the case for *Callithrix* species whose ranges are in part limited by rivers; for example in *C. jacchus* and *C. penicillata* the ranges are largely separated by the São Francisco River (Rylands et al., 1993). 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 as well as the rest of the *Callithrix* genus.

The Rio de Janeiro and Petrolina-Juazeiro hybrid zones. A comparison of patterns of genetic diversity between the PJ and RJ hybrid zones offers an interesting look at the dynamics of current marmoset hybrid zones, one at a species contact zone and another with direct anthropogenic origins. The PJ hybrid zone shows much higher haplotype diversity, a larger number of haplotypes, and higher nucleotide diversity than that found in the 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 little if any flow of new genetic variation coming into the hybrid zone as it is far removed from the natural ranges of either parental species. On the other hand, since the PJ zone is located at the edges of parental species' distributions, it more plausibly represents the flow of new genetic variation into the zone with the northern side being in contact with *C. jacchus* and the southern side being in contact with *C. penicillata*.

Our findings on the geographic distribution of phenotype categories within the RJ hybrid zone are similar to the pattern found by de Morais Jr. (2010). De Morais Jr. (2010) argues that such a phenotypic pattern evidences multiple marmoset introductions into the RJ hybrid zone given that certain phenotypes are confined to specific regions of the zone. Genetic data from our study also show evidence of at least three different female lineages being introduced into this hybrid zone. These data suggest the occurrence of multiple founder events from marmosets introduced to the area as illegal pets, along with reduced genetic variation and representation of the dual founding species. Considering the possibility of continued gene flow into the hybrid zone through continued release of illegally trafficked marmosets in the RJ zone, any such newly introduced genetic variation would probably be lost to genetic drift due to the relatively small effective population size of mitochondrial DNA (Ballard and Whitlock, 2004).

Significant genetic structure is evident between marmoset populations on the northern and southern sides of each zone, with the São Francisco River dividing the PJ zone, and highway BR-101 separating the two sides of the RJ zone. Genetic analyses of haplotypes from the PJ zone are essentially comparing a large number of sequences from two different species that our analysis shows have significant genetic structure. 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.

Another difference between the PJ and RJ hybrid zones is the availability of conspecific breeding mates. In the PJ zone, where we identified many of our sampled animals as either pure common or black-tufted marmosets, an individual probably has a much higher chance of finding a conspecific mating partner. On the other hand, marmosets in the RJ hybrid zone are limited to animals descended from the original *C. jacchus* or *C. penicillata* exotic populations introduced to the area*,* and in general, we identified a very low number of non-admixed individuals in our sample. While hybrid marmosets sampled within the two studied hybrid zones are obviously fertile, interspecific breeding between marmosets may carry fitness costs, as is sometimes the case with hybridization (Arnold 1997). While marmoset hybrid fitness is yet to be investigated, Coimbra-Filho et al. (1993) report a degree of reduced fertility in the captive hybridization of different marmoset species. They note this is especially true for matings between *C. jacchus* and *C. aurita,* which according to the phylogeny of Perelman et al. (2011) probably represent the youngest and oldest members of the *Callithrix* genus, respectively.

Hybridization and forest fragmentation. The two hybrid zones described in this current study occupy distinct Brazilian biomes, Caatinga for the PJ zone (Oliveira et al., 2012) and the Atlantic Forest for the RJ zone (Ribeiro, 2009). The Caatinga houses native *C. jacchus* and *C. penicillata,* the majority of *C. penicillata* is found in the Cerrado, and Atlantic Forest contains *C. jacchus* and *C. penicillata* in addition to the remaining *Callithrix* species (Rylands et al., 1993). All three biomes have undergone high levels of deforestation and fragmentation due to anthropogenic activity. The Caatinga occupies about 750,000 km^2 of the Brazilian northeast (Castenelli et al., 2004) and has a long history of agricultural land use (Mamede et al., 2008), with about 27.5% of the biome already converted to pastureland, and remaining undisturbed portions existing as fragmented "islands" (Castelletti et al., 2004). Although data about the status of these undisturbed fragments are scarce, Castelletti et al. (2004) estimated through simulations that the number of fragments is between 172 and 243, that between 27-30% are less than 50 km^2 (5,000 ha), and that between 9 and 11 fragments are larger than 10,000 km^2 (1,000,000 ha). For the Atlantic Forest biome, its remaining original forest cover is estimated to be only about 11%, with 80% of fragments sized at <50 ha, and a distance of 1440 m between fragments (Ribeiro et al., 2009). The study of da Cunha et al. (2007) exemplifies fragmentation in the Cerrado. The authors looked at 21.43 % of the state of Goiás and found that fragment sizes ranges from 0.0625 ha to 65,536 ha, but that 38.48%

of fragments were smaller than 1 ha. Home range sizes among the eastern marmosets varies, with the smallest seen among *C. jacchus* (0.5-6.5 ha), *C. penicillata* (1.25-13.1 ha), and *C. geoffroyi* (6 ha), but much larger ranges reported for *C. aurita* (11.5-35 ha), *C. kuhlii* (10-38 ha), and *C. flaviceps* (15-35.5 ha) (Ryland and de Faria, 1993; Raboy et al., 2008).

We reanalyzed sequences from the Faulkes et al. (2003) study to estimate levels of genetic diversity found within a 180 ha (Nisia Floresta) and 390 ha forest fragment (Tapacura) of the Atlantic Forest. Nucleotide diversity within both fragments was found to be 0.01 and haplotype diversity was 0.644 at Tapacura and 0.900 at Nisia Floresta, respectively. These levels are slightly lower than what we observed in general for *C. jacchus* genetic diversity indices. However, observed genetic diversity at these two fragments suggests that their size probably does not preclude marmoset gene flow, especially when considering the small home range sizes required by both *C. jacchus* and *C. penicillata.*

Although specific figures of fragment size are not available for the PJ zone, visual inspection of satellite imagery of the area (Google Maps, 2013) shows sprawling urbanization intersected by the São Francisco River that later gives way to an agricultural landscape. Sampled marmosets populations were predominately found within a thin line of relatively continuous forest edge that hugs the banks of the São Francisco River. Small breaks in the forest edge do exist, with most breakage found within urban regions of the riverbanks, and large continuous patches found around rural regions of the riverbanks. We find similar levels of nucleotide and haplotype diversity between *C. jacchus* and *C. penicillata* origin haplotypes from within the PJ zone and from *C. jacchus* haplotypes from the two large size Atlantic Forest fragments studied by Faulkes et al. (2003). While the São Francisco River seems to serve as an important (but leaky) north to south dispersal barrier within the PJ zone, connectivity between forest fragments, in addition to genetic introgression, probably facilitates gene flow between social groups. As a result of these processes, relatively high levels of genetic diversity can be maintained within the hybrid zone.

The landscape of the RJ hybrid zone is characterized by forest patches whose average size is 50 ha, and distances between patches to the north of highway BR-101 are about 250 m (personal observation, Carlos Ruiz-Miranda). 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 input of parental genomes into the fragment.

Our current data sets from the RJ and PJ zones do not include information on when the process of deforestation began within those zones. Therefore, specific ages of forest fragments within the hybrid zones are not known, nor do we know for how long populations may have been isolated within forest fragments. This historical perspective is needed to make a full appraisal of the effects of forest fragmentation on marmoset diversity within and outside of hybrid zones. Thus, this remains an open area of research within marmoset evolutionary biology and further research is needed to help elucidate how forest fragmentation and hybridization may affect genetic diversity of marmoset populations over time.

Implications for *Callithrix* biodiversity and genetic integrity. The current situation of habitat loss within the three biomes occupied by *Callithrix* species is certainly a threat to this genus, as sizes of many fragments seem to be near the minimum of adequate marmoset group home range size. Additionally, the average distance between fragments in the Atlantic Forest may serve as a significant dispersal barrier for marmosets. Further, *C. aurita* is 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 01 May 2013). 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. As already mentioned, *C. jacchus* and *C. penicillata* seem to be the most frequently introduced exotic species into the ranges of other native marmoset species, for which we have already discussed the occurrence of hybridization. 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 occurs, 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, the genetic locus we examined was a neutral marker, and 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 zones are much 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, implications for outcomes of hybridization within other marmoset hybrid zones are complex. 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 only 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.

CHAPTER 2

NATURAL AND ANTHROPOGENIC HYBRIDIZATION IN TWO SPECIES OF EASTERN BRAZILIAN MARMOSETS (*CALLITHRIX JACCHUS* AND *C. PENICILLATA*)

While animal hybridization is historically regarded as an “evolutionary dead-end” (Mayr, 1963; Arnold, 1997; Arnold 2006), modern research takes a more multi-faceted view, particularly in terms of natural versus anthropogenic hybridization. Here, we define hybridization as successful interbreeding between individuals from different populations possessing distinguishable heritable characteristics (modified from Arnold, 1997). We also differentiate between natural and anthropogenic hybridization with the latter as population interbreeding resulting from human-induced environmental change. Allendorf et al. (2001) perhaps best summarize current views regarding hybridization, where the authors acknowledge its importance in animal evolutionary history within a natural context, but emphasize its negative impact on modern biodiversity due to anthropogenic factors. This dichotomy in thought regarding hybridization makes conservation decisions especially difficult, particularly when distinguishing between the two processes is not simple. Accordingly, Allendorf et al. (2001) further highlight the importance of understanding the evolutionary role of hybridization in light of management of interbreeding taxa.

Amongst differing modern viewpoints on hybridization, natural hybrid zones are often heralded as “nature’s evolutionary laboratories, ” especially as genetic evidence is increasing for the importance of animal hybridization in speciation, introgression (gene transfer between species), and development of genetic novelties (e.g., Dowling and

Demarais, 1993; The Heliconius Genome Consortium, 2012; Levy, 2012; Mallet, 2005; Richards et al., 2008, Seehausen, 2004). Natural hybridization may also be a regular part of species divergence in young taxa and occurs in approximately 10% of animal species (Mallet, 2005). On the other hand, biodiversity decline and erosion of species genetic integrity are often linked to anthropogenic hybridization (Rhymer and Simberloff, 1996; Seehausen et al., 2008). A number of instances of hybrid swarming, or highly admixed populations where unique parental gene combinations were lost, have also been attributed to anthropogenic hybridization (e.g., Seehausen et al., 1997; Taylor et al., 2006). Surprisingly, some cases of anthropogenic hybridization may increase biodiversity through hybrid speciation and transgressive segregation (Crispo et al., 2011). Additionally, hybrid swarm theory predicts that introgressive hybridization can generate new biodiversity through adaptive radiation (Seehousen, 2004).

Understanding the evolutionary role of hybridization between various species of eastern Brazilian marmosets (genus *Callithrix*) certainly encompasses many of the challenges described by Allendorf et al. (2001). *Callithrix* contains six species, *C. jacchus, C. penicillata, C. kuhlii, C. flaviceps, C. aurita,* and *C. geoffroyi*, with population numbers of the last three species in decline (www.iucnredlist.org). This is a young genus, aged at about 2.5 million years, with *C. penicillata* and *C. jacchus* diverging as sister species less than 1 million years ago (Perelman et al., 2011). Experimental hybridization in captivity shows incomplete reproductive isolation between various members of *Callithrix* (Coimbra-Filho et al., 1993). All species inhabit separate ranges within the Caatinga, Cerrado, and Brazilian Atlantic Forest biomes of Brazil, but inter-specific points of contact do occur at species boundaries (Ryalnds et al., 1993;

Rylands et al., 2009). The biomes inhabited by marmosets have experienced a high level of deforestation and fragmentation (e.g., Castelletti et al., 2004; da Cunha et al., 2007), particularly the Atlantic Forest (Ribeiro et al., 2009). Such habitat disturbance is implicated in creating new areas of sympatry between historically separated marmoset species (Rylands et al., 1993). *Callithrix jacchus* and *C. penicillata* have also been introduced together in areas outside of their respective distributions, and into the native ranges of other *Callithrix* species. Marmoset hybridization occurs at many contact points and areas of human introduction (e.g., Alonso et al., 1987, Mendes, 1997, Passamani et al., 1997), but how much hybridization is natural and how much is anthropogenic remains unclear. Anthropogenic hybridization is a potential threat to marmoset genetic integrity, especially for the three declining marmoset species. Further, management of marmoset hybridization is complicated because the genetic and evolutionary implications of interbreeding between *Callithrix* species are not yet well understood.

Two *C. penicillata* x *C. jacchus* hybrid zones offer a unique opportunity to study both natural and anthropogenic aspects of marmoset interbreeding to improve understanding of its role in marmoset evolution. We previously described these two hybrid zones in Chapter One, one located at a natural species boundary and the other to which both species were anthropogenically introduced, using mitochondrial (mtDNA) D-loop sequence data. Because mtDNA is passed on to offspring by females, it only characterizes interspecific gene flow for one sex. Complementing mtDNA data with multiple independent, bi-parentally inherited autosomal loci reveals a more comprehensive view of the history of a population that includes both sexes (Thalmann et al., 2006). Here, we report microsatellite data from pure and hybrid wild marmosets to address the following questions:(1) What are the patterns of genetic diversity and differentiation inside and outside of *C. jacchus x C. penicillata* hybrid zones? (2) Do patterns and levels of genetic admixture and introgression differ between natural and anthropogenic marmoset hybrid zones? (3) What are the evolutionary implications of natural and anthropogenic marmoset hybridization?

Methods

Sample populations and hybrid zones. Between 2010 and 2011, biological samples were obtained from captive and wild populations of pure and hybrid *C. jacchus* and *C. penicillata*. Sampling information is listed in Table 8 and latitude/longitude coordinates of the collection site for each individual are given in Table S2. Detailed information about collection permits, sample collection, storage, sampling sites/facilities, and DNA extraction from biological samples has been provided in Chapter 1. The municipalities of Silva Jardim and Rio Bonito in Rio de Janeiro state, Brazil make up the Rio de Janeiro hybrid zone, abbreviated here as the "RJ zone." The cities of Petrolina, PE and Juazeiro, BA make up the Petrolina-Juazeiro zone, or the "PJ zone." The PJ zone lies at a natural species border between *C. jacchus* and *C. penicillata,* while marmosets in the RJ zone are descendants of introduced *C. jacchus* and *C. penicillata* present in the area since at least the mid-1980s (Ruiz-Miranda et al., 2000).

Marmoset chimersim and genetic markers. Marmoset chimerism (i.e. possession of at least two genomic lineages by a single individual), occurs as a result of frequent twinning in these primates and *in utero* exchange of stem cells by twins (Benirshke et al., 1962). As a result, marmoset tissues can be derived from self and

Table 8.

Summary of Sampled Individuals from Captive and Wild Pure Populations and Wild Hybrid Zones

Populations	**Type**	**Source**	**Year Collected**	**Individuals Sampled**
C. jacchus	Captive	CRC[a], Omaha, NE, US	2011	2
	Wild	IBAMA CETAS[b], Recife, PE, Brazil	2011	27
	Captive	NEPRC[c], Southborough, MA, US	2010	10
	Wild	Parque Dois Irmãos & Tapacurá Reserve, PE, Brazil[d]	2005	42
C. penicillata	Captive	CRC[a], Omaha, NE, US	2011	8
	Wild	Muriaé, MG; Brasilía, DF; Goiânia, GO, Brazil	2011	29
	Captive	IBAMA CETAS[b], Recife, PE, Brazil	2011	3
	Wild	IBAMA CETAS[b], Goiânia, GO, Brazil	2011	5
C .jacchus x C. penicillata hybrids	Wild	Silva Jardim and Rio Bonito Municipalities, RJ, Brazil	2011	46
C .jacchus x C. penicillata hybrids	Wild	Petrolina, PE and Juazeiro, BA, Brazil	2011	42
	Captive	CEMAFAUNA, Petrolina, PE	2011	3

[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 Borstelmanna de Oliveira
[e] Center for Management of Fauna of the Caatinga

sibling embryonic cell lineages (Ross et al., 2007). Ross et al. (2007) showed that chimersim levels differ by tissue in *C. kuhlii*, with epithelial tissue having some of the lowest levels (12% chimeric) and blood-derived tissue having some of the highest levels (50% chimeric). We carried out a preliminary microsatellite genotyping analysis in *C. jacchus* using blood and epithelial tissue donated by the New England Primate Research Center. We genotyped 10 unrelated individuals at two microsatellite loci, caja1 and caja5 (Raveendran et al., 2008). The two loci were amplified in separate 24 uL polymerase chain reactions (PCR) using the AmpliTaq Gold PCR Kit with Buffer II (Life Technologies) within the following reagents at final concentrations of 1X Buffer II, 0.8 mM total dNTPs, 1.5 mM $MgCl_2$, 0.5 uM of each forward and reverse primer, and 0.025 U/uL taq DNA polymerase. Thermocyclers settings were as follows: (1) 94.5°C for 5 minutes, (2) 94.5°C for 45 sec, (3) 48°C for 30 seconds for caja5 or 55°C for 30 seconds for caja1, (4) 72°C for 30 seconds, (5) repeat steps 2-4 a total of 36 times for caja5 or 35 times for caja1, and (6) 72°C for 1:30 minutes. Most side-by-side comparisons of genotypes amplified from blood and skin samples from the same individual were consistent with each other, though blood samples occasionally showed 3 allele genotypes when skin genotypes showed 2 alleles. Thus, we only used epithelial tissues from wild and captive marmosets that were part of our main dataset.

We incorporated caja1 and caja5 into a larger panel as recommended by Vaha and Primmer (2006) to differentiate between hybrid and pure individuals. For the larger microsatellite panel, we tested a total of 50 dinucleotide markers developed for marmosets and lion tamarins (Nievergelt et al.,1998; Galbusera and Gillemot, 2008; Katoh et al., 2008; Raveendran et al., 2008). Six loci were excluded due to poor amplification, and the remaining 44 loci were polymorphic in both *C. jacchus* and *C. penicillata.* These 44 loci were amplified in 15 multiplex reactions (Table S3), each at a 10 uL volume, using the Qiagen Multiplex PCR Kit following manufacturer directions at a modified annealing temperature of 64°C. A fluorescent dye labeled the 5' end of the forward primer for each locus. PCR products were separated by size on an ABI 3730 sequencer with GeneScan 500 LIZ (Life Technologies) size standard. Alleles sizes were determined using GENEMARKER (Softgenetics) and checked manually.

Main data set analysis. Allele frequencies at each locus for the two parental species and within the hybrid zones were calculated with GENEPOP 4.2 (Roussett, 2008). The "exact test" (Haldane, 1954; Weir, 1996; Guo and Thompson, 1992) in GENEPOP was carried out to test each locus within each species and hybrid zone for deviation from Hardy-Weinberg equilibrium (HWE) using the MCMC method with 10,000 dememorization steps, and 1000 batches, and 10,000 iterations per batch. The same software was used to test pairwise linkage disequilibrium (LD) within each species and hybrid zone under the same MCMC settings as for HWE. *P*-values for LD and HWE tests were adjusted with the Bonferroni correction for multiple comparisons (Rice, 1989). FSTAT 2.9.3.2 (Goudet, 1995) was used to determine the number of observed alleles, allelic richness (R), and F_{IS} (Weir and Cockerham, 1984) for each locus within the two species and hybrid zones. Observed (H_O) and expected heterozygosity (H_E) (Nei, 1973) were determined with GENODIVE (Meirmans and Van Tienderen, 2004).

Possible presence of null alleles within the dataset was examined by MICROCHECKER (Oosterhout et al., 2004), which also checks for other genotyping errors such as stuttering and short allele dominance. As our dataset likely contains null alleles (discussed further in Results), we calculated locus null allele frequency (r) within each population using 10,000 iterations of the EM algorithm (Dempster et al., 1977) as implemented in the program FREENA (Chapuis and Estoup, 2007; Chapuis et al., 2008). Chapuis and Estoup (2007) found the EM method to be the to the best r estimator among three commonly used estimators. Additionally, these authors found only a weak effect of null alleles on H_E across a large range of r. Thus, we did not make corrections for any of the within-population analyses discussed above.

We next examined population differentiation between the two study species as well as subpopulations found on the respective northern and southern sides of each hybrid zone. FREENA can calculate F_{ST} values that are corrected and unbiased for null alleles (Chapuis and Estoup, 2007), and we used the software to obtain uncorrected and corrected values of the statistic. The corrected F_{ST} value is based on Weir (1996) and includes only visible allele sizes. Statistical population differentiation analysis was also carried out in GENODIVE using AMOVA R_{ST} statistics, based on the stepwise mutation model for microsatellites, and 10,000 permutations.

We applied two Bayesian clustering approaches to determine levels of *C. jacchus* and *C. penicillata* admixture within the RJ and PJ hybrid zones. Genotypes of pure species caught outside of hybrid zones were used in both methods as reference samples upon which cluster allele frequencies were estimated. The reference samples also aided in ancestry estimation and identification of hybrids among individuals sampled within the two hybrid zones. Adapting the approach of Godinho et al. (2011), we made the *a priori* assumption that the number of clusters (K) is two, i.e. that there are two ancestral populations contributing to the gene pool in either hybrid zone. Based upon the simulation studies of Vaha and Primmer (2006), we considered an individual a hybrid if $0.10 < q < 0.90$ for that individual (the fraction of the individual's genome inherited from population *k*). First we set up 10 independent runs using STRUCTURE 2.3.4 software, (Pritchard et al., 2000) under the USEPOPINFO model to allow for use of pre-defined parental species groups to aid classification of hybrid samples following conditions per run with MIGRPRIOR=0.05 (default value). *Callithrix penicillata* individuals were indicated by Popflag=1, *C. jacchus* individuals were identified by Popflag=2, and all individuals sampled from hybrid zones were identified by Popflag=0. The PFROMPOPFLAGONLY option was turned on for allele frequency estimation only based on the parental species. Admixture levels of hybrids were estimated for hybrid zone individuals under that admixture ancestry model which assumes that some fraction of an individual's genome comes from the two parental clusters. This model was used with the default setting of an inferred alpha initially set to 1.0. The default correlated allele frequency model was used for all runs, and each run consisted of 80,000 burn-in steps followed by 8,000,000 MCMC iterations. The ten runs were checked for consistency in summary statistics and convergence of parameter values. Average *q*-values for hybrid samples across the independent runs were determined with the full-search algorithm of CLUMPP 1.1.2 (Jakobsson and Rosenberg, 2007). DISTRUCT 1.1 (Rosenberg, 2004) was used to produce a graphical display of hybrid zone admixture as determined by STRUCTURE. Null allele corrections were not applied during STRUCTURE analyses, as Carlsson (2008) found that the presence of null alleles at microsatellite loci "would probably not alter the overall outcome of assignment testing."

Second, we took a similar approach with BAPS 6.0 (Corander et. al., 2006;

Corander et al., 2008; Tang et al., 2009) by conducting an admixture analysis based on pre-defined common and black-tufted marmosets clusters of individuals from outside of marmoset hybrid zones. Genotypes of pure individuals were used to define allele frequencies. Each BAPS run was executed with the following conditions: minimum population size was set to the default size of 5, there were 300 iterations per run, 200 reference individuals, and the number of iterations for admixture estimations of reference individuals was set at 10. Five independent BAPS runs were carried out and consistency of ancestry estimation for hybrid zone samples was verified between run replicates. Null allele corrections were not applied to BAPS analyses, as simulations studies by Chapuis et al. (2008) found that assignment results for this software actually improve in the presence of null alleles at microsatellite loci.

Because the assumed priors and efficiency (proportion of correctly identified hybrids out of a total number of actual hybrids in a sample (Vaha and Primmer, 2006)) of the above analyses cannot be assessed statistically, we conducted simulation studies to evaluate the power of our reference dataset to detect hybrids and to estimate *q.* We simulated 10 STRUCTURE datasets and 5 BAPS datasets composed of all reference samples, 10 F1 hybrids, 10 F2 hybrids, 10 *C. jacchus* x F1 backcross hybrids, and 10 *C. penicillata* x F1 backcross hybrids. All simulated hybrid classes were created with HYBRIDLAB 1.0 (Nielsen et al., 2006) and analyzed with STRUCTURE and BAPS as described for actual data sets. Then efficiency was calculated as in Vaha and Primmer (2006).

We also employed one non-Bayesian method to study the partition amongst hybrid and pure groups. GENODIVE was used to carry out a principle component analysis (PCA) to identify axis which contribute to population structure. The PCA used a covariance matrix based on population-level allele frequencies. We used the first and second components of the PCA to summarize differences microsatellite allele frequency data between the four population groups as a final analysis of population clustering of pure species and hybrid zone samples.

Results

Deviations from Hardy-Weinberg equilibrium and linkage disequilibria.

Deviations from HWE varied among loci within the parental species and the hybrid zones (Table S4). For the parental species, 20 out of 44 *C. penicillata* loci and 5 out of 44 *C. jacchus* loci were in disequilibrium. MICROCHECKER indicated possible presence of null alleles for almost all loci out of HWE in *C. penicillata,* but within-species pooling of samples from isolated captive and geographical subpopulations may have also caused loci to be out of HWE (Walhund effect). Null allele presence indicated by MICROCHECKER and Walhund effect for *C. jacchus* loci may also explain some of the significant heterozygote deficiencies. In the hybrid zones, 12 out of 44 loci in the PJ Zone and 6 out of 44 loci in the RJ zone were in disequilibrium. All loci out of HWE had positive F_{IS} values indicating heterozygote deficiencies in pure and hybrid populations. MICROCHECKER flagged most hybrid zone loci not in HWE for the likely presence of null alleles. For the PJ zone, pooling of samples from two parapatric and genetically differentiated forms separated by a significant dispersal barrier (the São Francisco River) may explain part of the observed significant heterozygote deficiency. In the RJ zone, pooling of several subpopulations separated by a dispersal barrier (highway BR-101) may explain observed Hardy-Weinberg disequilibrium.

The majority of markers used in this study map to different common marmoset chromosomes of the common marmoset published genome (calJac 3 build, genome.ucsc.edu), and those located on the same chromosome should have a large enough physical distance to prevent linkage. Nonetheless, significant linkage disequilibrium was found in some pairwise locus comparisons out of a total of 946 comparisons within each group as follows: (1) pairs of markers where each locus was found on a different chromosome totaled 29 in *C. penicillata*, 8 in *C. jacchus*, 34 in the PJ zone, and 14 in the RJ zone, (2) and marker pairs with both loci located on the same chromosome totaled 1 in *C. penicillata*, 3 in *C. jacchus*, 3 in the PJ zone, and 11 in the RJ zone. Demographic factors such as population structure may help explain inflated levels of LD, particularly in the case of *C. jacchus* and *C. penicillata* where we are pooling samples from different captive and geographic origins. For the RJ zone, the high number of physically linked loci in disequilibrium probably indicates recombination has not yet broken these loci apart and they continue to segregate within their ancestral genetic background.

Allele frequencies, genetic diversity, and population differentiation. An averaged summary of various genetic diversity measures for pure and hybrid groups is shown in Table 9 and expanded out for individual loci in Table S4. *C. penicillata* generally exhibited higher allele numbers (mean 10.864) and allelic richness

Table 9.

Averages of Various Genetic Diversity Indices for Species and Hybrid Groups

Group	**N**	***A***	**R**	**r**	**H_o**	**H_E**	**F_{IS}**
C. penicillata	37.841	10.864	10.276	0.09	0.62	0.79	0.216
C. jacchus	55.568	8.386	7.12	0.037	0.618	0.673	0.082
PJ Hybrid Zone	39.386	8.295	7.707	0.071	0.581	0.703	0.173
RJ Hybrid Zone	41.295	6.75	6.427	0.046	0.634	0.724	0.125

Note: N is number of individuals sampled at a locus, A is the number of alleles at a locus, R is allelic richness, r is EM null allele frequency, H_o is observed heterozygosity, H_E is expected heterozygosity, F_{IS} is the inbreeding coefficient.

(mean 10.276) at these microsatellite loci than *C. jacchus* (mean number of alleles per locus= 8.386, mean allelic richness = 7.120). H_E levels are broadly similar between the two species, but for many *C. penicillata* loci H_O levels are lower than expected, while H_E and H_O are similar for *C. jacchus.* Measures of genetic diversity in the PJ zone are similar to values seen in the parental species. The number of observed alleles and allelic richness within the RJ zone was the lowest out of the four groups, but H_E end H_O were comparable to the other three groups. Loci with lower H_O than H_E are also loci with a significant heterozygote deficit and those that may contain null alleles. Thus allele drop-out may cause deflation of H_O relative to H_E at such loci, especially in *C. penicillata*.

Allele frequencies uncorrected and corrected for null allele presence across the 44 microsatellite loci are shown in Table S5 for parental and hybrid populations. No true diagnostic loci fixed between *C. jacchus* and *C. penicillata* were found. *C. penicillata* had on average 4.84 private alleles and *C. jacchus* 2.65 private alleles. Most private alleles in *C. penicillata* were found together in a continuous size range whereas *C. jacchus* private alleles were found as singletons. The remaining alleles overlapped in the two species but allele frequencies differed interspecifically. Alleles present in the hybrid zones were a mosaic of those alleles found in the parental population, but the PJ zone contained a much larger representative sample of parental alleles than did the RJ zone. The PJ zone had an average of 1.52 private alleles per locus, mostly as singletons and the RJ zone had an average of 1.36, also mostly singletons.

F_{ST} values corrected and uncorrected for null allele presence were similar for each pairwise comparison (Table 10). *C. jacchus* and *C. penicillata* showed a moderate

Table 10.

Pair-Wise F_{st} Indices

Pair-Wise Comparison	$^{U}F_{st}$	$^{C}F_{st}$
C. penicillata-C. jacchus	0.182965	0.170626
PJ N- PJ S	0.204969	0.192092
RJ N- RJ S	0.172477	0.170218

Note: N and S indicate northern and southern portions of each hybrid zone.

Table 11.

Eigenvalues from PCA of Genetic Variation Between Parental Species and Populations from Hybrid Zones

Principle Component	**Eigenvalue**	**%Variance**	**Cumulative**
1.00	3.03	47.71	47.71
2.00	2.18	34.40	82.11
3.00	1.14	17.89	100.00

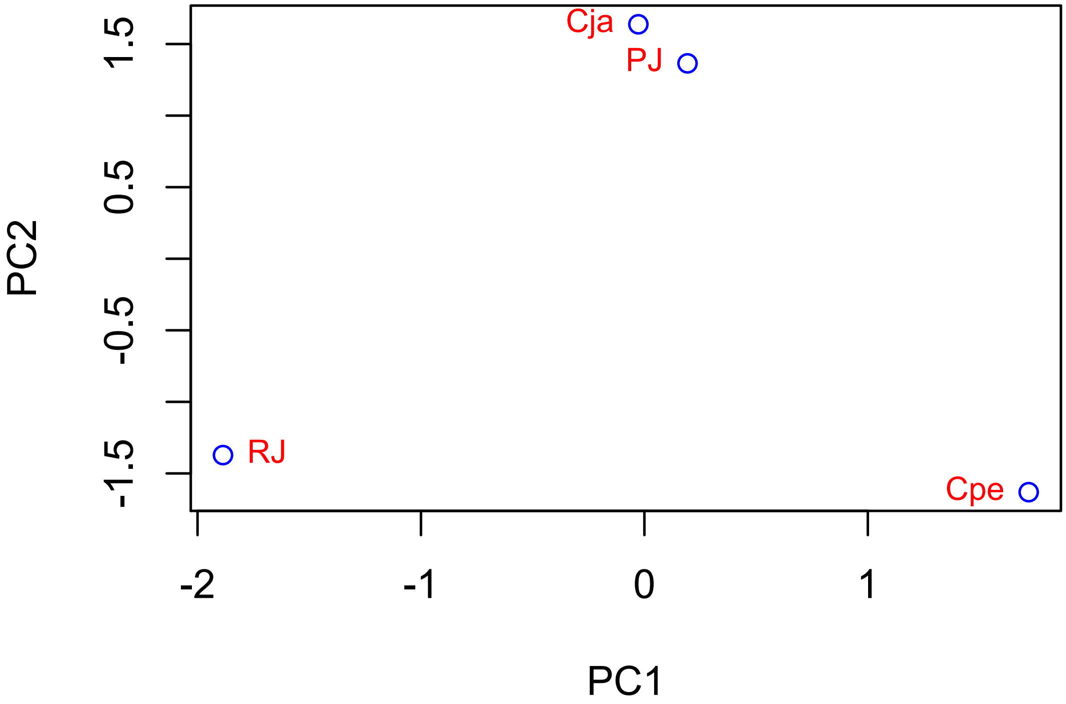

Figure 11. Principle components analysis of microsatellite allele frequencies. Plot of first and second components from the principle components analysis showing genetic differences in terms of microsatellite frequencies between parental species (Cja indicates *C. jacchus* and Cpe indicates *C. penicillata*). PJ and RJ refer to the two hybrid zones.

level of differentiation (uncorrected F_{st} = 0.183). AMOVA analysis revealed that a significant portion (*P*-value=0.000) of genetic variation occurred at the species level (32% Rst-based). The northern and southern sides of the respective hybrid zones also showed significant pairwise levels of genetic variation (*P*-value=0.000) at a similar level observed for parental populations. AMOVA indicated that 26% of variation in the PJ zone is found between northern and southern subpopulations (*P*-value=0.000, Rst-based**).** In the RJ zone, 14% of genetic variation was found between population separated by highway BR-101.

In the PCA (Figure 11 and Table 11), the first and second component accounted for 82.11% of the total variation in population allele frequencies. A bivariate plot shows that the first and second components define differences between *C. jacchus* and *C. penicillata*. On the other hand, strong similarity in allele frequencies between *C. jacchus* and PJ hybrid zone marmosets is observed along bivariate plot axes. The RJ hybrid zone population was distinct from the parental species along the first component, but similar to *C. penicillata* along the second component.

Hybrid zone admixture patterns. For all cluster analyses, admixture coefficients are relative to *C. penicillata* ancestry with *q*=1.0 indicating full ancestry and *q*= 0.0 indicating no *C. penicillata* ancestry. All runs conducted in STRUCTURE were concordant for *C. jacchus* and *C. penicillata* admixture levels in hybrid individuals*,* and results averaged across 10 runs by CLUMPP are shown in Figure 12. The 90% confidence intervals for *q*-values of hybrid individuals are shown in Figure 13. Within both the PJ and RJ zones, all sampled individuals had admixture coefficients within the range of $0.10<q<0.90$, indicating a strong possibility that most of these marmosets were

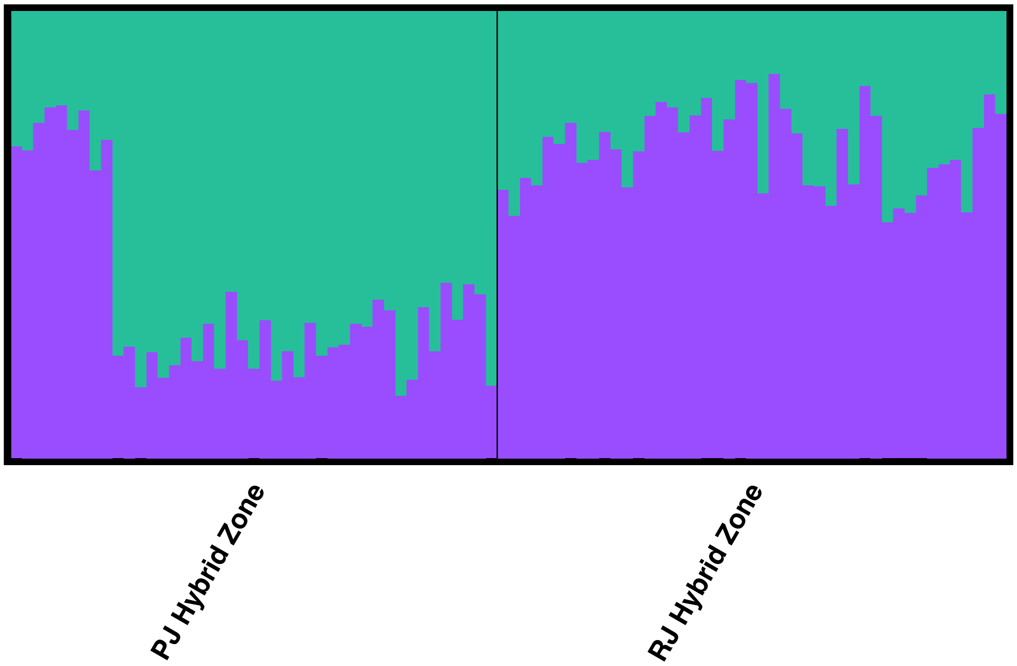

Figure 12. Plot of *C. jacchus* and *C. penicillata* admixture within hybrid zones as assigned by STRUCTURE. STRUCTURE only calculated admixture coefficients for non-reference samples. Purple and green bar proportions indicate ancestry attributed to *C. penicillata* and *C. jacchus,* respectively. First half of the figure contains individuals sampled within the PJ hybrid zone and the second half of the figure contains individuals sampled within the RJ hybrid zone.

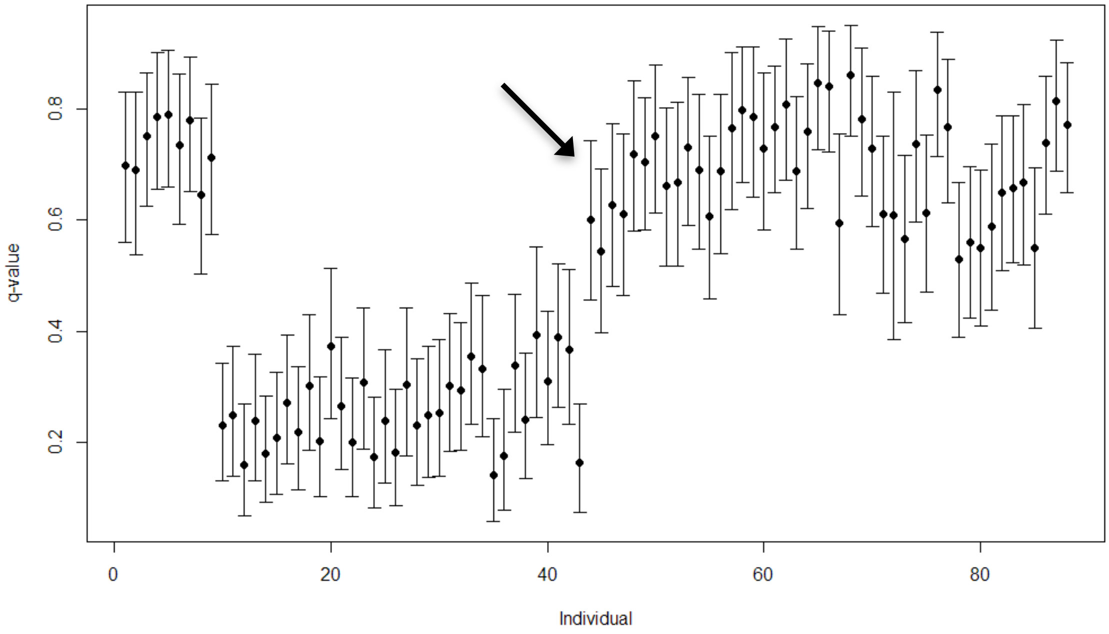

Figure 13. Plot of STRUCTURE *q*-values and 90% confidence intervals for individuals sampled in the RJ and PJ zones. *Q*-values are in reference to *C. penicillata* (0 indicates no *C. penicillata* ancestry and 1 indicates full *C. penicillata* ancestry). The PJ and RJ zones compose the first and second respective halves of the plot. The black arrow represents the first RJ zone individual.

C. penicillata x *C. jacchus* hybrids. The average admixture coefficient for the entire PJ zone was *q*=0.36, ranging from 0.14 to 0.79. Marmosets showed on average *q*=0.75 on the south side of the river within the PJ zone, and an average of 0.26 on the north side of the river. Thus, this indicates that the former possess mostly ancestry from *C. penicillata* and the latter possess ancestry mostly from *C. jacchus.* Ten PJ zone individuals had 90% confidence *q*-value intervals that fell into the non-admixed range, with 7 marmosets sampled north of the river possessing full *C. jacchus* ancestry and 3 marmosets sampled south of the river possessing full *C. penicillata* ancestry. The average admixture for marmosets in the RJ zone was *q*=0.69, with a range of 0.53 to 0.86. Average *q*-values on the north side of this zone were 0.75 and on the south side average values were 0.60, thus overall, ancestry on both sides of the RJ zone tends more towards *C. penicillata* than *C. jacchus.* Nine RJ zone marmosets, all from the northern side, showed 90% confidence interval ranges that fell into the range of *q*-values of pure *C. penicillata.*

Admixture coefficients calculated by BAPS were concordant between replicate runs, but BAPS coefficient values for all hybrid zone individuals (Figure 14) were lower than those calculated by STRUCTURE. BAPS considered 19 individuals within the PJ zone as pure *C. jacchus.* The average BAPS coefficient in the PJ zone was *q*=0.20, ranging from 0 to 0.68, and the average RJ zone BAPS coefficient was *q*=0.53, with a range from 0.31 to 0.77. As with STRUCTURE estimates for the north PJ side, ancestry tended towards *C. jacchus* (average *q*=0.09) and on the south side ancestry was biased towards *C. penicillata* (average *q*=0.63). BAPS *q*-values, in contrast to STRUCTURE, indicated pure *C. jacchus* ancestry for 18 individuals on the north PJ zone side. The

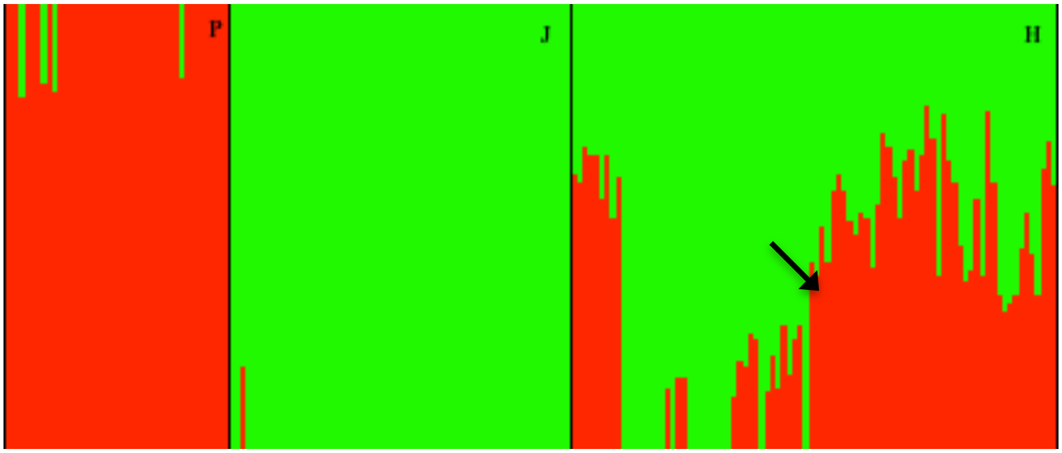

Figure 14. Plot of *C. jacchus* and *C. penicillata* BAPS admixture probabilities. Figure is divided by reference *C. penicillata* individuals (P), reference *C. jacchus* (J), and hybrid zones (H). The PJ and RJ zones compose the first and second respective halves of the H partition. The black arrow represents the first RJ zone individual. Red and green bar proportions indicate ancestry attributed to *C. penicillata* and *C. jacchus,* respectively.

average north-side RJ zone BAPS *q*-value was 0.61, while the south-side average was 0.41, a trend that agreed with that of STRUCTURE *q*-values. No marmosets within the RJ hybrid zone were of pure *C. jacchus* or *C. penicillata* ancestry according to BAPS *q*-values.

Simulation results. Simulation files contained the full set of pure *C. jacchus* and *C. penicillata* multilocus genotypes, plus 10 each of F1 hybrids, F2, hybrids, and backcross hybrids for each species. Table S6 shows summary information across 10 simulated STRUCTURE runs, averaged across 10 replicate runs each. These data show that STRUCTURE classified all simulated hybrids with 100% efficiency at the 0.9 *q*-value threshold. Further, 90% confidence intervals for *C. jacchus* and *C. penicillata* backcross hybrids stayed within our *q*=0.90 threshold for hybrid classification. Thus our dataset has 100% power to detect F1, F2, and backcross hybrids of both species using the STRUCTURE clustering algorithm. Table S7 shows a summary of *q*-values and *q*-value ranges for simulated BAPS runs averaged across 5 repetitions per simulated dataset. BAPS showed a 100% efficiency of correct hybrid assignment for F1, F2 and *C. jacchus* backcross hybrids. BAPS calculated full *C. penicillata* ancestry for 1 out of 10 *C. penicillata* backcross hybrids in 2 out of 5 datasets, giving it an average of 96% efficiency of correct assignment for this hybrid class. STRUCTURE *q*-values tended to be higher than BAPS *q*-values. However, our reference data set of pure individuals allowed both programs to estimate, on average, appropriate admixture values expected for F1, F2, and back-cross hybrids. STRUCTURE and BAPS estimated admixture levels within the PJ and RJ zones are proportionally similar to simulated early and later generation hybrids.

Discussion

Differentiation between *C. jacchus* and *C. penicillata*. We genetically characterized *C. penicillata* and *C. jacchus* interbreeding at a natural and anthropogenic hybrid zone using a large panel of 44 autosomal microsatellite loci and reference samples from both parental species. While the reference dataset contained no species-specific loci between the two study species, there were differences in frequencies of overlapping alleles and also some private alleles within species. Given the very recent divergence date estimated by Perelemen et al. (2011) for *C. penicillata* and *C. jacchus,* diagnostic loci between species will probably emerge with time. Our microsatellite panel combined markers from several previous studies of wild and captive *C. jacchus* (e.g., Nievergelt et al., 2001; Raveendran et al., 2008; Katoh et al., 2009). There have not been any published microsatellite data for *C. penicillata*. Thus this combined marker set enabled us expand previous reports of *C. jacchus* genetic diversity and give an initial report of *C. penicillata* genetic diversity by using a large number of microsatellite loci in a large number of samples from both species. Averages at microsatellite loci were slightly higher in *C. penicillata* in than *C. jacchus* for the number of total observed alleles, private allele number, allelic richness, and expected heterozygosity. Similar differences in diversity between the two species were also observed at the mtDNA CR as described in Chapter One. Nonetheless, if our reference sample covered a larger area of the *C. jacchus* range, reported genetic diversity indices may have been closer to that reported for *C. penicillata.* We found evidence for moderate levels of differentiation in the parental species using various population genetic and clustering techniques, and post-hoc simulations of the reference dataset indicated it is powerful enough to detect hybrids with varied levels of admixture.

Genetic diversity and admixture within hybrid zones. Genetic diversity indices within the PJ and RJ hybrid zones were broadly similar to those found in the two parental species. Nonetheless, cluster analyses as well as PCA painted a more complex picture of the two hybrid zones. The general observed geographical distribution of admixture patterns within the PJ zone match the geographical boundary of *C. jacchus* to the north of the São Francisco River and *C. penicillata* to the south (Rylands et al., 1993 and 2009). Observed admixture patterns and PCA groupings also suggest that northern subpopulations receive more gene flow from parental *C. jacchus,* and subpopulations to the south exchange genes mostly with parental *C. penicillata* populations. Thus, this pattern suggests an important evolutionary role for the São Francisco River as a barrier to gene flow between *C. jacchus* and *C. penicillata.* However, we do see evidence of two way gene flow admixture between individuals on either side of the river, as some *C. jacchus* ancestry exists in the south bank and some *C. penicillata* genetic input was observed in the north.

The admixture patterns above beg the question of a proximate mechanism of the São Francisco River as a leaky barrier to gene flow between marmoset species. It is thought that gene flow between organisms on either side of the river is bridged by islands and peninsulas shaped through time by the river that occasionally come into contact with the river banks (personal observation, L.C.M. Pereira). Our mtDNA data showed that individuals at the Bom Jesus site on the *C. jacchus* side of the PJ zone possessed *C. pencillata* D-loop haplotypes and these haplotypes were closely related to haplotypes possessed by marmosets found directly across the way on the *C. penicillata* side of the zone. A large island was found between the two sides of the river at this particular spot. Many Bom Jesus marmosets also had admixed phenotypes that ranged from intermediate to *C. jacchus*-like (Chapter One). Admixture coefficients for the Bom Jesus marmosets show among the highest components of *C. penicillata* ancestry for marmosets sampled on the northern side of the PJ zone. Thus, both our autosomal and nuclear data show evidence favoring islands and peninsulas as corridors for gene flow between the northern and southern banks of the São Francisco River.

Cluster admixture coefficients showed that individuals sampled on the southern side the PJ zone had mostly *C. penicillata* ancestry. However, the majority of our PJ zone samples came from the northern size of the zone (largely the result of limited access to sampling sites on privately held farms on the south side), and there was a dominant *C. jacchus* ancestry component in these individuals. Grouping patterns within the PCA corroborate our PJ zone cluster data. Since our genetic sampling within the PJ zone focused primarily on the *C. jacchus* north side of the São Francisco River, we most likely overrepresented the *C. jacchus* ancestry component within the PJ zone as a result of an unintended sampling bias. Extending sampling along the south bank of the São Francisco River would probably continue to support the observed pattern of majority *C. penicillata* ancestry for marmosets found on the south side of the PJ zone.

Analyses of the RJ hybrid zone indicated more intermediate levels of *C. jacchus* and *C. penicillata* admixture, with a slight bias towards *C. penicillata* ancestry. The RJ zone contained, on average, a somewhat lower number of alleles and allelic richness than that observed in either parental population, most likely a consequence of the lack of direct gene flow between this zone and parental populations. PCA indicated that alleles found within the RJ zone were closer to alleles found *C. penicillata* than in *C. jacchus* and alleles frequencies differed from parental populations. Surprisingly, heterozygosity levels within the RJ zone were comparable to those of parental populations, despite the isolated location of this zone in relation to the distribution of *C. jacchus* and *C. penicillata.*

Interestingly, our mtDNA results paint a different picture of genetic variation within the RJ zone, although they also show a stronger component of *C. penicillata* ancestry than that of *C. jacchus* in the zone. We recovered only 2 *C. penicillata* and 1 *C. jacchus* mtDNA D-loop haplotypes within the RJ zone. It is sometimes assumed that populations of introduced animals are depauperate of genetic variation due to the effect of drift on small founder populations, but multiple introductions can boost genetic diversity in introduced populations (Facon et al., 2006; Hufbauer, 2008). Given the zone's various, albeit few, mtDNA CR haplotypes and high heterozygosity at microsatellitle loci, it is possible that multiple introductions of both parental species occurred in the RJ hybrid zone. MtDNA may have already been strongly affected by genetic drift within the RJ zone, as mtDNA has a smaller effective population size (Ballard and Whitlock, 2004). On the other hand, autosomal loci may still be buffered from drift due to their larger effective population size, but with time we would also expect heterozygosity levels at nuclear loci to degrade in the RJ zone. How fast this may occur is not clear, but marmoset populations within this zone tend to be isolated from one another amongst forest fragments and are probably cut off from gene flow between fragments as well as from parental populations. This level of isolation probably threatens RJ zone subpopulations with increasing levels of inbreeding.

Implications for marmoset evolution, conservation, and futureesearch. Genetic signatures of hybridization can vary for each hybrid zone as evolutionary and ecological dynamics can also differ between any set of interbreeding taxa. Bimodal hybrid zones at geographical contact points between different populations contain individuals that are genotypically similar to one or the other parental taxa with few intermediates, usually requiring evidence of strong linkage disequilibrium and indicating strong assortative mating and pre-zygotic reproduction barriers (Jiggins and Mallet, 2005). Intermediate genotypes dominate in a unimodal contact hybrid zone where assortative mating is not as strong (Jiggins and Mallet, 2005). In the PJ zone, ancestry can be bimodally classified as either mostly *C. jacchus* or *C. penicillata,* whereas the highly admixed subpopulations of the RJ zone possess a more unimodal genotype distribution. Compared to the RJ zone, we also observed much higher levels of Hardy-Weinberg disequilibrium and LD within the PJ zone. The conditions observed within the PJ zone are similar to what Arias et al. (2008) observed in a *Heliconius erato venus* and *Heliconius erato chestertonii* butterfly hybrid zone, which the authors presented as strong evidence of incipient speciation in these taxa. Reproductive isolation between various *Callithrix* species is not complete (Coimbra-Filho et al., 1993), but the São Francisco River is an important geographical, albeit porous, reproductive barrier in the PJ zone that drives assortative mating within the two distinct lineages found on the northern and southern river banks.

Geographical barriers are probably important to overall speciation in *Callithrix* given the historical geographic range separation of marmoset species described in Rylands et al. (1993&2009). On the other hand, data from the PJ zone suggests that some level of hybridization which does not disrupt the genetic integrity of species may be part of the evolutionary history of *Callithrix.* Thus, geographical barriers probably serve as important buffers against levels of hybridization that would erode the genetic integrity of seperate marmoset lineages. Such barriers are not present within the RJ zone, where we essentially observed complete collapse of genetic integrity within *C. penicillata* and *C. jacchus.* Our autosomal data indicate that individuals sampled within the RJ hybrid zone represent a hybrid swarm where parental genomes have been replaced with highly admixed hybrid genomes. From an evolutionary point of view, pure *C. jacchus* and *C. penicillata* genomes are probably extinct within the RJ hybrid zone.

Other areas where *C. jacchus* and *C. penicillata* have been introduced outside of their natural distributions occur in the state of Rio de Janeiro, as well as in the Brazilian states of São Paulo and Minais Gerais (personal observation, I. de Oliveira e Silva and Vanner Boere). These areas include ranges of the three previously described endangered *Callithrix* species. *Callithrix jacchus* and *C. penicillata* may pose an ecological as well as genetic threat to other marmoset species, since they are more morphologically specialized than other marmosets to exploit disturbed habitats (Rylands and de Faria, 1993), which characterizes much of the Brazilian Atlantic Forest (Ribeiro et al., 2009). In the RJ zone, hybrid marmosets also may pose a threat to the endangered golden lion tamarin (*Leontopithecus rosalia*) (Ruiz-Miranda et al., 2000). These lion tamarins are part of the native biota of the RJ hybrid zone and share a similar biology and ecology to the introduced marmosets. Evidence from Ruiz-Miranada et al. (2006) indicates that the two taxa compete for similar resources.

Our data show that hybridization is likely a natural part of the evolution of marmosets, yet it can also threaten the genetic integrity of marmoset species under human-induced conditions. As recommended by Allendorf et al. (2001), the distinction between natural and anthropogenic hybridization should be made whenever possible in conservation decisions regarding marmosets. Additionally, demographic factors need to be taken into account regarding admixed populations, as marmoset species and populations can vary between combinations of invasive/native and threatened/stable. We recommend expanding genetic research on marmoset hybridization within both anthropogenic and natural contexts because such comparative data will be valuable in establishing the evolutionary and conservation values of pure and admixed populations. These data will become particularly indispensable if reports of recent marmoset hybridization continue to rise and increase our need to understand the effects and to assess the value of admixed populations evolving under variable contexts and conditions.

CHAPTER 3

APPLICATION OF RESTRICTION-SITE ASSOCIATED DNA-SEQUENCING TO THE STUDY OF GENOMIC DIVERSITY AND DIVERGENCE IN EASTERN BRAZILIAN MARMOSETS

The eastern Brazilian *Callithrix* marmosets are compelling models for the study of primate evolution, particularly from a genetic and genomic perspective, due to their singular mix of unique biological traits and adaptive specializations. For example, as members of the Neotropical primate family Callitrichidae, *Callithrix* marmosets possess a number of unique primate characteristics including cooperative breeding and social modulation of female reproductive function (Digby et al., 2007; Smith et al., 1997). *Callithrix,* particularly *C. jacchus* and *C. penicillata,* also exhibits a highly specialized morphology for gummivory (Ferrarri, 1993). While species-level relationships between *Callithrix* species are not yet fully resolved, there is an emerging picture of a complex past and recent demographic history for this genus (see Chapter One). *Callithrix* is a recently diverged genus (Barroso et al, 1997; Marroig et al, 2004; Pereleman et al., 2911; Schneider et al., 2012), and past interspecific interbreeding (or hybridization) may be common during divergence of young taxa (Mallet, 2005). Further, reports of recent natural and anthropogenic (i.e., driven by human induced environmental change) hybridization have been increasing in the primatological literature (Malukiewicz, 2012; Mendes, 1997a&b; Passanami et al., 1996; Ruiz-Miranda et al., 2000; Ruiz-Miranda et al., 2006).

Two main challenges exist in approaching the study of marmoset evolutionary history from genetic and genomic perspectives: (1) the occurrence of chimerism in

Callithrix (Ross et al., 2007; J.Malukewicz personal observation) and (2) a limited number of available genetic markers. *In utero*, callitrichid twins exchange stem cells, especially those derived from blood tissues, due to fusion of placentas, which results in individuals possessing self and sibling genomes in tissues developed from shared stem cells (Ross et al., 2007). Such "genome sharing" can complicate genotyping for genetic studies. Use of low-chimerism tissues such as skin and other parts of the epithelium can help side-step genetic challenges brought on by chimerism. The employment of traditional molecular techniques such as cloning and Sanger sequencing can also help with chimerism challenges. Finding a large number of genetic markers for *Callithrix,* as it is recommended for genetic studies of young species (e.g., Vaha and Primmer, 2006), is perhaps the more challenging problem, as availability of genetic markers for marmosets is limited. Currently available markers include several regions of mitochondrial DNA (mtDNA) (e.g., Tagliaro et al., 1997; Tagliaro et al., 2000) and nuclear microsatellite loci (e.g., Galbusera and Gillemot, 2008; Nievergelt et al., 1998; Raveendran et al., 2008). However, the application of these traditional markers to evolutionary genetic studies can be very time consuming, expensive, and generates relatively limited genetic information. Fortunately, recent advances in cost- and time-effective next generation sequencing technologies (NGS) can substantially increase power and resolution relative to the above traditional markers for addressing evolutionary biology questions.

One novel NGS technique is called restriction associated DNA sequencing (RADseq), which has already been applied to evolutionary genetics studies of several nonprimate animals (e.g., Catchen et al., 2013; Hohenlohe et al., 2011; The *Heliconius* Genome Gonsortium, 2012). RADseq is based on the simultaneous discovery and genotyping of a very large number of single nucleotide polymorphisms contained within short DNA sequences (or tags) that are located close to restriction enzyme recognition sites (described in more detail in Baird et al., 2008). The Illumina sequencing platform that underlies RADseq is carried out a manner that emulates molecular cloning and is very cost effective for studies aiming to examine a large number of individuals at a large number of genetic loci.

Here, we apply RADseq in the characterization of intra- and interspecific genomic variation as well as divergence between two marmoset species, *C. jacchus* and *C. penicillata*. These two species are the youngest members of the *Callithrix* genus, having diverged as sister species less than a million years ago (Perelmen et al., 2011). This study respresents the first application of this technique to the *Callithrix* genus, and RADseq holds great potential in addressing several open issues regarding evolution of the species including: (1) resolving interspecific evolutionary relationships, (2) identifying genes important to divergence within *Callithrix*, and (3) studying the evolutionary dynamics of marmoset hybridization. The main focus of the study is to establish the utility of RADseq in performing a genomic scan of *C. jacchus* and *C. penicillata* and to begin to address questions about population history and structure. Resulting patterns of genomic variation and divergence between these two species will provide a foundation for future genomic studies of more species that address larger questions of demographic and evolutionary history in the *Callithrix* genus.

Methods

Table 12.

Sex, species, WGA[a] Status, Origins and Associated Latitude/Longitude Coordinates when Applicable for Wild Samples

Sample	Sex	Species	WGA[a]	Origin	Lat/Long
cja013	F	*C. jacchus*	NO	CRC[b], Omaha, NE, US	NA
cja014_a	M	*C. jacchus*	NO	CRC[b], Omaha, NE, US	NA
cja014_b	M	*C. jacchus*	YES	CRC[b], Omaha, NE, US	NA
cja030	F	*C. jacchus*	YES	IBAMA CETAS[c], Recife, PE, Brazil	NA
cja031	M	*C. jacchus*	NO	IBAMA CETAS[c], Recife, PE, Brazil	NA
cja034	M	*C. jacchus*	YES	IBAMA CETAS[c], Recife, PE, Brazil	NA
cja041	F	*C. jacchus*	YES	IBAMA CETAS[c], Recife, PE, Brazil	NA
cpe001	F	*C. penicillata*	YES	CRC[b], Omaha, NE, US	NA
cpe009	M	*C. penicillata*	YES	Muriaé, MG, Brazil	21° 7' 15.60"S 42° 22' 2.50"W
cpe018	M	*C. penicillata*	YES	Brasilía, DF, Brazil	15°45'1.15"S 47°50'34.10"W
cpe022	F	*C. penicillata*	NO	Brasilía, DF, Brazil	15°51'56.46"S 47°58'13.94"W
cpe023	F	*C. penicillata*	NO	Brasilía, DF, Brazil	15°42'33.45"S 47°54'44.80"W
cpe026	F	*C. penicillata*	YES	Brasilía, DF, Brazil	15°54'38.07"S 47°57'10.64"W
cpe041	M	*C. penicillata*	YES	IBAMA CETAS[c], Goiânia, GO, Brazil	NA

[a] WGA refers to "whole genome amplified"

[b] Callitrichid Research Center, University of Nebraska at Omaha

[c] Wild Animal Triage Center, Brazilian Institute of the Environment and Natural Resources

Sample collection and whole genome amplification (WGA). Due to the occurrence of chimerism in marmosets (discussed above), which can complicate genotype determination of nuclear markers, only DNA derived from epithelial skin tissues was used. Skin tissue is a relatively low-chimerism tissue, and details of chimerism are further discussed in Chapter Two. The opportunistically selected samples for this study consisted of 6 *C. jacchus* and 7 *C. penicillata* collected from captive and wild marmoset populations. Information on species, sex, whole genome amplification status WGA (explained below), and origin of sampled individuals is given in Table 12. More detailed information about collection permits, sample collection, storage, sampling sites/facilities, and DNA extraction from these biological samples has been provided in Chapter One. DNA concentrations of these 13 samples were determined with a Qubit 2 Fluorometer (Life Technologies) using the Qubit dsDNA BR Assay Kit (Life Technologies). Three wild *C. jacchus* samples, a single captive *C. penicillata* sample, and 4 wild *C. penicillata* samples initially showed DNA amounts too low for further processing. DNA amounts of these samples were increased through WGA with the Repli-g Mini Kit (Qiagen) using manufacturer's instructions for purified genomic DNA. DNA concentrations of WGA samples were rechecked with the Qubit dsDNA BR Assay Kit.

RADseq library preparation and sequencing analysis. A single RADseq library was prepared following a modified version of the RAD pair-ended protocol of Etter and Johnson (2012). First 1000 ng of each DNA sample was digested with 0.5 uL of *Sbf*I-High Fidelity (20 U/uL, New England BioLabs) restriction enzyme, 5 uL of 1X NEB buffer 4 (New England BioLabs), and H_20 in a total reaction volume of 50 uL. Each digestion was incubated at 37°C for 60 minutes, enzyme activity was inactivated at 65°C for 20 min, and reactions were allowed to cool from 30 to 60 minutes. A unique 5-base pair barcode was ligated to each DNA sample for identification in later downstream library preparation and analyses. All barcodes differed from each other by three bases, and each barcode was located within *Sbf*I restriction-site specific P1 adaptors (pg. 146, Etter and Johnson (2012)) that were compatible to the ends of the digested genomic DNA samples. P1 adaptors were ligated to digested DNA samples in a reaction of 1.0 uL 10x New England BioLabs buffer 2 (NEB), 0.6 uL 100 mM rATP (Promega), 0.5 uL concentrated T_4 ligase (2,000,000 U/mL, NEB), 50 uL DNA, 1.15 uL 250 nM P1 adaptor and 6.75 uL H_20 in a total reaction volume of 60 uL. The ratio of P1 adaptor to genomic compatible ends to use in the ligation reaction should strike a balance between a ratio too low for sufficient library amplification and a ratio high enough to promote library contamination with P1 adaptor (further detail is described in note 13 pg. 148 of Etter and Johnson (2012)). Thus, amount of P1 adaptor used in the ligation reaction was optimized empirically by systematically varying the above ratio in ligation reactions between 2.5:1 and 20:1. Ligation reactions were incubated from 30 minutes to overnight at room temperature and then heat-inactivated for 10 min at 65°C. Results of enzyme restrictions and adaptor ligations were checked visually through gel electrophoresis using 1.25% agarose, 5 uL of each sample, 6x Orange DNA Loading Dye (Thermo Scientific), and 2 uL Generuler 100 bp DNA Ladder Plus (Thermo Scientific). Eight separate combinations were made of barcoded DNA samples containing equimolar amounts for a total of 1432.9 ng of DNA in a total volume of 86 uL. Both non-WGA and WGA samples of one captive *C. jacchus* individual (cja014) were included in the library to check for consistency in sequence amplification between the WGA sample and the non-WGA sample. The 86 uL combined genomic DNA samples were sheared chemically to an average size of 500 bp with NEBNext dsDNA Fragmentase (New England BioLabs) using 10 uL 10x Fragmentase Reaction buffer, 1 uL 100X BSA and 3 uL dsDNA Fragmentase enzyme. Fragment size results for chemical shearing reactions can vary between individual reactions, thus shearing reactions were carried out at two different incubation times. Manufacturer's instructions were followed for chemical shearing reactions with a modification of incubation time to 7:30 min for three reactions and 7:00 min for another three reactions. Reactions were deactivated with 5uL 0.5 M EDTA. Shearing reactions were concentrated in 20 uL in 1x EB buffer (10 mM Tris-Cl, pH 8.5) with DNA Clean and Concentrator-5 capped columns (Zymo Research), and then mixed with 6X Orange DNA Loading Dye and loaded on a 1.25% agarose electrophoresis gel along with 2 uL of Gene Ruler Generuler 100 bp DNA Ladder Plus. Slices spanning 300 bp to 800 bp of each of each reaction were cut out of the gel and cleaned with the MinElute Gel Purification Kit (Qiagen), with melting of gel slices in QG buffer done at room temperature.

Each cleaned reaction was then concentrated in 20 uL of 1x EB buffer. Initially, four reactions were combined together into a single library, and then subsequently two remaining reactions were combined into another library. Both libraries were processed in the same manner as described below. Each library was cleaned with AMPure XP beads (Agencourt) following manufacturer's directions with a volume of beads equal to 0.65x of each library volume, 70% ethanol, and an upright magnetic bead stand for eppdendorf microcentrifuge tubes. We followed the remaining protocol of Etter and Johnson (2012) to blunt 5' or 3' overhangs of sheared DNA, add an "A" base overhang to the 3' end of blunted DNA library, and ligate P2 adaptors to library DNA fragments. Between each step, the libraries were cleaned with AMPure XP beads as above.

The libraries were eluted in 50 uL 1x EB buffer after the final bead cleaning, and the library concentrations were checked with the Qubit dsDNA BR Assay Kit. Libraries were enriched by polymerase chain reaction (PCR) with the Phusion High-Fidelity PCR Master Mix with HF buffer (New England BioLabs). A test amplification was first carried out using 6 ng (1.88 uL) of each DNA library, 12.5 uL of Phusion High-Fidelity PCR Master Mix, 1 uL 10 uM mix of RAD forward and reverse primers (Etter and Johnson, 2012), and 10.5 uL H_20. The PCR thermocycler program consisted of 30 s at 98°C, 18 cycles of [10 s at 98°C, 30 s at 65°C, 30 s at 72°C], and 5 min at 72°C. The full PCR volumes were loaded next to each unenriched library on a 1.0% agarose gel with 6X Orange DNA Loading Dye, and 2 uL Gene Ruler 100 bp DNA Ladder Plus. The amplified products were 4x brighter than the library template and thus we proceeded with two larger volume PCR library enrichments. The first enrichment used 36 ng of DNA library template originally made from the four shearing reactions (11.2 uL) along with 25 uL of Phusion High-Fidelity PCR Master Mix, 2 uL of10 uM a mix of RAD forward and reverse primers, and 11.8 uL H_20. The second enrichment used 64.4 ng of DNA library template from the four shearing reactions (20 uL) along with 25 uL of Phusion High-Fidelity PCR Master Mix, 2 uL of 10 uM a mix of RAD forward and reverse primers, and 3 uL H_20. The third used 118.33 ng of DNA library template from the last two shearing reactions (34.5 uL) along with 37.5 uL of Phusion High-Fidelity PCR Master Mix, and 3 uL of10 uM a mix of RAD forward and reverse primers. The three PCRs used the same thermocycler program as above but with the total number of cycles reduced to 14. PCR products then were concentrated in 1x EB buffer using DNA Clean and Concentrator-5 capped columns. The concentrated PCR products were run out on 1.25% agarose, in 6X Orange DNA Loading Dye along with 2 uL Generuler 100 bp DNA Ladder Plus. A slice of each PCR product spanning 350 bp to 850 bp was cut out from the gel, cleaned with the MinElute Gel Purification, with the melting of agarose carried out at room temperature. The three PCR products were eluted in 1x EB and combined together for a total volume of 56 uL. DNA concentration of the final library was determined with the Qubit 2 dsDNA BR assay and submitted for sequencing on a single lane of an Illumina HiSeq 2500 sequencer for a total of 100 cycles to produce paired-end reads. The library contained a low complexity region due to all fragments in the library possessing the sequence for the *Sbf*I after unique barcodes at the same set of sites along the length of all fragments. Such low diversity regions may cause data loss during sequencing on HiSeq machines (Krueger et al., 2011). To prevent data loss due to low-diversity fragment regions within the library, the library was mixed with a PhiX control (Illumina) prior to loading onto the HiSeq sequencer. The spiked mixture loaded onto the sequencer consisted of 60% submitted library and 40% of the PhiX control.

Data analysis. Illumina sequence reads were filtered for duplicated PCR sequences using the *clone_filter* program part of STACKS 1.04 software package (Catchen et al., 2013). Read counts form the basis of RADtag genotyping and PCR duplicates may bias base calls as non-independent repeats of the same region (Hohenlohe, 2013). The STACKS *process_radtags* program was used to demultiplex sequence reads by unique identifier barcodes. *Process_radtags* also filters low quality data by dropping reads with a phred score < 10 and any reads possessing uncalled bases.

Reads from individual marmosets were aligned to the most recent version of the marmoset genome (calJac3, genome.ucsc.edu) with BOWTIE2 (Langmead and Salzberg, 2012) under default settings. Only reads that mapped uniquely to the marmoset genome were retained for further analyses. The *mpileup* program within the SAMTOOLS package (Li et al., 2009) under settings of -BQ0 and -d10000000 produced the number of sequenced nucleotides per individual and depth of coverage per nucleotide per individual. Custom bash shell and python3 scripts were used to calculate the average of those two measures for each individual. Then the STACKS *pstacks* program was used to organize reads from each individual into RADtags or "RAD loci", which comprised reads that mapped to the same genomic location of a *Sbf*I recognition site. *Pstacks* was also used to genotype individuals at RAD loci without specifying any minimum depth of coverage for each RAD locus. Coverage of RAD loci across a genome is highly variable due to biases introduced library preparation that include variation of restriction fragment length, restriction site heterozygosity, and PCR GC content (Davey et al., 2013). *Pstacks* uses a maximum-likelihood algorithm that accounts for variation in coverage across RAD loci which implicitly sets its own threshold value for depth of sequencing coverage (Hohenlohe et al., 2011). Next, the STACKS *cstacks* program produced a catalogue of RAD loci across all samples. *Sstacks* compared individual loci against the catalogue to determine an individual's allelic status at the catalogued loci. Due to the availability of the marmoset published genome, *csstacks* and *sstacks* matching was based on genomic position.

The STACKS *populations* program was used to calculate measures of genomic divergence and diversity, requiring variant loci to be present in 70% of samples for a species for intraspecific analyses and in 70% of samples from both species for interspecific analysis. *Populations* produced genomic diversity indices of nucleotide diversity (π), observed heterozygosity (H_O), expected heterozygosity (H_E), and F_{IS} for each variant site. Measures of these indices were based on sites that may have been present in one species and not the other as per the *population* settings above. F_{ST} values measuring genetic divergence between the two species were based on a reduced set of variant loci that were present in both *C. jacchus* and *C. penicillata*. Kernel-smoothed genome wide values of π and F_{ST} were also produced using a sliding window analysis using a Gaussian function that weighed SNPs found within a 150 KB window size (Catchen et al., 2013). Statistical significance of kernel-smoothed F_{ST} indices was determined by 100,000 repetitions of bootstrap resampling. All plots for kernel-smoothed indices were produced by the R software package (R Core Team, 2013).

The R *adegent* 1.3.7 package (Jombart and Ahmed, 2011) was used for further interspecific analyses using the same reduced set of loci as was used in STACKS for calculation of F_{ST} values. First, a principle components analysis was used to assess overlap in allele frequencies between *C. jacchus* and *C. penicillata.* Missing values within the data set were replaced with mean allele frequencies prior to PCA (http://cran.r-project.org/web/packages/adegenet/vignettes/adegenet-basics.pdf). The *adegent* package also used to conduct an analysis of molecular variance (AMOVA) between the two species. Significance of the AMOVA was assessed with 16,000 resampling permutations and based on Euclidian genetic distances between samples.

Table 13.

Listing of Total Number of Sequencing Reads Obtained for Each Individual, How Many of those Reads Did Not Map to the CalJac3 Marmoset Genome, Number of Reads that Mapped to the CalJac3 Marmoset Genome, the Percentage of All Reads that Mapped to the CalJac3 Marmoset Genome, and How Many Reads Mapped Only Once to the CalJac3 Genome

Sample	**Total Read Count**	**Number of Unmapped Reads**	**Number of Reads Mapping to calJac3**	**% of All Reads Mapped that Mapped to calJac3**	**Number of Uniquely Mapped Reads**
cja013	1124293	50190	1074103	95.54%	892187
cja014_a	1046818	42240	1004578	95.96%	838120
cja014_b	1357022	107175	1249847	92.10%	1041251
cja030	1718919	350983	1367936	79.58%	1139404
cja031	1884681	77366	1807315	95.90%	1475358
cja034	4662523	4492530	169993	3.65%	154189
cja041	2103837	326989	1776848	84.46%	1430297
cpe001	727253	93890	633363	87.09%	511540
cpe009	1729574	97276	1632298	94.38%	1298563
cpe018	2822518	274786	2547732	90.26%	2033948
cpe022	1905000	81402	1823598	95.73%	1529167
cpe023	2260980	108278	2152702	95.21%	1798167
cpe026	1662269	87518	1574751	94.74%	1284841
cpe041	6441167	6432166	9001	0.14%	7451
Total	31446854	12622789	18824065	N/A	15434483

Table 14.

Total Number of Nucleotide Bases Sequenced per Individual and Average Depth of Sequencing Coverage per Nucleotide per Individual

Sample	**Number of Bases Sequenced**	**Mean Base Coverage**
cja013	11213708	7.56
cja014_a	N/A	N/A
cja014_b	9302953	10.63
cja030	10708110	10.10
cja031	10921903	12.83
cja034	N/A	N/A
cja041	20878064	6.51
cpe001	8923721	5.44
cpe009	10778912	11.44
cpe018	22196487	8.70
cpe022	12208252	11.90
cpe023	12686727	13.46
cpe026	11506045	10.61
cpe041	N/A	N/A
Average	**13040297.9**	**9.93**

Results

Sequencing results and coverage. The total number of sequencing reads obtained from pooling several restriction digested *C. jacchus* and *C. penicillata* genomes for sequencing on a single HiSeq 2500 lane was 54,528,646. The application of the *clone_filter* program to these sequences filtered the number of sequences to 48,186,141. The total number of sequencing reads demultiplexed by individual barcodes and changes in read numbers after application of various filters is shown in Table 13. STACKS *process_radtags* filtered the total number of sequences to 31,446,854. On average, 79% of all reads mapped to the marmoset calJac3 genome. However, the majority of sequences from two individuals, cja034 and cpe041, failed to align to the marmoset genome and were excluded from further analyses. Without those two individuals, a mean of 92% of sequences per individual aligned to the marmoset genome. There were a total of 15,434,483 sequences across all sampled individuals that mapped uniquely to the marmoset genome, which made up 49% of all reads that passed the *process_radtagps* filters. To check for amplification bias in WGA samples, several randomly chosen sequences from the same RAD locus were compared between WGA and non-WGA samples of cja014. No differences were observed between these two samples, and thus the non-WGA cja014 was excluded from further analyses. Considering only uniquely mapped sequence reads from the remaining 11 individuals listed in Table 14, there was an average of 13040297.9 nucleotides sequenced per individual at an average depth of coverage of 9.93x, ranging from 5.44x to 13.46x.

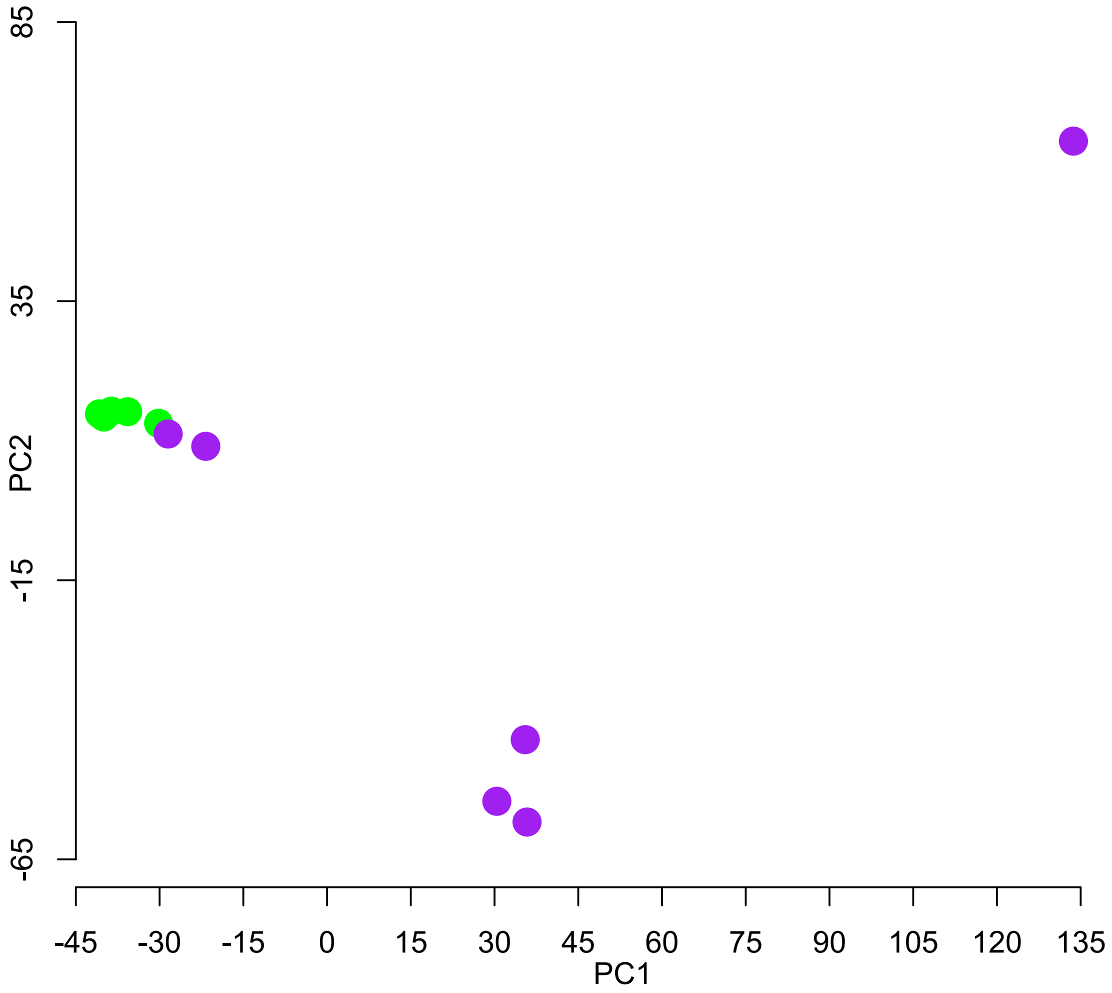

Figure 15. PCA plot of the first two components of genetic variation between *C. jacchus* and *C. penicillata.* Individual *C. penicillata* are represented in purple and individual *C. jacchus* are shown in green.

Table 15.

Eigenvalues of Principle Components of Genetic Variation of 3549 Loci

	Eigenvalue	**% Variance**
1	2687.87	0.43
2	1236.27	0.20
3	678.02	0.11
4	645.35	0.10
5	258.48	0.04
6	225.39	0.04
7	177.12	0.03
8	145.55	0.02
9	106.14	0.02
10	92.56	0.01

Intraspecific genetic diversity and interspecific divergence. A comparison of *C. jacchus* and *C. penicillata* patterns of intraspecific genetic diversity shows higher levels of variation in the latter than in the former, and these patterns are summarized in Table S8. When considering only "variant sites", or those nucleotide positions, which are polymorphic in at least one species, the average major allele frequency in *C. jacchus* was 0.933 (+/- 0.002SE) and 0.709 (+/- 0.002SE) in *C. penicillata.* The observed heterozygosity (H_O) for variant sites was 0.101 (+/- 0.004SE) for *C. jacchus* and 0.411 (+/- 0.003SE) for *C. penicillata.* While all variant sites were polymorphic within *C. penicillata,* only 19.49% were polymorphic within *C. jacchus*. Variant nucleotide diversity levels were higher for *C. penicillata* at 0.423 than for *C. jacchus* at 0.099.

When considering "all positions." which represent all nucleotides across RAD tags regardless whether a site is polymorphic between or within species or not, there is an expected drop in values of genetic diversity indices in both species. *C. penicillata* H_O is lowered to 2.5100E-04 and *C. jacchus* H_O becomes 5.7488E-05. Major allele frequency for both species becomes 0.999. Levels of observed heterozygosity were close to expected values at both the "variant" and "all" position levels. Nucleotide diversity changed to 5.5677E-05 in *C. jacchus* and to 2.5900E-04 in *C. penicillata.* Neither species showed F_{IS} values at the "variant" or "all" levels that greatly deviated from zero. *C. jacchus* showed no private alleles, while *C. penicillata* exhibited a total of 2929 private alleles.

The average F_{ST} value across all variant sites present in both *C. jacchus* and *C. penicillata* was 0.08. An AMOVA showed that 35% of genetic variation was partitioned between the two species and the remainder was partitioned within the two species (*P*-

value = 0.0172). Figure 15 shows a PCA plot of the first two components of genetic variation between *C. jacchus* and *C. penicillata*. Eigenvalues of all components are shown in Table 15, and the first two PCs account for 63% of genetic variation between the study species. The six *C. penicillata* show a greater level of intraspecific variation than the five *C. jacchus* as the former spread over 3 separate quadrants and the latter are concentrated into the same quadrant. The low levels of overall genetic divergence shown by the above F_{ST} value average are corroborated by the clustering of *C. jacchus* with two *C. penicillata* individuals.

Genomic architecture of *C. jacchus* and *C. penicillata* nucleotide diversity, and genetic differentiation. Kernel-smoothing sliding window analyses were performed genome-wide in *C. jacchus* and *C. penicillata* on nucleotide diversity and F_{ST} measures of genetic divergence (Figures 16-18). Genome-wide nucleotide diversity values throughout the *C. jacchus* genome tended towards zero and generally indicated low levels of intraspecific variation (Figure 16). Patterns of nucleotide diversity did increase in a few regions of each *C. jacchus* chromosome. On the other hand, levels of nucleotide diversity across the *C. penicillata* chromosome resembled a pattern of “rolling peaks and valleys” (Figure 17). Visual inspection of Figures 16 and 17 shows some homologous genomic regions in both species have relatively high levels of nucleotide diversity. Examples of these regions are located on chromosome 1 around 200 MB, chromosome 12 around the 40 MB mark, and between 30 and 40 MB on chromosome 22.

In terms of population divergence, most F_{ST} values across the *C. jacchus* and *C. penicillata* tended towards zero (Figure 18). However, there were several regions across

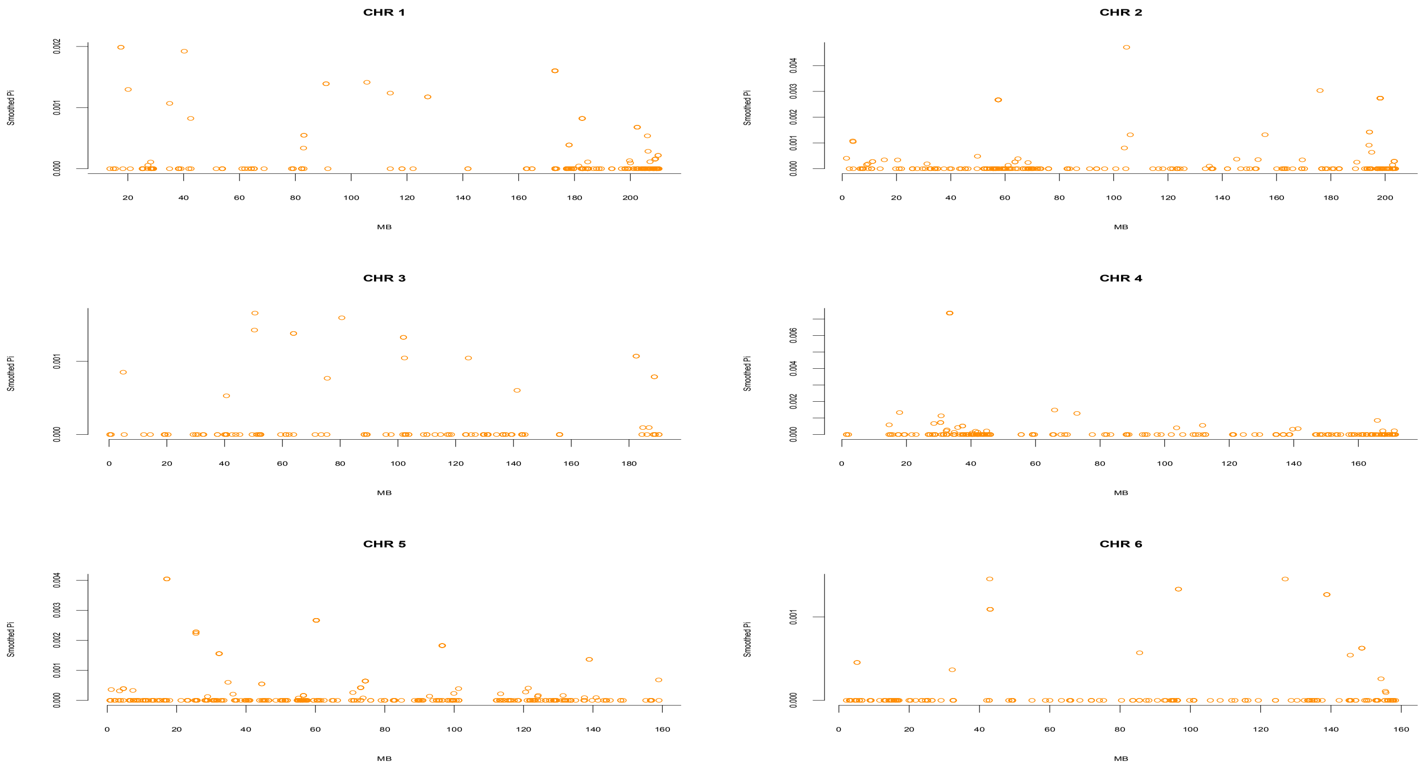

Figure 16. Genome-wide distribution of kernel-smoothed nucleotide diversity across all *C. jacchus* chromosomes. The *x*-axis shows the length of each chromosome in megabases of sequence. The *y*-axis shows kernel-smoothed measures of nucleotide diversity.

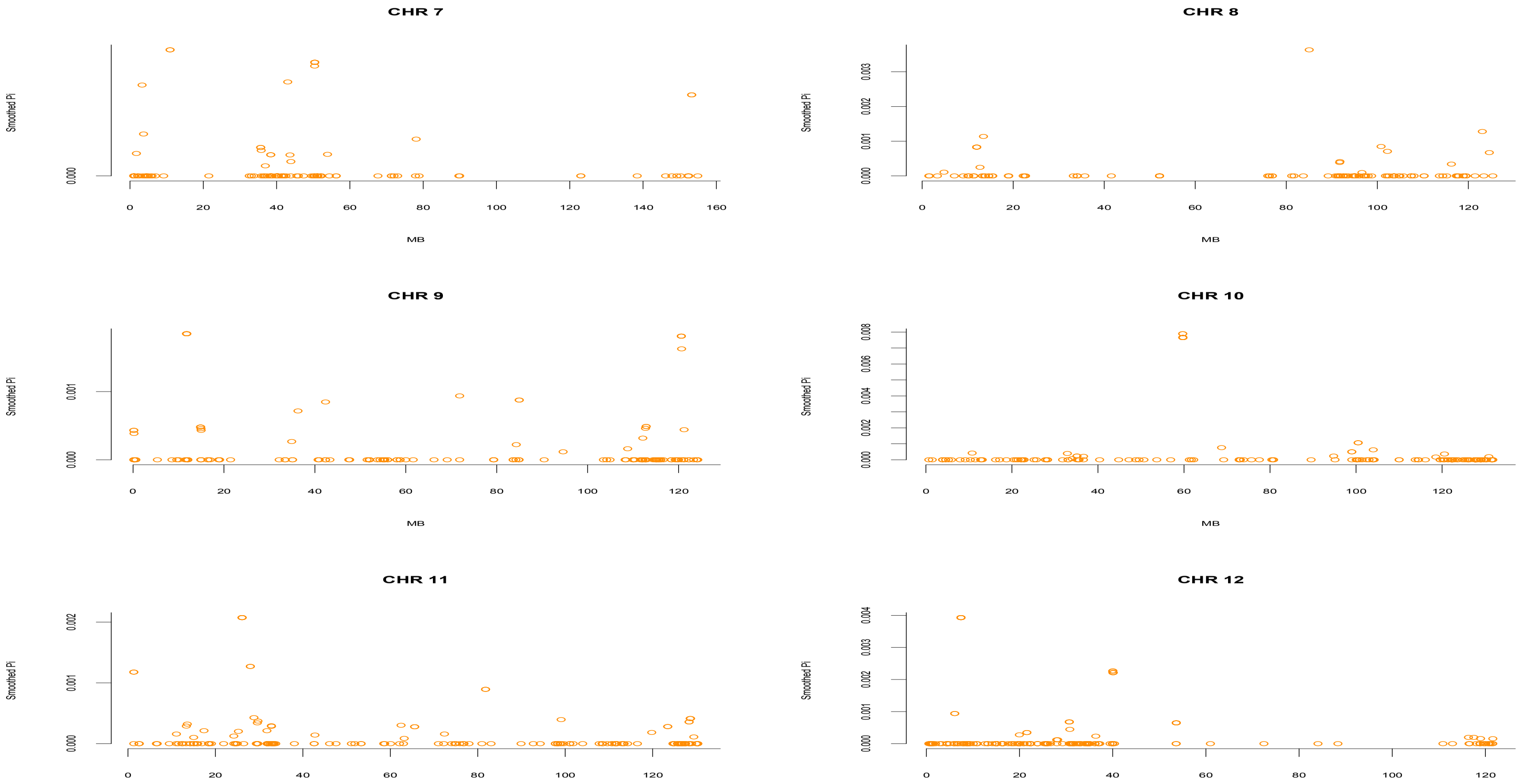

Figure 16 cont'd. Genome-wide distribution of kernel-smoothed nucleotide diversity across all *C. jacchus* chromosomes. The *x*-axis shows the length of each chromosome in megabases of sequence. The *y*-axis shows kernel-smoothed measures of nucleotide diversity.

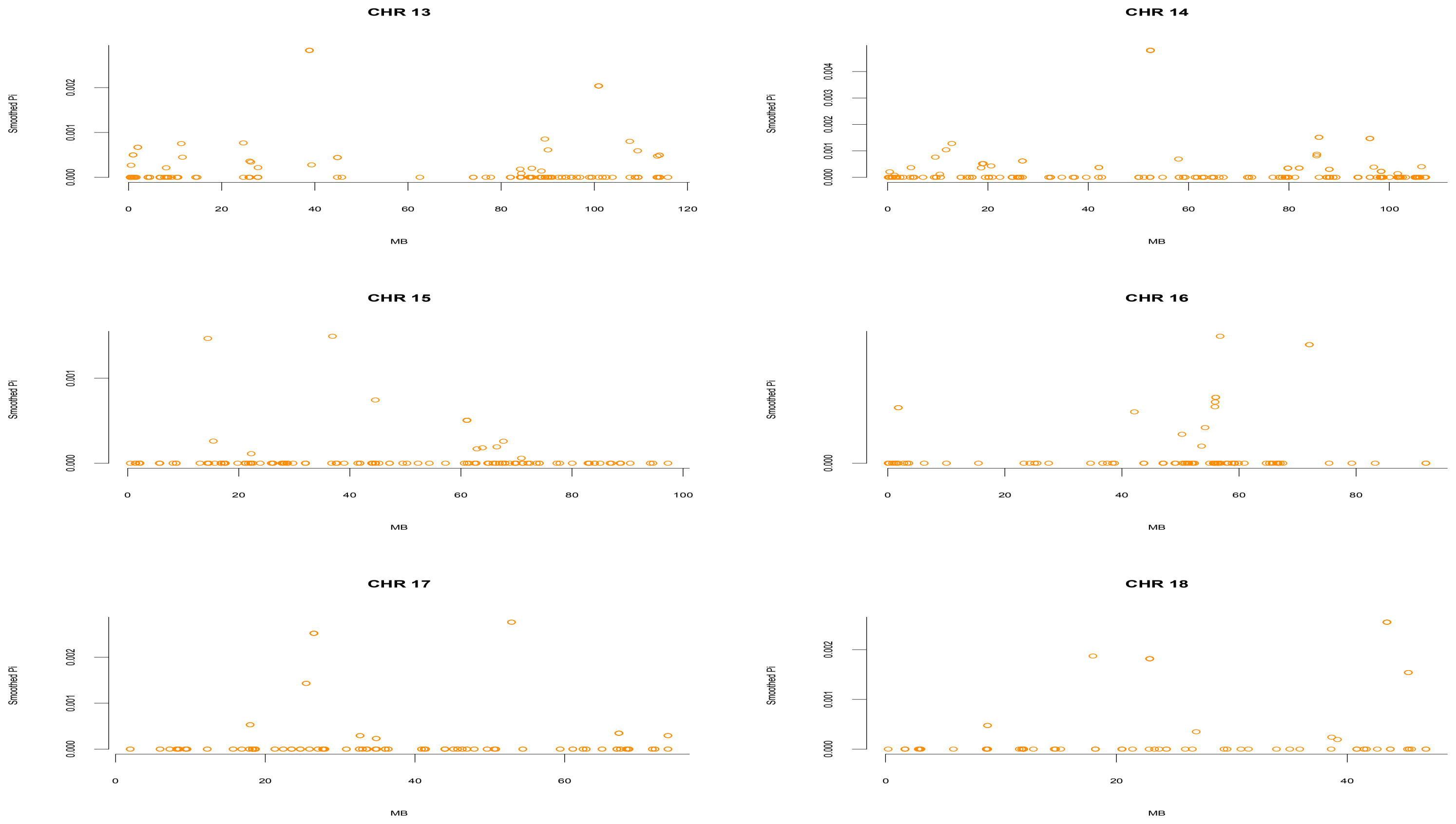

Figure 16 cont'd. Genome-wide distribution of kernel-smoothed nucleotide diversity across all *C. jacchus* chromosomes. The *x*-axis shows the length of each chromosome in megabases of sequence. The *y*-axis shows kernel-smoothed measures of nucleotide diversity.

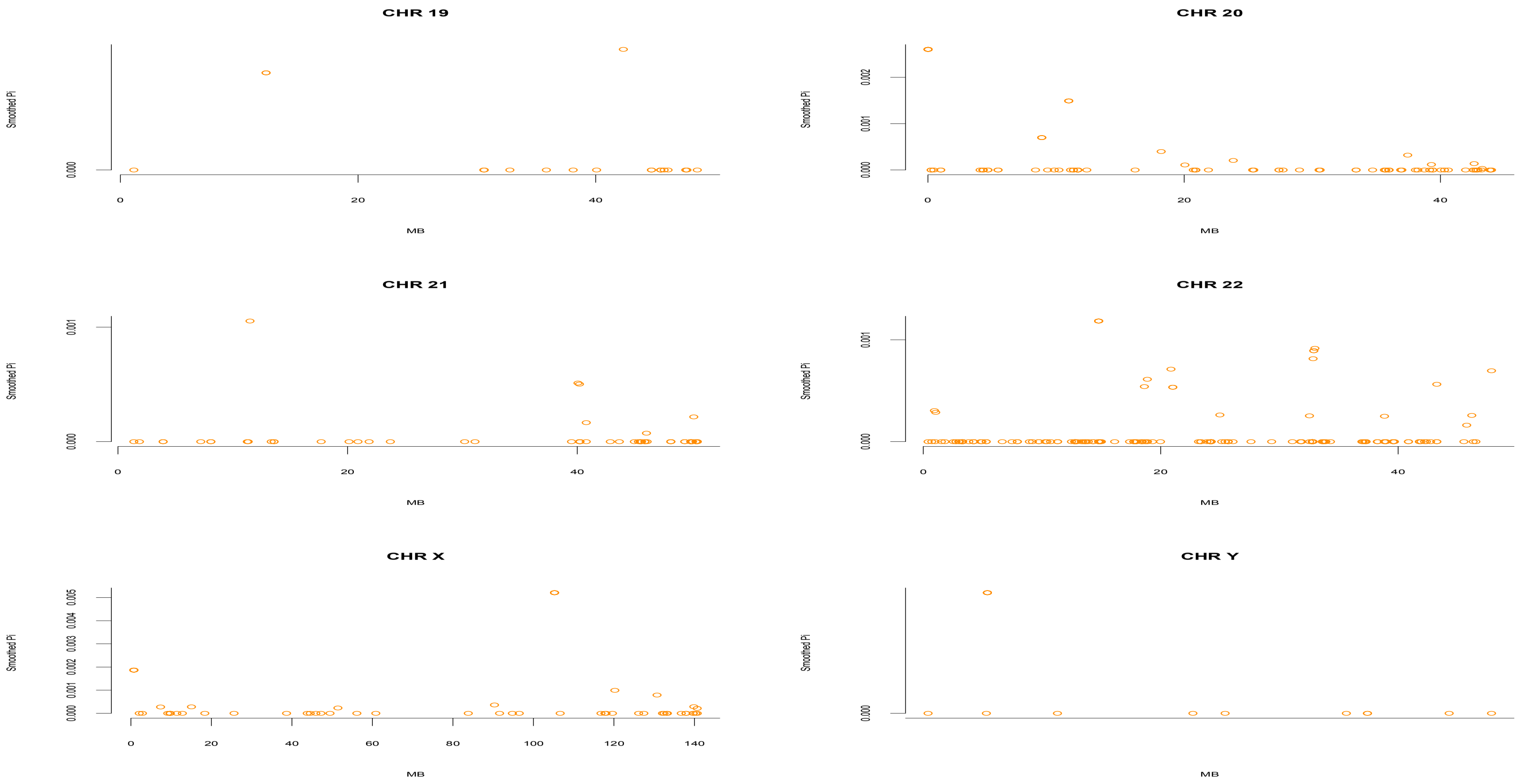

Figure 16 cont’d. Genome-wide distribution of kernel-smoothed nucleotide diversity across all *C. jacchus* chromosomes. The *x*-axis shows the length of each chromosome in megabases of sequence. The *y*-axis shows kernel-smoothed measures of nucleotide diversity.

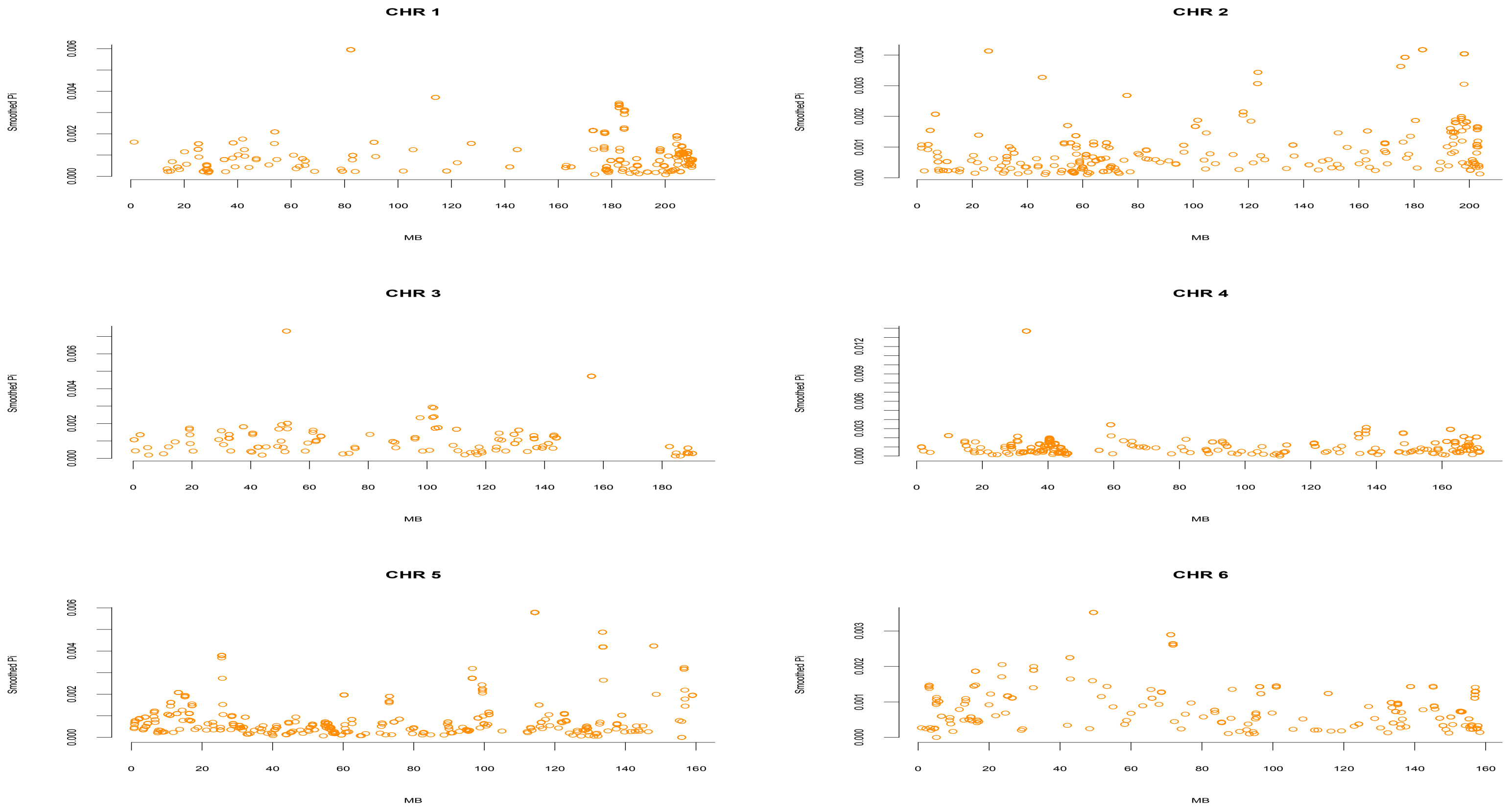

Figure 17. Genome-wide distribution of smoothed nucleotide diversity across all *C. penicillata* chromosomes. The *x*-axis shows the length of each chromosome in megabases of sequence. The *y*-axis shows kernel-smoothed measures of nucleotide diversity.

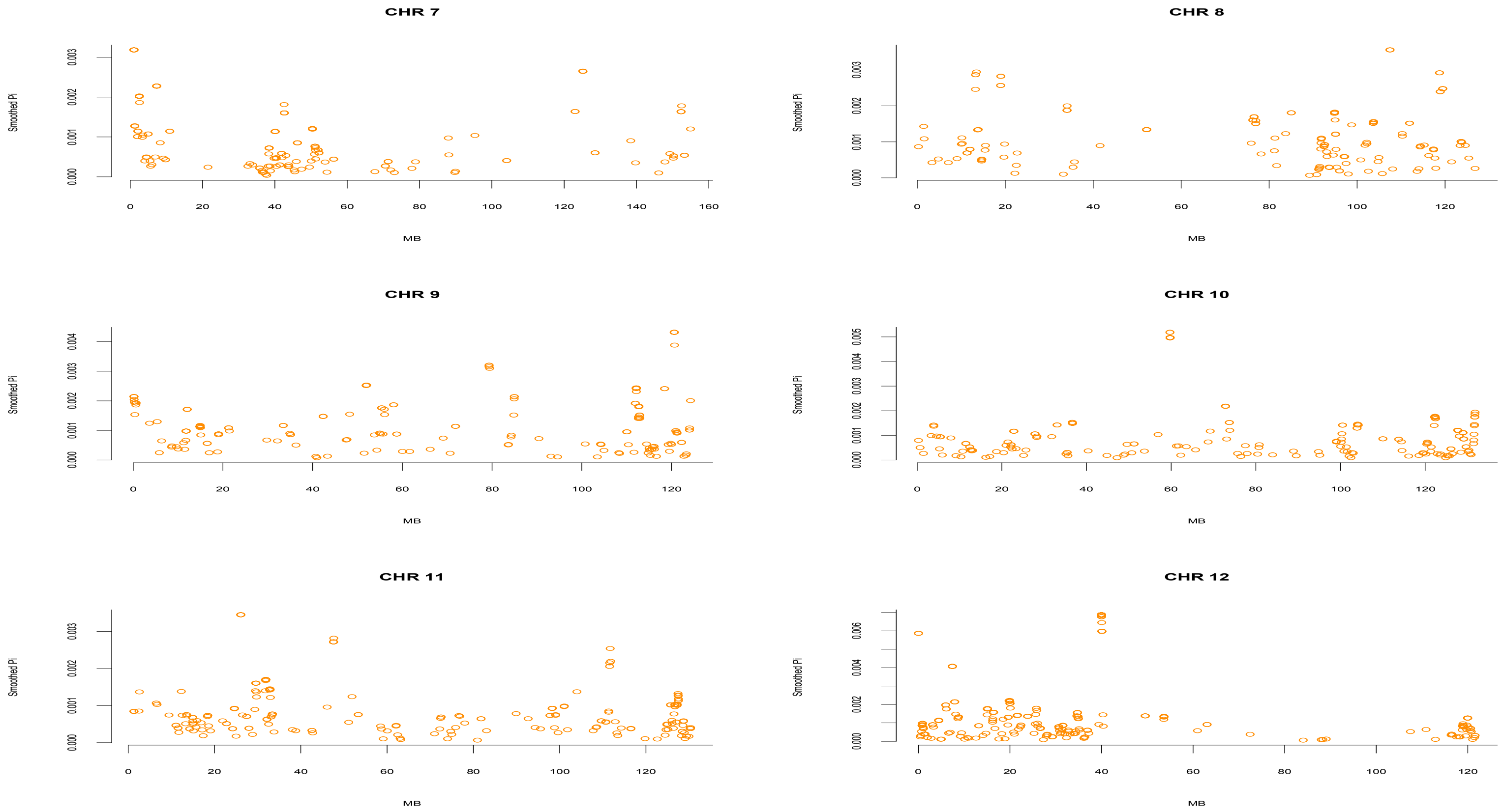

Figure 17 cont'd. Genome-wide distribution of smoothed nucleotide diversity across all *C. penicillata* chromosomes. The *x*-axis shows the length of each chromosome in megabases of sequence. The *y*-axis shows kernel-smoothed measures of nucleotide diversity.

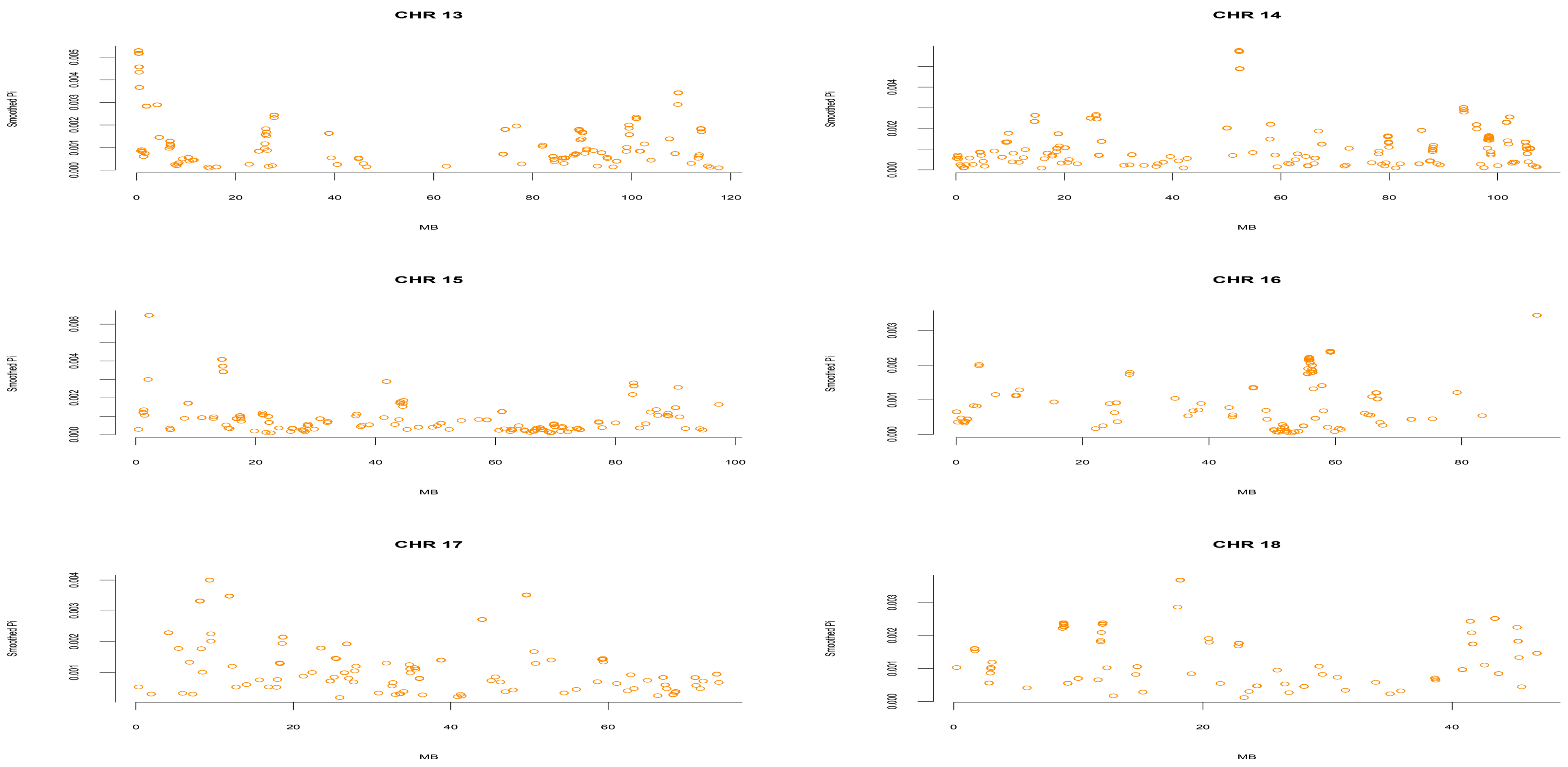

Figure 17 cont'd. Genome-wide distribution of smoothed nucleotide diversity across all *C. penicillata* chromosomes. The *x*-axis shows the length of each chromosome in megabases of sequence. The *y*-axis shows kernel-smoothed measures of nucleotide diversity.

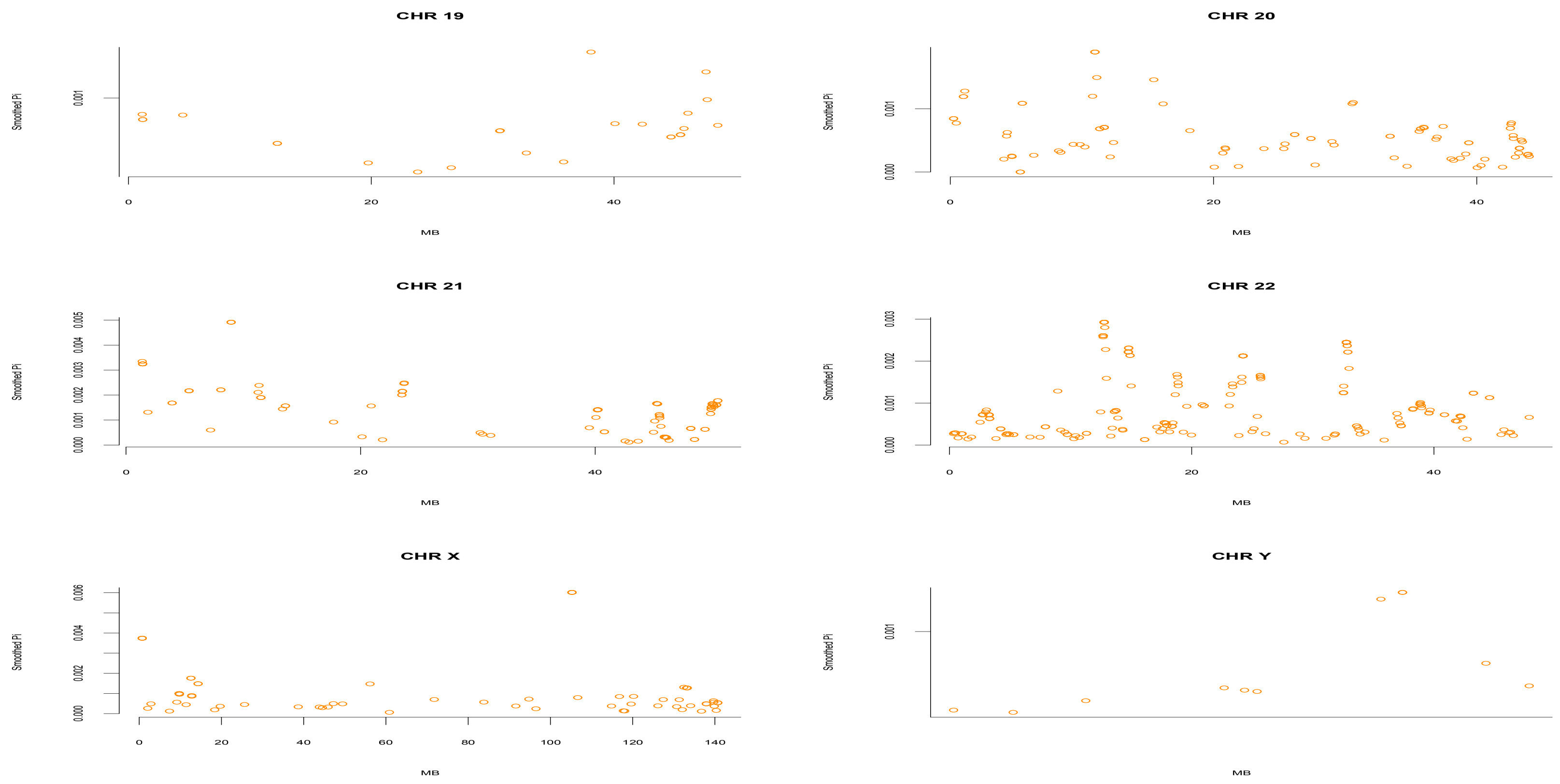

Figure 17 cont'd. Genome-wide distribution of smoothed nucleotide diversity across all *C. penicillata* chromosomes. The *x*-axis shows the length of each chromosome in megabases of sequence. The *y*-axis shows kernel-smoothed measures of nucleotide diversity.

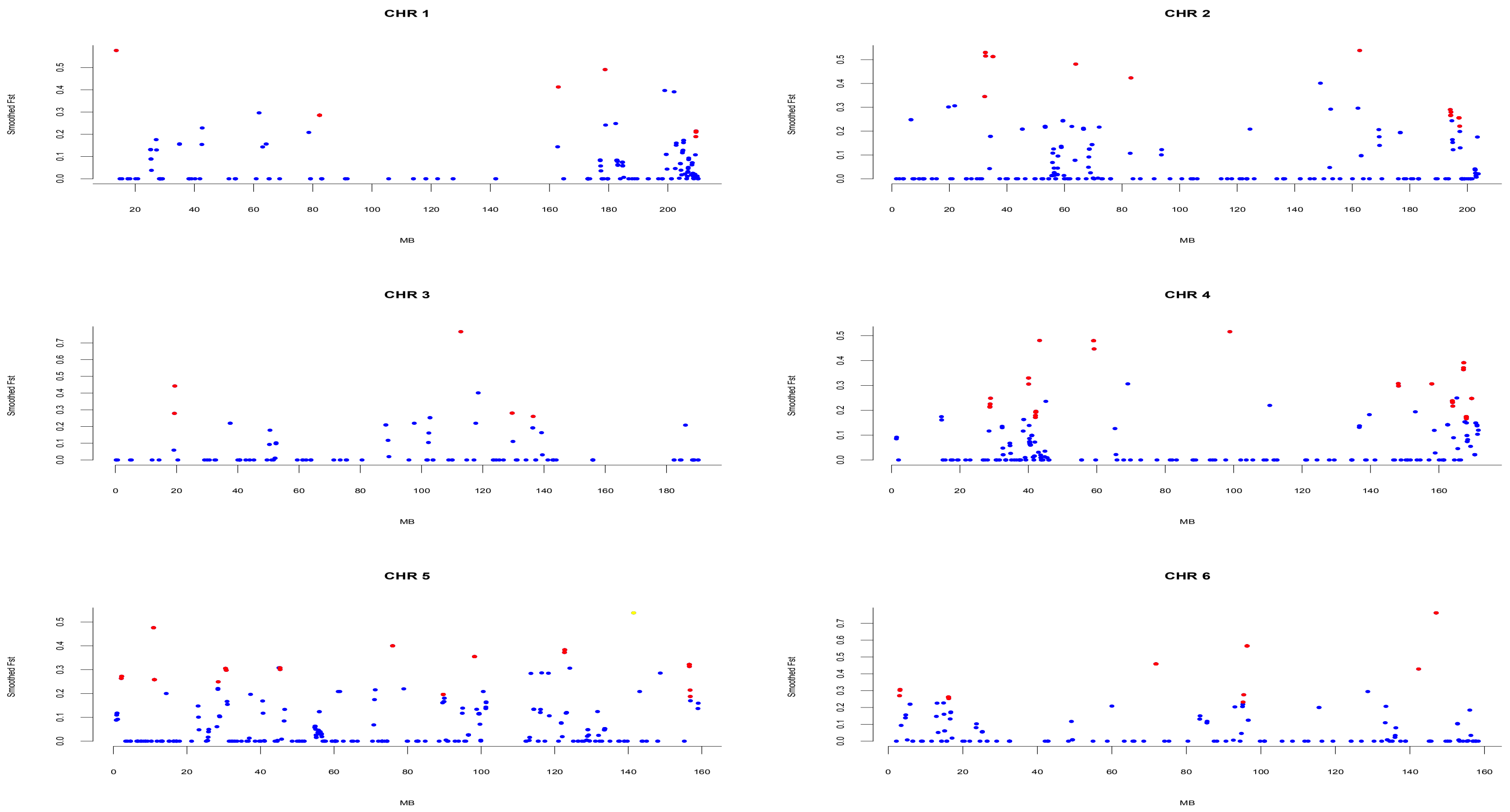

Figure 18. Genome-wide distribution of smoothed F_{ST} across all *C. penicillata* and *C. jacchus* chromosomes. The *x*-axis shows the length of each chromosome in megabases of sequence. The *y*-axis shows kernel-smoothed measures of F_{ST}. Red circles indicate F_{ST} significant at $p < 0.05$.

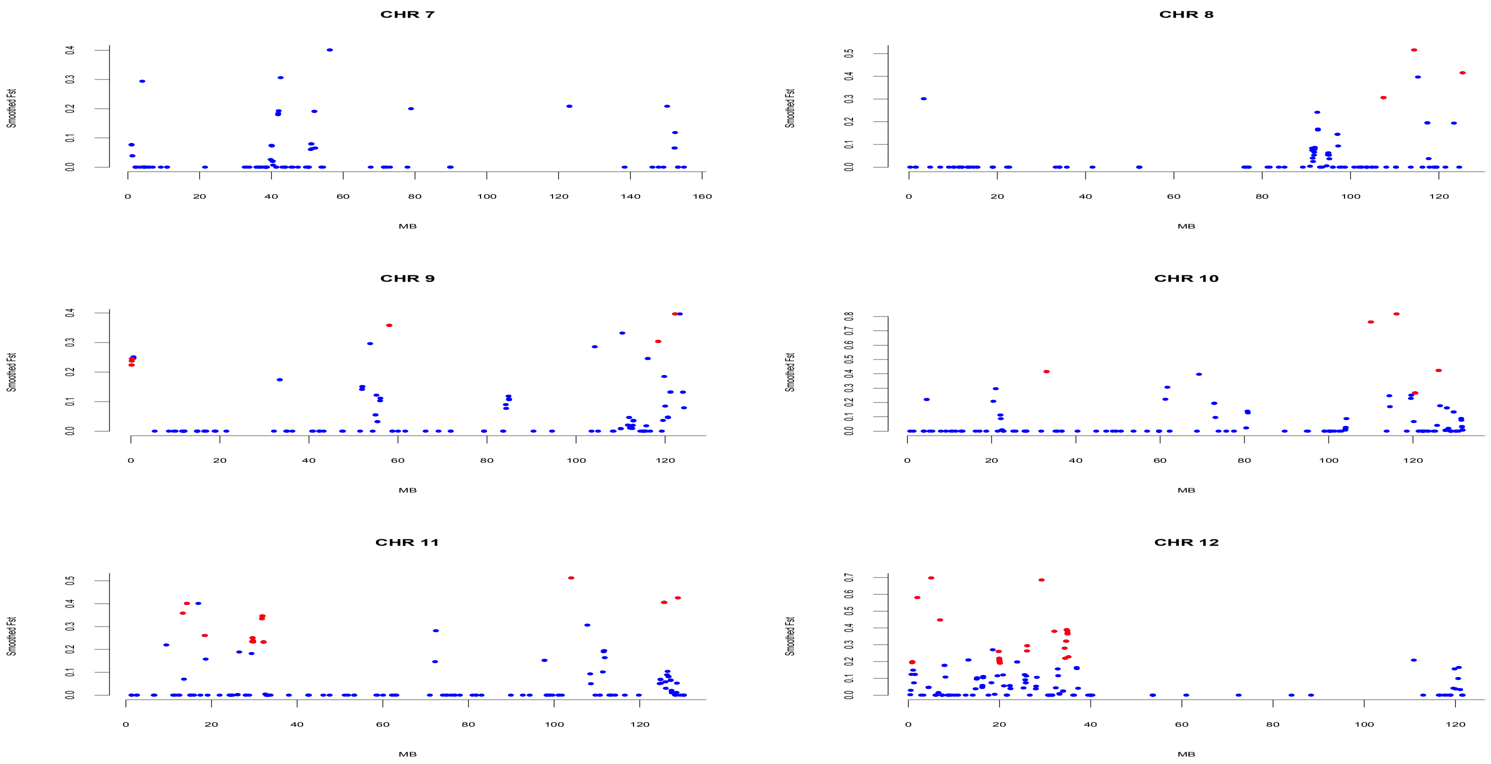

Figure 18 cont'd. Genome-wide distribution of smoothed F_{ST} across all *C. penicillata* and *C. jacchus* chromosomes. The *x*-axis shows the length of each chromosome in megabases of sequence. The *y*-axis shows kernel-smoothed measures of F_{ST}. The *y*-axis shows kernel-smoothed measures of F_{ST}. Red circles indicate F_{ST} significant at $p < 0.05$.

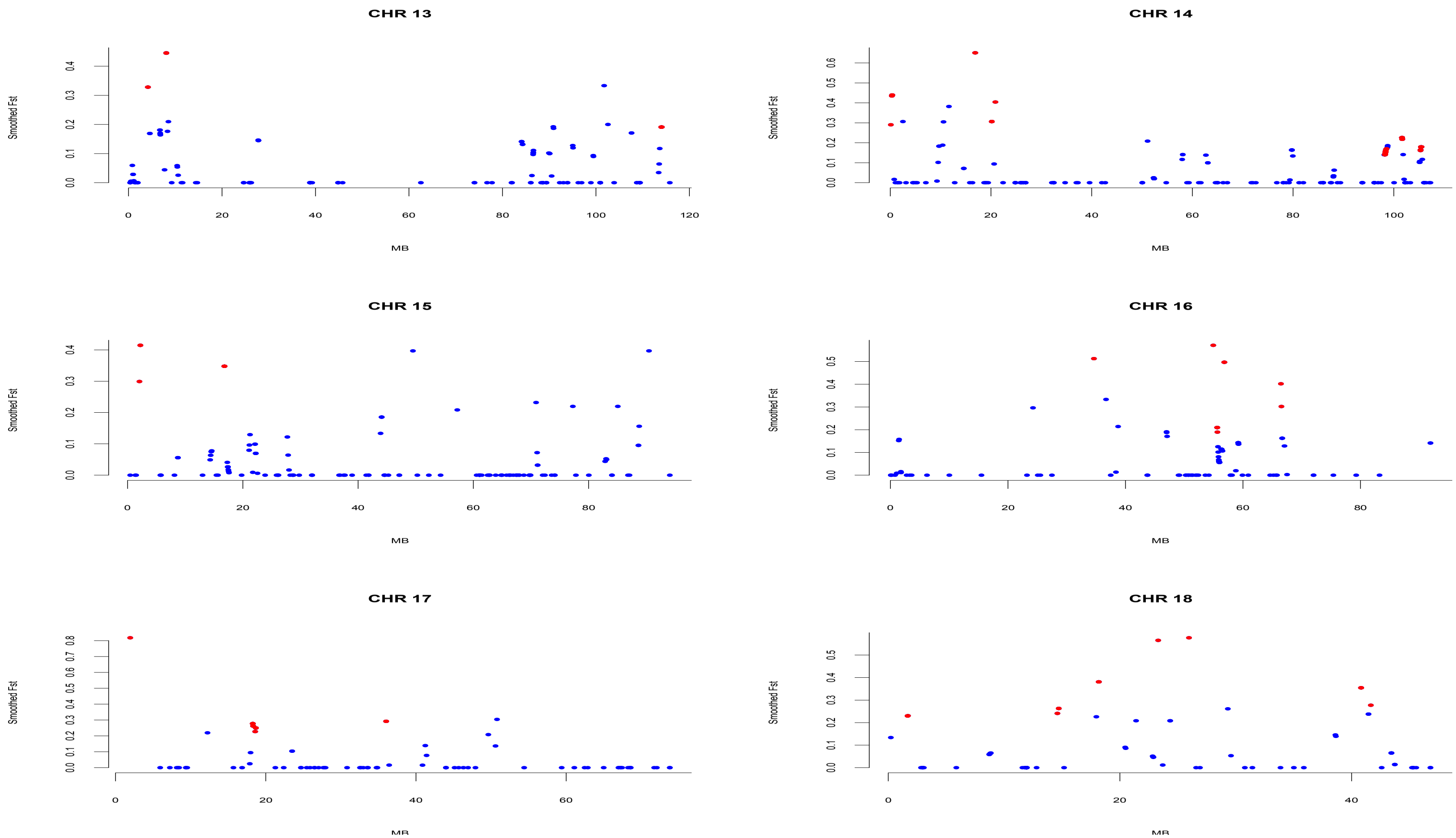

Figure 18 cont'd. Genome-wide distribution of smoothed F_{ST} across all *C. penicillata* and *C. jacchus* chromosomes. The *x*-axis shows the length of each chromosome in megabases of sequence. The *y*-axis shows kernel-smoothed measures of F_{ST}.The *y*-axis shows kernel-smoothed measures of F_{ST}. Red circles indicate F_{ST} significant at $p < 0.05$.

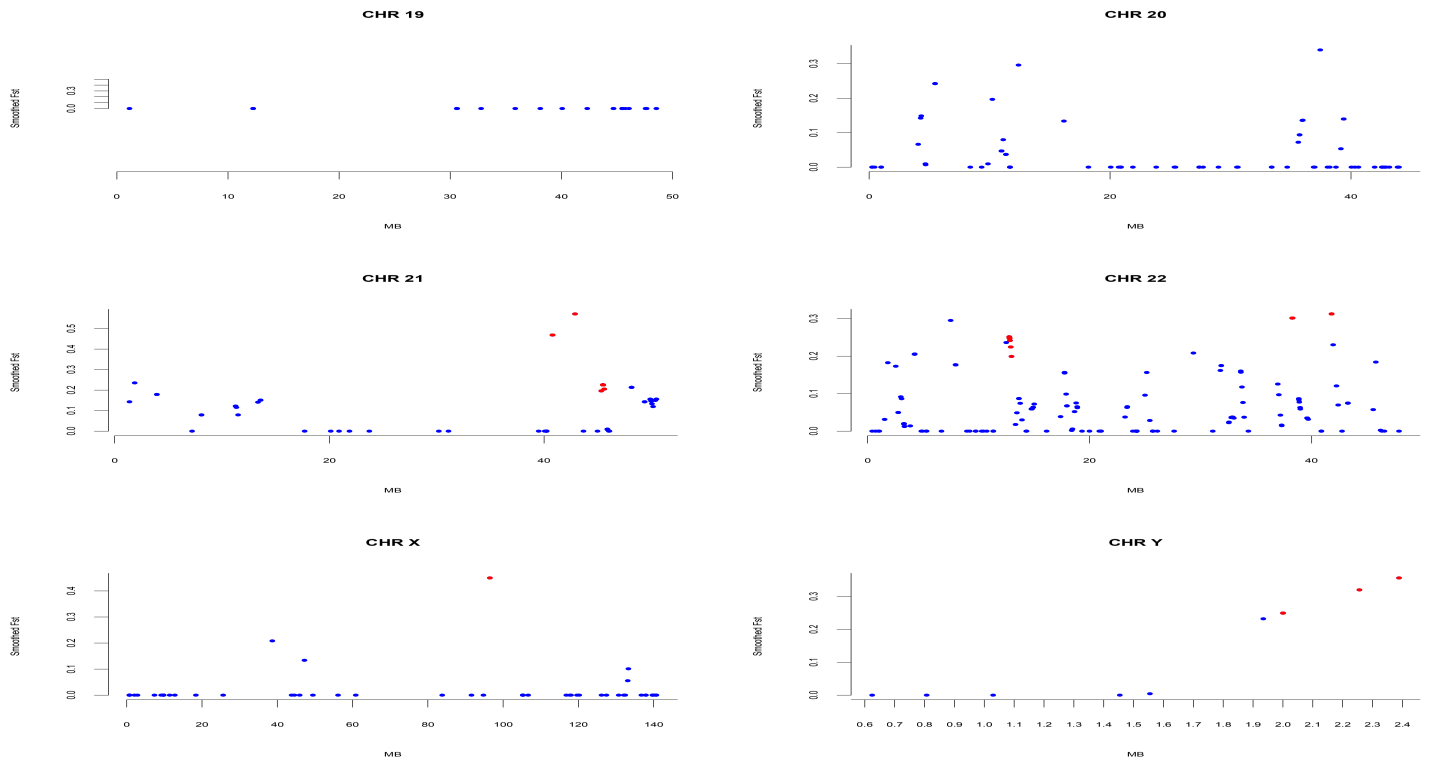

Figure 18 cont'd. Genome-wide distribution of smoothed F_{ST} across all *C. penicillata* and *C. jacchus* chromosomes. The *x*-axis shows the length of each chromosome in megabases of sequence. The *y*-axis shows kernel-smoothed measures of F_{ST}.The *y*-axis shows kernel-smoothed measures of F_{ST}. Red circles indicate F_{ST} significant at $p < 0.05$.

numerous chromosomes that showed a high level of significant divergence. Notable examples are chromosomes 2, 4, 11, 12, 14, and 22, which show several pockets of significant, elevated F_{ST} values. Notably on chromosome 19, the two species show no regions of species divergence.

Discussion

Using pooled genomic DNA samples from 6 *C. jacchus* individuals and 7 *C. penicillata,* a genomic scan of inter- and intraspecific genetic diversity as well as species divergence was performed using RADseq. The calJac3 marmoset genome was used as a reference for aligning reads resulting from RADseq library preparation and sequencing on the Illumina HiSeq 2500 platform. Although reads from a single *C. jacchus* and one *C. penicillata* failed to align to the marmoset reference genome, a high percentage of reads from the remaining individuals aligned successfully indicating a high level of similarity between sequenced reads and the calJac3 marmoset genome. Processing of aligned reads with STACKS software found a total of 4525 variable sites between *C. jacchus* and *C. penicillata* (of which 2929 were fixed in the latter species) that were applied to further population genomic analyses.

Genetic diversity of *C. jacchus* and *C. penicillata*. Population genomic analysis of *C. jacchus* and *C. penicillata* showed different patterns of intraspecific genetic diversity in the two species. Overall, *C. penicillata* showed a higher level of average nucleotide diversity and observed heterozygosity than *C. jacchus.* These patterns can also be seen in a genome wide comparison of kernel-smoothed values of nucleotide diversity between the two species. These values tended towards zero at most genomic sites in *C. jacchus* and exhibited a “rolling peaks and valleys” pattern of value changes in *C.*

penicillata. Similar genetic diversity patterns were observed in the two species at the mtDNA D-loop (Chapter One) and in a large panel of nuclear microsatellite loci (Chapter Two). Although *C. jacchus* and *C. penicillata* are sister species, varying genetic diversity patterns within the two species may be due to differences in effective population size and other demographic factors, or differing selective pressures across the genome. Interestingly, no private alleles were found for *C. jacchus* within this current RADseq data set. This again may be due to demographic or selective factors or a result of the small sample size used in the current study.

Genetic divergence between *C. jacchus* and *C. penicillata*. The overall level of divergence between *C. jacchus* and *C. penicillata* was low at the variant sites recovered by RADseq analysis. However, there are a number of genomic regions that showed high levels of statistically significant divergence between the two species. Such regions are found on almost all chromosomes, with various levels of divergence present at these regions. In comparison, nuclear microsatellite markers showed higher level of divergence between *C. jacchus* and *C. penicillata* (Chapter Two) than did RADseq-derived SNPs.

However, there are some important factors regarding differences between microsatellite and single nucleotide polymorphisms that need to be considered in making this comparison. Additional considerations regarding sample size of individuals and number of used loci also need to be made. Microsatellite mutation rates tend to be significantly higher than rates of single base mutations (Ellegren, 2000; Schlotterer, 2000). As faster mutating loci, more differences may accumulate at microsatellite loci than at loci with single nucleotide polymorphisms over the short time scale in which *C. jacchus* and *C. penicillata* have diverged. Interestingly, Helyar et al. (2011) point out that approximately 100 SNPs have the discriminatory power of 10-20 microsatellite markers. Estimates of divergence in Chapter Two were based on 42 microsatellite loci, whereas here divergence was based on thousands of RADseq variant loci, thus arguably the latter has more discriminatory power than the former in terms of locus number. It is important to consider that the results described are based on a small sample number for each species, thus the findings of this study are preliminary at best. Future work applying RADseq in the study of marmoset evolutionary history should employ a larger sample size per species studied to form more conclusive results.

Application of RADseq to future studies of marmoset evolutionary biology. In regards to species divergence, theory predicts that genomic regions important to speciation should show high levels of differentiation between members of different species (e.g. Beaumont and Nichols, 1996; Hohelenlohe et al., 2011). Thus, the genomic areas identified in this study as showing high levels of statistically significant levels of divergence between *C. jacchus* and *C. penicillata* may be such genomic “outliers.” Specifically, these outliers may represent loci important to speciation between these two species. For future studies employing a similar methodology along with a larger number of species samples, the published common marmoset genome allows for flagged genomic outliers to be used in the identification of potential marmoset speciation genes. Gene ontology analyses can be used to uncover the function of those genes.

Hybridization occurs both within natural and anthropogenic (human induced) contexts among *Callithrix* marmosets. While the six *Callithrix* species hold historically allopatric ranges (Ryland, 1993 and 2009), common and black-tufted marmosets have been introduced to areas outside of their borders through Brazil’s illegal pet trade (Ruiz-

Miranda et al., 2006). As a result, introduced common and black-tufted marmosets are now found in artificial sympatry with each other and other congeners. Extensive hybridization in wild *Callithrix* populations has been increasingly observed and reported (Mendes, 1997a&b; Passanami et al., 1996). Yet, the genetic and evolutionary consequences of marmoset hybridization remain largely unstudied. The application of RADseq of marmoset hybridization studies can help elucidate those consequences, as well as help build an understanding of marmoset species divergence.

For hybridization studies, genome scans of admixed populations within hybrid zones will show low levels of introgression (or exchange) between hybridizing species (Gompert and Buerkle, 2011). Observance of such introgression patterns represent the theoretical basis of the “genomic clines” technique (Gompert and Buerkle, 2011; Gompert and Buerkle, 2012), which measures expected genotype frequencies along an admixture gradient, and helps identify such genomic “outliers.” One challenging aspect of the genomic clines technique is it that requires a large number of genetic loci. The technique can also be used to identify the type of natural selection (e.g. over-dominance vs. under-dominance) acting within a hybrid zone. For the study of the genomic signatures of hybridization, the high number and density of genetic markers developed by RADseq can certainly overcome the methodological challenge of genomic clines analysis. RADseq markers developed from genetic samples collected within hybrid zones and from pure wild species populations can thus be used under the genomic clines framework to address natural selection and speciation aspects of marmoset hybridization.

Ascertainment bias, or ‘the systematic deviation from the expected allele frequency distribution that occurs because of the sampling processes used to find marker loci' (Heylar et al., 2011), is a concern for SNP-based analyses (Garvin et al., 2010; Marvin et al., 2004). This concern is applicable to situations where SNPs are first discovered in a subset of a population and then applied to a different subpopulation for assessment of population structure and genetic diversity. Ascertainment bias may skew estimates of diversity in the new subpopulation depending on how representative genetic patterns of the original representative reference data set are relative to the rest of the population (Morin et al., 2004). One of the advantages in applying RADseq to the study of genetic diversity patterns within a given set of taxa, is that SNPs are simultaneously discovered and genotyped, which eliminates the need for pre-screening SNPs in a prior reference dataset. However, an informative set of data from reference parental species will be important to the application of RADseq in studies of marmoset hybridization. The reference samples will be used to assess either diagnostic loci representative of each parental species or determine patterns of parental allele frequencies. Otherwise, biases may arise in estimation of contributions of parental populations to the ancestry of admixed hybrid populations or estimation of introgression across hybrid zones.

Overall, the preliminary results of this study suggest great potential in the application of RADseq to the study of marmoset evolutionary biology. The large number of SNPs uncovered in this current study suggests that RADseq high statistical power for future studies of *Calithrix* evolutionary genetics and demographic history. Such studies will help build on previous studies based on more traditional markers such as mtDNA and microsatellites, while allowing researchers to apply cost- and time-effective NGS technology. This study also shows RADseq also holds strong potential for the study of wild primate genetics, especially when combined with whole genome amplification techniques. Despite low starting amounts of DNA collected from wild marmosets, we successfully sequenced DNA from such samples under a modified RADseq protocol after subjecting samples to WGA. Thus, our preliminary data suggest that RADseq is applicable in the study of wild primate genetics and genomics at a potentially higher power and resolution than previously possible.

SUMMARY AND CONCLUSION

Modern attitudes toward the evolutionary important of hybridization have changed significantly since the early 20th century (Arnold, 1997), and in this current age of immense human development, dubbed the "Anthropocene," human driven environmental changes have promoted contact between formerly separated species and enabled interbreeding between these species (Thomas, 2013). Natural hybridization is also common in animal and plant species, especially recently diverged ones (Mallet, 2005). Our increased awareness of the ubiquity of hybridization warrents further study of the evolutionary importance of species interbreeding in both the natural and anthropogenic contexts.

This work explored the genetic diversity and species origins of two *C. penicillata* and *C. jacchus* hybrid zones, as well as genetic diversity and divergece in the two parental species. Use of the mitochondrial (mtDNA) control region and a large panel of 44 autosomal microsatellites showed that *C. penicillata* is more genetically diverse than *C. jacchus.* The two species also have different demographic histories based on mtDNA data, with *C. jacchus* experiencing a past population expansion and *C. penicillata* evolving under constant population size. The mtDNA and microsatellite data revealed the existence of a previously undocumented natural hybrid zone along the São Francisco River in NE Brazil and confirmed species origins of an anthropogenic zone in Rio de Janeiro state. Median-joining network analysis and a Bayesian phylogeny of mtDNA data showed two largely distinctive mtDNA control region clades for each species, with haplotypes from hybrid zones clustering within one of these two clades. The mtDNA and microsatellite data sets showed much lower levels of admixture within the natural hybrid zone than in the anthropogenic zone. Further, the data suggested that São Francisco River is an important geographic barrier to gene flow in the natural hybrid zone. On the other hand, admixture patterns within the anthropogenic hybrid zone suggested collapse of reproductive barriers, and the formation of a hybrid marmoset swarm (an admixed population from which unique combinations of parental genes have been lost). Further, hybrids from the anthropogenic zone showed a reduced level of genetic diversity than hybrids from the natural zone in comparison to parental species populations. Thus, the results of this work suggested different evolutioanry dynamics in anthropogenic vs. natural animal hybrid zones.

Open issues of *Callithrix* evolution and hybridization still include questions broached by this research regarding marmoset species relationships, genetic admixture and species origins of hybrid zones. Beyond these issues, other open questions pertain to: (1) hybrid fitness within hybrid and anthropogenic zones, (2) genome-wide effects of hybridization, (3) the underlining genetic basis, or genetic architecture, of marmoset speciation, and (4) assessment of morphological signatures of species interbreeding. Future work on marmoset hybridization and evolution should include research that will address such issues as above with a combined genomic and morphological approach, given the breath of these issues. Thus, we recommend that future marmoset research should utilize a next generation sequencing technique like RADseq for population genomic phylogenetic questions, but also techniques that collect morphological data to better understand basis of phenotypic variation among *Callithrix* species.

REFERENCES

Affonso, A.G., Ruiz-Miranda, C.R., & Beck, B. (2004). Interações ecológicas entre ico leão dourado (*Leontopithecus rosalia linnaeus*,1766) reintroduzido e mico-estrela (*Callithrix jacchus linnaeus,*1758) introduzido em fragmentos de Mata Atlântica, R.J. *A Primatologia no Brasil, 8*,123-134.

Alonso, C., de Faria, D.S., Langguth, A., & Santee, D.F. (1987). Variação da pelagem na área de intergradação entre *Callithrix jacchus* e *Callithrix penicillata*. *Revista Brasileira de Biologia, 47*, 465-470.

Allendorf, F., Leary, R., Spruell P., & Wenburg J. (2001). The problems with hybrids: Setting conservation guidelines. *Trends in Ecology & Evolution*, *16*, 613-622.

Arnold, M.L. (1997). *Natural hybridization and evolution*. New York: Oxford University Press.

Arnold, M.L., & Meyer A. (2006). Natural hybridization in primates: One evolutionary mechanism. *Zoology*, *109*, 261-276.

Arias, C.F., Munoz, A.G., Jiggins, C.D., Mavarez, J., Bermingham, E., & Linares, M. (2008). A hybrid zone provides evidence for incipient ecological speciation in *Heliconius* butterflies. *Molecular Ecology*, *17*, 4699-4712.

Aguiar, L. M., Mellek, D. M., Abreu, K. C., Boscarato, T. G., Bernardi, I. P., Miranda, J. M., et al. (2007). Sympatry between *Aloutta caraya* and *Alouatta clamitans* and the rediscovery of free-ranging potential hybrids in southern Brazil *Primates, 48*, 245-248.

Baele, G., Lemey, P., Bedford, T., Rambaut, A., Suchard, M.A., & Alekseyenko, A.V. (2012). Improving the accuracy of demographic and molecular clock model comparison while accommodating phylogenetic uncertainty. *Molecular Biology and Evolution*, *29*, 2157-2167.

Baele, G., Li, W.L.S., Drummond, A.J., Suchard, M.A., & Lemey, P. (2013). Accurate model selection of relaxed molecular clocks in Bayesian phylogenetics. *Molecular Biology and Evolution, 30*, 239-43.

Baird, N.A., Etter, P.D., Atwood, T.S., Currey, M.C., Shiver, A.L., Lewis, Z.A., Selker, E.U., Cresko, W.A., & Johnson, E.A. (2008) Rapid SNP discovery and genetic mapping using sequenced RAD markers. *PLOS One*, *3(10),* e3376.

Ballard, J. & Whitlock, M. (2004). The incomplete natural history of mitochondria. Molecular Ecology 13, *4,* 729-44.

Bandelt, H.J., Forster, P., Rohl A. (1999). Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution*, *16*, 37-48.

Barroso, C.M.L., Schneider, H., Schneider, M.P.C., Sampaio, I., Harada, M.L., Czelusniak, J., & Goodman, M. (1997). Update on the phylogenetic systematics of New World monkeys: further evidence for placing the pygmy marmoset (Cebuella) within the genus Callithrix. *International Journal of Primatology*, *18(4),* 651-674.

Bensasson, D., Feldman, M.W., & Petrov, D.A. (2003). Rates of DNA duplication and mitochondrial DNA insertion in the human genome. *Journal of Molecular Evolution, 57, 343-354.*

Benirshke, K., Anderson J.M., & Brownhill, L.E. (1962). Marrow chimerism in marmosets. *Science, 138,* 384-393.

Bicca-Marques, J. C., Prates, H. M., Aguiar, F. R. C., & Jones, C. B. (2008). Survey of *Alouatta caraya*, the black-and-gold howler monkey, and *Alouatta guariba clamitans,* the brown howler monkey, in a contact zone, state of Rio Grande do Sul, Brazil: Evidence for hybridization. *Primates, 49*(246), 252.

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*). *Journal of Heredity, 100*(2), 158-169.

Brown, W.M., George Jr., M., & Wilson, A.C. (1979). Rapid evolution of animal mitochondrial DNA. *PNAS*, *76*,1967-1971.

Bynum, E. L., Bynum, D. Z., Froehlich, J. W., & Supriatna, J. (1997). Revised geographic ranges and hybridization in *Macaca tonkeana* and *Macaca hecki*. *Tropical Biodiversity, 4*(3), 275-283.

Canavez, F.C., Moreira, M.A.M, Simon, F., Parham, P., & Seuanez, H.N. (1999). Phylogenetic relationships of the Callitrichinae (Platyrrhini, primates) based on beta(2)-microglobulin DNA sequences. *American Journal of Primatology, 48,* 225-236.

Carlsson, J. (2008). Effects of microsatellite null alleles on assignment testing. *Journal of Heredity, 99*, 616-23.

Carnaval, A.C., & Moritz, C. (2008). Historical climate modelling predicts patterns of current biodiversity in the Brazilian Atlantic Forest. *Journal of Biogeography*, *35*, 1187-1201.

Castelletti, C.H.M., Silva J.M.C., Tabarelli, M., & Santos, A.M.M. (2004). Quanto ainda resta da Caatinga? Uma estimativa preliminar. Pages 91–100 in J. M. C. Silva, M. Tabarelli, M. Fonseca and L. Lins, editors. Biodiversidade da Caatinga: áreas e ações prioritárias para a conservação. Ministerio do Meio Ambiente, Brasília (in Portuguese).

Chapuis, M., & Estoup, A. (2007). Microsatellite null alleles and estimation of population differentiation. *Molecular Biology and Evolution*, 24, 621-31.

Coimbra-Filho, A.F., & Mittermeier, R.A. (1973). New data on the taxonomy of the Brazilian marmosets of the genus *Callithrix* erxleben, 1777. *Folia Primatologica*, *20(241),* 264.

Coimbra-Filho, A.F., Mittermeier, R.A., Rylands, A.B., Mendes, S.L., Kierulff, M.C.M., & de S.Pinto, LP. (2006). The taxonomic status of Wied's black-tufted-ear marmoset, *Callithrix kuhlii* (Callitrichidae, primates). *Primate Conservation, 21,*1-24.

Coimbra-Filho, A.F., Pissinatti A., & Rylands A.B. (1993). Experimental multiple hybridism and natural hybrids among *Callithrix* species from eastern Brazil. In A.B. Rylands(Ed.). *Marmosets and tamarins*: *Systematics, behaviour, and ecology* (pp. 95-120). New York: Oxford University Press.

Corander, J., & Marttinen, P. (2006). Bayesian identification of admixture events using multi-locus molecular markers. *Molecular Ecology*, *15*, 2833-2843.

Corander, J., Marttinen, P., Sirén J., & Tang J. (2008). Enhanced Bayesian modeling in BAPS software for learning genetic structures of populations. *BMC Bioinformatics*, *9*, 539.

Cortes-Ortiz, L., Duda, T. F., Jr., Canales-Espinosa, D., Garcia-Orduna, F., Rodriguez-Luna, E., & Berminghamt, E. (2007). Hybridization in large-bodied New World primates. *Genetics, 176*(4), 2421-2425.

Crispo, E., Moore, J., Lee-Yaw, J.A., Gray, S.M., & Haller, B.C. (2011). Broken barriers: human-induced changes to gene flow and introgression in animals. *Bioessays, 33*, 508-18.

Da Cunha, H.F., Ferreira A.A., & Brandão, D. (2007). Composição e fragmentação do Cerrado em Goías usando sistema de informação geográfica (SIG). *Boletim Goiano De Geografia 27*, 139-152.

Darriba, D., Taboada, G.L., Doallo, R., & Posada, D. (2012). jModelTest 2: More models, new heuristics and parallel computing. *Nature Methods, 9*,772.

De Morais Jr, M.M. (2010) Os saguis (*Callithrix* spp. ERXLEBEN, 1777) exóticosinvasores na Bacia do Rio São João, Rio de Janeiro: biologia populacional e padrão de distribuição em um paisagem fragmentada. Universidade Estadual do Norte Fluminense, Campos Dos Goytacazes, RJ, Brazil.

Dempster, A. P., Laird, N. M. & Rubin, D. B., 1977. Maximum Likelihood from incomplete data via the EM algorithm (with discussion). *Journal of the Royal Statistical Society B, 39*, 1–38.

Detwiler, K. M., Burrell, A. S., & Jolly, C. J. (2005). Conservation implications in hybridization in African cercopithecine monkeys. *International Journal of Primatology, 26*(3), 661-684.

Digby, L.J., Ferrari, S.F., & Saltzman, W. (2007). Callitrichines: The role of competition in cooperatively breeding species. In C.J. Campbell, A. Fuentes, K.C. MacKinnon, and others, Eds., *Primates in perspective* (pp. 85-106). New York: Oxford University Press.

Drummond, A.J., Rambaut, A., Shapiro, B., & Pybus, O.G. (2005). Bayesian coalescent inference of past population dynamics from molecular sequences. *Molecular Biology and Evolution*, *22*,1185-1192.

Drummond, A.J., Suchard, M.A., Xie, D., & Rambaut, A. (2012). Bayesian phylogenetics with BEAUti and the BEAST 1.7. Molecular Biology and Evolution, *29*, 1969-1973.

Edgar, R.C. (2004). Muscle: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, *32(5),* 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. *Systematic Biology*, *60*, 541-561.

Ellegren, H. (2000). Microsatellite mutations in the germline: implications for evolutionary inference. *Trends in Genetics, 16(12),* 551-558.

Etter, P.D., Bassham, S., Hohenlohe, P.A., Johnson, E.A., & Cresko, W.A. (2011). SNP discovery and genotyping for evolutionary genetics using RAD sequencing. In V. Orgogozo & M.V. Rockman (Eds.), Molecular Methods for Evolutionary Genetics, Methods in Molecular Biology vol. 72 (pp. 156-178). New York: Humana Press.

Excoffier, L., & Lischer, H.E.L. (2010.) Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resoures*, *10*, 564-567.

Facon, B., Genton, B., Shykoff, J., Jarne, P., Estoup, A., & David, P. (2006). A general eco-evolutionary framework for understanding bioinvasions. *Trends in Ecology & Evolution, 21*, 130-135.

Faulkes, C.G, Arruda M.F., & Monteiro da Cruz M.A.O. (2003). Matrilineal genetic structure within and among populations of the cooperatively breeding common marmoset, *Callithrix jacchus. Molecular Ecology, 12*, 1101-1108

Faulkes, C.G., Arruda, M.F., & Monteiro da Cruz, M.A.O. 2009. Genetic structure within and among populations of the common marmoset, *Callithrix jacchus:* Implications for cooperative breeding. In S.M. Ford, L.M. Porter, L.C. Davis, (Eds.), The Smallest Anthropoids: The Marmoset/Callimico Radiation (pp. 103-117). New York: Springer.

Fu, X. (1997). Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics*, *147*,915-925

Funk, D.J., & Omland, K.E. (2003). Species-level paraphyly and polyphyly: frequency, causes, and consequences, with insights from animal mitochondrial DNA. *Annual Review of Ecology, Evolution, and Systematics, 34*, 397-423.

Ferrari, S.F. (1993) Ecological differentiation in the Callitrichidae. In A. B. Rylands (Ed.), *Marmosets and Tamarins: Systematics, Behaviour, and Ecology* (pp. 314-328). New York: Oxford University Press.

Galbusera, P.H.A., & Gillemot, S. (2008). Polymorphic microsatellite markers for the endangered golden-headed lion tamarin, *Leontopithecus chrysomelas* (Callitrichidae). *Conservation Genetics*, *9*, 731-773

Garvin, M.R., Saitoh, K., & Gharrett, A.J. (2010). Application of single nucleotide polymorphisms to non-model species: A technical review. *Molecular Ecology Resources*, *10(6),* 915-934.

Godinho, R., Llaneza, L., Blanco, J.C., Lopes, S., Alvares. F., Garcia, E.J., Palacios, V., Cortes, Y., Talegon, J., & Ferrand, N. (2011). Genetic evidence for multiple events of hybridization between wolves and domestic dogs in the Iberian peninsula. *Molecular Ecology*, *20*, 5154-5166.

Goudet, J. (1995). FSTAT (version 1.2): A computer program to calculate F-statistics. *Journal of Heredity*, *86*,485-486.

Google Maps. (2013). [Petrolina, Brazil][Satellite map] Retrieved https://maps.google.com/maps?q=petrolina&hl=pt&ll=-9.408335,-40.499897&spn=0.023836,0.031843&sll=-8.054278,-34.881256&sspn=0.382768,0.509491&t=h&hnear=Petrolina+-+Pernambuco,+Brasil&z=15.

Guindon, S, & Gascuel, O. (2003). A simple, fast and accurate method to estimate large phylogenies by maximum-likelihood. *Systematic Biology*, *52*, 696-704.

Guo, S. W. & Thompson, E. A. (1992). Performing the exact test of Hardy-Weinberg proportion for multiple alleles. *Biometrics*, 48, 361–372.

Haldane, J. B. S., (1954). An exact test for randomness of mating. *Journal of Genetics, 52*, 631–635.

Helyar, S.J., Hemmer-Hansen, J., Bekkevold D, Taylor, M.I., Ogden, R., Limborg, M.T., Cariani, A., Maes, G.E., Diopere, E., Carvalho G.R., & Nielsen, E.E. (2011). Application of SNPs for population genetics of nonmodel organisms: new opportunities and challenges. *Molecular Ecology Resources, 11*, 123-36.

The *Heliconius* Genome Consortium. (2012). Butterfly genome reveals promiscuous exchange of mimicry adaptations among species. *Nature, 487*, 94-98.

Hershkovitz, P. (1968). Metachromism or the principle of evolutionary change in mammalian tegumentary colors. *Evolution, 22,* 556-575.

Hershkovitz, P. (1975). Taxonomy of Brazilian marmosets (*Callithrix*, Callitrichidae) - comments. *Folia Primatologica, 24*, 137-172.

Hershkovitz, P. (1977). *Living New World Monkeys (Platyrrhini): With an Introduction to Primates.* Chicago: University of Chicago Press.

Hewitt, G. (1988). Hybrid zones - natural laboratories for evolutionary studies. *Trends in Ecology & Evolution, 3*(7), 158-167.

Huelsenbeck, J. P., & Ronquist, F. (2001). MRBAYES: Bayesian inference of phylogeny. *Bioinformatics, 17*, 754-755.

Hufbauer, R.A. (2008). Biological invasions: Paradox lost and paradise gained. *Current Biology*, *18*, R246-247.

Jakobsson, M., & Rosenberg, N.A. 2007. CLUMPP: A cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. *Bioinformatics*, *23*, 1801-1806.

Jombart, T., & Ahmed, I. (2011). Adegenet 1.3-1: New tools for the analysis of genome-wide SNP data. *Bioinformatics, 27*, 3070-3071.

Jiggins, C., & Mallet, J. (2000). Bimodal hybrid zones and speciation. *Trends in Ecology & Evolution, 15*, 250-255.

Kanthaswamy, S., Satkoski, J., George, D., Kou, A., Erickson, B. J. A., & Smith, D. G. (2008). Hybridization and stratification of nuclear genetic variation in *Macaca mulatta* and *M. fascicularis*. *International Journal of Primatology, 29*, 1295-1311.

Kass, R.E., & Raftery, A.E. (1995). Bayes factors. *Journal of the American Statistical Association, 90*, 773-795.

Kelaita, M. A., & Cortes-Ortiz, L. (2013). Morphological variation of genetically confirmed *Alouatta pigra x A. palliata* hybrids from a natural hybrid zone in Tabasco, Mexico. *American Journal of Physical Anthropology, 150*(2), 223-234.

Keller, C., Roos, C., Groeneveld, L. F., Fischer, J., & Zinner, D. (2010). Introgression hybridization in southern African baboons shapes patterns of mtDNA variation. *American Journal of Physical Anthropology,* (142), 125-136.

Kinzey, W.G. (1982). Distribution of primates and forest refuges. In G.T. Prance (Ed.), *Biological diversification in the tropics* (pp 455-485). New York: Columbia University Press.

Kocher, T.D., Thomas, W.K., Meyer, A., Edwards, S.V., Paabo, S., Villablanca, F.X., & Wilson, A.C. (1989). Dynamics of mitochondrial-DNA evolution in animals - amplification and sequencing with conserved primers. *PNAS*, *86*, 6196-6200.

Langmead, B., & Salzberg S. (2012) Fast gapped read-alignment with Bowtie 2. *Nature Methods,* 9, 357-359.

Lecoq, Y. Michalakis, A. Loiseau, G. A. Sword, S. Piry, & A. Estoup. (2008) Do outbreaks affect genetic population structure? A worldwide survey in *Locusta migratoria,* a pest plagued by microsatellite null alleles. *Molecular Ecology,*17, 3640-3653.

Leal, I.R., Da Silva, J.M.C., Tabarelli, M., & Lacher, T.E. (2005). Changing the course of biodiversity conservation in the Caatinga of northeastern Brazil. *Conservation Biology, 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 in Ecology & Evolution*, 20, 229-37.

Malukiewicz, J., Grativol, A.D., Ruiz-Miranda, C.R., & Stone, A.C. (2012). Almost Carioca: hybridization between introduced populations of *Callithrix jacchus* and *C. penicillata* in Rio de Janeiro State, Brazil. *American Journal of Physical Anthropology, 147(s54),* 202.

Marroig, G., Cropp, S., & Cheverud, J. M. (2004). Systematics and evolution of the jacchus group of marmosets (Platyrrhini). *American Journal of Physical Anthropology, 123(1),* 11-22.

Mayr, E. (1963) *Animal Species and Evolution.* Cambridge, Mass.: Harvard University Press.

Mamede, M.A., & Araujo, F.S. (2008). Effects of slash and burn practices on a soil seed bank of caatinga vegetation in Northeastern Brazil. *Journal of Arid Environments, 72*, 458-470.

Meirmans, P.G., & P.H. Van Tienderen: (2004). GENOTYPE and GENODIVE: two programs for the analysis of genetic diversity of asexual organisms. *Molecular Ecology Notes, 4*, 792-794.

Mendes, S. L. (1997)a. Hybridization in free-ranging *Callithrix flaviceps* and the taxonomy of the Atlantic Forest marmosets. *Neotropical Primates, 5(1),* 6-8.

Mendes, S.L. (1997)b. Padrões biogeográficos e vocais em *Callithrix* do grupo *jacchus* (Primates, Callithricidae). PhD Dissertation, Unversidade Estadual de Campinas, Campinas, São Paulo.

Moreira, M.A.M., & Seuanez, H.N. (1999). Mitochondrial pseudogenes and phyletic relationships of *Cebuella* and *Callithrix* (Platyrrhini, primates). *Primates*, *40*, 353-364.

Morin., P., Luikart, G., Wayne, R., & SNP Workshop Grp. (2004). SNPs in ecology, evolution and conservation. *Trends in Ecology & Evolution*, *19(4),* 208-16.

Mundy, N.I., Pissinatti, A., & Woodruff, D.S. (2000). Multiple nuclear insertions of mitochondrial cytochrome b sequences in callitrichine primates. *Molecular Biology and Evolution*, *17*, 1075-1080.

Nagel, U. (1973). Comparison of anubis baboons, hamadryas baboons and their hybrids at a species border in Ethiopia. *Folia Primatologica, 19*(2-3), 104-165.

Nei, M. (1977). F-statistics and analysis of gene diversity in subdivided populations. *Annals of Human Genetics, 41*, 225-33.

Nielsen, E.E.G., Bach, L.A., & Kotlicki, P. (2006). HYBRIDLAB (version 1.0): A program for generating simulated hybrids from population samples. *Molecular Ecology Notes, 6*, 971-973.

Nievergelt, C. M., Mundy, N. I., & Woodruff, D. S. (1998). Microsatellite primers for genotyping common marmosets (*Callithrix jacchus)* and other callitrichids. *Molecular Ecology*, *7,* 1432-1434.

de Oliveira, G., Araujo, M.B., Rangel, T.F., Alagador, D., & Diniz-Filho, J.A.F. (2012) Conserving the Brazilian semiarid (Caatinga) biome under climate change. *Biodiversity and Conservation, 21,*2913-2926.

Passamani, M., Agular, L., Machado, R.B., & Figueirdo, E. (1996). Hybridization between *Callithrix geoffroyi* and *C. penicillata* in southern Minas Gerais, Brazil. *Neotropical Primates, 5*, 9-10.

Pastorini, J., Zaramody, A., Curtis, D. J., Nievergelt, C. M., & Mundy, N. I. (2009). Genetic analysis of hybridization and introgression between wild mongoose and brown lemurs. *Bmc Evolutionary Biology*, *9*, 32.

Perelman, P., Johnson, W.E., Roos, C., Seuanez HN, Horvath JE, Moreira MAM, Kessing B, Pontius J, Roelke M, Rumpler Y, & others. (2011). A molecular phylogeny of living primates. *Plos Genetics*, *7*, e1001342.

Pereira, D.M. (2010). Densidade, genética e saúde populacional como ferramentas par propor um plano de controle e erradicação de invasão biológica: o caso de *Callithrix aurita* (Primates) no Parque Nacional da Serra dos Órgãos, RJ, Brasil. Universidade do Estado do Rio de Janeiro, Rio de Janeiro, RJ, Brazil.

Phillips-Conroy, J. E., & Jolly, C. J. (1981). Sexual dimorphism in two subspecies of Ethiopian baboons (*Papio hamadryas*) and their hybrids. *American Journal of Physical Anthropology, 56*, 115-129.

Phillips-Conroy, J. E., Jolly, C. J., Nystrom, P., & Hemmalin, H. A. (1992). Migration of male hamadryas baboons into anubis groups in the Awash National Park, Ethiopia. *International Journal of Primatology, 13*(4), 455-476.

Posada, D., & Buckley, T.R. (2004). Model selection and model averaging in phylogenetics: advantages of akaike information criterion and bayesian approaches over likelihood ratio tests. *Systematic Biology, 53*, 793-808.

Posada, D., & Crandall, K.A. (2001). Intraspecific gene genealogies: Trees grafting into networks. *Trends in Ecology and Evolution, 16,* 37-45.

Pritchard, J.K., Stephens, M., & Donnelly, P. (2000) Inference of population structure using multilocus genotype data. *Genetics, 155*, 945-959.

R Core Team (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/.

Raveendran, M., Tardif, S., Ross, C.N., Austad, S.N., Harris, R.A., Milosavljevic, A., & Rogers, J. (2008). Polymorphic microsatellite loci for the common marmoset (Callithrix *jacchus)* designed using a cost- and time-efficient method. *American Journal of Primatology, 70,* 906-910.

Rambaut. A., & Drummond, A.J. (2007). Tracer v1.4, Available from http://beast.bio.ed.ac.uk/Tracer

Raboy, B.E., Canale, G.R., & Dietz, J.M. (2008). Ecology of *Callithrix kuhlii* and a review of eastern Brazilian marmosets. *International Journal of Primatology, 29,* 449-467.

Ribeiro, M.C., Metzger, J.P., Martensen, A.C., Ponzoni, F.J., & Hirota, M.M. (2009). The Brazilian Atlantic Forest: How much is left, and how is the remaining forest distributed? Implications for conservation. *Biological Conservation, 142,*1141-1153.

Rice, W.R. (1989). Analyzing tables of statistical tests. *Evolution, 43*, 223-225.

Rhymer, J. & Simberloff, D. (1996). Extinction by hybridization and introgression. *Annual Review of Ecology and Systematics, 27,* 83-109.

Rogers, A.R. & Harpending, H. 1992. Population-growth makes waves in the distribution of pairwise genetic-differences. *Molecular Biology and Evolution*, *9*, 552-569.

Ronquist, F. & J. P. Huelsenbeck. (2003). MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics, 19*, 1572-1574.

Rosenberg N. (2004). DISTRUCT: A program for the graphical display of population structure. *Molecular Ecology Notes, 4*,137-8.

Ross, C. N., French, J. A., & Orti, G. (2007). Germ-line chimerism and paternal care in marmosets (*Callithrix kuhlii*). *PNAS, 104,* 6278-6282.

Rousset, F. (2008). GENEPOP'007: A complete re-implementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Resources, 8*,103-6.

Ruiz-Miranda, C.R., Affonso, A.G., Martins, A., Beck, B. (2000). Distribuição do sagui (*Callithrix jacchus*) nas áreas de ocorrência do mico-leão-dourado (*Leontopithecus rosalia*) no estado do Rio de Janeiro. *Neotropical Primates*, *8,* 98-101.

Ruiz-Miranda, C., Affonso, A., de Morais, M., Verona, C., Martins, A., Beck, B. 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. *Brazilian Archives of Biology and Technology, 49*, 99-109.

Rylands, A.B. & de Faria, D.S. (1993). Habitats, feeding ecology, and home range size in the genus *Callithrix.* In A.B. Rylands (Ed.), *Marmosets and tamarins: Systematics, behaviour, and ecology* (pp 262-272). New York: Oxford University Press.

Rylands AB, Coimbra-Filho AF, Mittermeir RA. 1993. Systematics, geographic distribution, and some notes on the conservation status of the Callithrichidae. Rylands (ed Rylands, AB), pp 9-77.In: Marmosets and Tamarins: Systematics, Behaviour, and Ecology. Oxford University Press, New York, New York.

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 S.M. Ford, L.M. Ford, & L.C. Davis (Eds.) *The Smallest Anthropoids: The Marmoset/Callimico radiation* (pp. 23-61). New York: Springer.

Rylands, A.B., & Mittermeier, R,A. (2009). The diversity of the new world primates (Platyrrhini): An annotated taxonomy. In P.A. Garber, A. Estrada, J.C. Bicca-Marques, E.W. Heymann, & K.B. Strier (Eds). *South American primates: Comparative Perspectives in the Study of Behavior, Ecology, and Conservation.* Springer.

Sambrook, J., & Russel, D.W. (2001). *Molecular Cloning 3rd ed*. Cold Spring Harbor: CSHL Press.

Samuels, A., & Altmann, J. (1986). Immigration of a *papio-anubis* male into a group of *papio-cynocephalus* baboons and evidence for an *anubis-cynocephalus* hybrid zone in Amboseli, Kenya. *International Journal of Primatology, 7*(2), 131-138.

Schlotterer, C. (2000) Evolultionary dynamics of microsatellite DNA. *Chromosoma, 109,* 365-371.

Schneider, H., Bernardi, J.A.R., da Cunha, D.B., Tagliaro, C.H., Vallinoto, M., Ferrari, S.F., & Sampaio, I. (2012). A molecular analysis of the evolutionary relationships in the Callitrichinae, with emphasis on the position of the dwarf marmoset. *Zoologica Scripta, 41,* 1-10.

Seehausen, O., van Alphen, J.J.M., &Witte, F. (1997) Cichlid fish diversity threatened by eutrophication that curbs sexual selection. *Science, 277*, 1808-1811.

Seehausen O. (2004). Hybridization and adaptive radiation. *Trends in Ecology & Evolution, 19*, 198-207.

Sena, L., Vallinoto, M., Sampaio, I., Schneider, H., Ferrari, S.F., & Schneider, M.P.C. (2002). Mitochondrial COII gene sequences provide new insights into the phylogeny of marmoset species groups (Callitrichidae, primates). *Folia Primatologica, 73*, 240-251.

Sherwin, W.B., & Moritz, C. (2000). Managing and monitoring genetic erosion. In A. G. Young & G.M. Clarke (Eds). *Genetics, demography, and viability of fragmented populations* (pp9-34). Cambridge: Cambridge University Press.

Shurtliff, Q.R. (2013). Mammalian hybrid zones: a review. *Mammal Review, 43,* 1-21.

Smith, T.E., Schaffner, C.M., & French, J.A. (1997). Social and developmental influences on reproduction in female Wied's black tufted-ear marmosets (*Callithrix kuhli). Hormones and Behavior, 31,* 159-168.

Soares, P., Ermini. L., Thomson, N., Mormina, M., Rito ,T., Roehl, A., Salas, A., Oppenheimer, S., Macaulay, V., & Richards, M.B. (2009). Correcting for purifying selection: An improved human mitochondrial molecular clock. *American Journal of Human Genetics, 84*, 740-759.

Stevison, L. S., & Kohn, M. H. (2009). Divergence population genetic analysis of hybridization between rhesus and cynomolgus macaques. *Molecular Ecology, 18*(11), 2457-2475.

Suchard, M.A., Weiss, R.E., Sinsheimer, J.S. (2001). Bayesian selection of continuous-time markov chain evolutionary models. *Molecular Biology and* Evolution, 18, 1001-1013.

Tagliaro, C.H., Schneider, M.P.C., Schneider, H., Sampaio, I.C., & Stanhope, M.J. (1997). Marmoset phylogenetics, conservation perspectives, and evolution of the mtDNA control region. *Molecular Biology and Evolution*, *14*, 674-684.

Tagliaro, C.H., Schneider, M.P.C., 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. *Genetics and Molecular Biology, 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. *Molecular Biology and Evolution, 28*,2731-9.

Tang, J., Hanage, W.P., Fraser, C., & Corander, J. (2009) Identifying currents in the gene pool for bacterial populations using an integrative approach. *PLoS Computational Biology*, *5,* e1000455.

Taylor, E.B., Boughman, J.W., Groenenboom, M., Sniatynski, M., Schluter, D., Gow, J.L. (2006). Speciation in reverse: morphological and genetic evidence of the collapse of a three-spined stickleback (*Gasterosteus aculeatus*) species pair. *Molecular Ecology, 15*, 343-355.

Thalmann, O. H., Fischer, A. H., Lankester, F. H., Paabo, S. H., & Vigilant, L. H. (2007). The complex evolutionary history of gorillas: Insights from genomic data. *Molecular Biology and Evolution, 24*, 146-158.

Thomas, C.D. (2013). The Anthropocene could raise biological diversity. *Nature, 502,* 7.

Turchetto-Zolet, A.C., Pinheiro, F., Salgueiro, F., & Palma-Silva, C. (2013). Phylogeographical patterns shed light on evolutionary process in South America. *Molecular Ecology, 22*, 1193-213.

Vaha, J. P., & Primmer, C. R. (2006). Efficiency of model-based Bayesian methods for detecting hybrid individuals under different hybridization scenarios and with different numbers of loci. *Molecular Ecology, 15*, 63-72.

Van Oosterhout, C., Hutchinson, W., Wills, D., & Shipley, P. (2004). MICRO-CHECKER: Software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes, 4,* 535-8.

van Roosmalen, M.G.M., & van Roosmalen, T. (2003). The description of a new marmoset genus, *Callibella* (Callitrichinae, primates) including its molecular phylogenetic status. *Neotropical Primates*, 11,1-10.

Weir, B. S. & Cockerham, C. C., (1984). Estimating F-statistics for the analysis of population structure. *Evolution, 38*, 1358–1370.

Wildman, D.E., Jameson, N.M., Opazo, J.C., & Yi, S.V. (2009). A fully resolved genus level phylogeny of Neotropical primates (Platyrrhini). *Molecular Phylogenetics and Evolution, 53*, 694-702.

Zhang D.X., & Hewitt G.M. (1996). Nuclear integrations: challenges for mitochondrial DNA markers. *Trends in Evolution and Ecology, 11*, 247-251.

Zinner, D., Groeneveld, L. F., Keller, C., & Roos, C. (2009). Mitochondrial phylogeography of baboons (*Papio* spp.) - indication for introgressive hybridization? *Bmc Evolutionary Biology, 9*, 83.

APPENDIX A

SUPPLEMANTRY TABLES

Table S1.

Locations and Latitude/Longitude Coordinates for Captive and Wild Samples

Taxa	**Sampling Location**	**Latitude/Longitude Coordinates**	**Samples**
RJ Hybrids	Boa Esperança	22°39'5.40"S 42°26'26.92"W, 22°39'19.98"S 42°26'24.16"W	RJ024, RJ028-RJ032
	Fazenda Afetiva	22°37'57.32"S 42°28'1.67"W, 22°37'56.52"S 42°28'3.69"W	RJ005-RJ013
	Fazenda dos Tamarins	22°36'6.33"S 42°23'34.21"W	RJ016-RJ023
	House U	22°41'2.81"S 42°29'7.39"W	RJ001-RJ004
	Pesque Pague	22°36'22.73"S 42°23'47.13"W, 22°36'22.28"S 42°23'47.75"W	RJ014,RJ015, RJ033, RJ034, RJ044-RJ046
	Ponto do Camarão	22°36'23.09"S 42°24'12.47"W	RJ025-RJ027
	Rio Vermelho I	22°42'58.02"S 42°33'52.27"W	RJ035-RJ037
	Rio Vermelho II	22°43'18.36"S 42°34'50.64"W, 22°43'15.12"S 42°35'4.02"W	RJ038-RJ043
PJ Hybrids	CEMAFAUNA[e]	N/A	PJ035-PJ037
	Chácara Bom Jesus	9°26'52.53"S 40°33'29.25"W	PJ064-PJ070
	Chácara do Senhor dos Santos	9°24'33.76"S 40°30'47.06"W	PJ033
	Chácara Galo da Briga	9°20'47.85"S 40°25'18.30"W	PJ058-PJ063
	Recanto do Sossego	9°27'58.52"S 40°33'31.95"W	PJ030-PJ032
	Rio Verde	9°14'31.59"S 40°18'39.96"W	PJ038-PJ042
	Sítio Caranaíba	9°16'12.97"S 40°22'59.73"W	PJ050, PJ052-PJ056

	Sítio Picos	9°15'50.69"S 40°19'19.24"W	PJ048, PJ049, PJ051, PJ057
	Sítio Porto da Cruz	9°13'5.72"S 40°18'13.74"W	PJ043-PJ047
	Universidade do Estado da Bahia	9°25'16.63"S 40°28'58.73"W	PJ028, PJ029,PJ034
C. penicillata	Brasília Zoo, Brasília, DF	15°50'40.12"S 47°56'35.83"W	CPE016, CPE017
	Callitrichid Research Center [a]	N/A	CPE001-CPE008
	Clube do Congresso, Brasília, DF	15°46'12.90"S 47°49'59.83"W	CPE012, CPE013
	Condominio Ouro Vermelho, Brasília, DF	15°52'28.65"S 47°46'14.94"W	CPE027, CPE028
	CONTAG, Brasília, DF	15°51'56.46"S 47°58'13.94"W	CPE020-CPE022
	Horto Florestal, MG	21° 7' 15.60"S 42° 22' 2.50"W	CPE009-CPE011
	IBAMA CETAS, Goiânia, Goiás[b]	16° 38' 5.55"S 49° 15'16.041"W	CPE039-CPE041
	Instituto Israel Pinheiro, Brasília, DF	15°48'11.03"S 47°47'57.49"W	CPE014, CPE015
	Jardim Botânico, Brasília, DF	15°51'40.15"S 47°49'43.47"W	CPE029-CPE035
	Parque dos Buritis, Goiânia, Goiás	16°40'55.22"S 49°15'43.08"W	CPE038
	Q19 Conjunto 6, Brasília, DF	15°45'1.15"S 47°50'34.10"W	CPE018, CPE019
	São Sebastião, Rua do Bosque, Brasília, DF	15°54'31.51"S 47°45'26.26"W	CPE024
	School Mistress House, Goiânia, Goiás	16°40'47.69"S 49°12'28.98"W	CPE036, CPE037
	SWPW Quadra 15, Conjunto 5, Brasília, DF	15°54'38.07"S 47°57'10.64"W	CPE025, CPE026
	Vila Weslyn, Brasília, DF	15°42'33.45"S 47°54'44.80"W	CPE023
C. jacchus	Callitrichide	N/A	CJA013, CJA014

	Research Center[a]		
	IBAMA CETAS, Recife, PE[b]	N/A	CJA018-CJA041
	NEPRC[c]	N/A	CJA002-CJA011
	Parque Dois Irmãos, Recife, PE, Tapacurá Reserve, PE[d]	N/A	CJA043-CJA080
C. geoffroyi	Callitrichide Research Center[a]	N/A	CGE002-CGE006
	Corielle	N/A	CGE001
C. kuhlii	Callitrichide Research Center[a]	N/A	CKU001-CKU04

[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 Borstelmanna de Oliveira, most samples were too degraded to amplify the mtDNA control region

[e] Center for Management of Fauna of the Caatinga

Table S2.

Locations and Latitude/Longitude Coordinates for Captive and Wild Samples

Taxa	**Sampling Location**	**Latitude/Longitude Coordinates**	**Samples**
RJ Hybrids	Boa Esperança	22°39'5.40"S 42°26'26.92"W, 22°39'19.98"S 42°26'24.16"W	RJ024, RJ028-RJ032
	Fazenda Afetiva	22°37'57.32"S 42°28'1.67"W, 22°37'56.52"S 42°28'3.69"W	RJ005-RJ013
	Fazenda dos Tamarins	22°36'6.33"S 42°23'34.21"W	RJ016-RJ023
	House U	22°41'2.81"S 42°29'7.39"W	RJ001-RJ004
	Pesque Pauge	22°36'22.73"S 42°23'47.13"W, 22°36'22.28"S 42°23'47.75"W	RJ014,RJ015, RJ033, RJ034, RJ044-RJ046
	Ponto do Camarão	22°36'23.09"S 42°24'12.47"W	RJ025-RJ027
	Rio Vermelho I	22°42'58.02"S 42°33'52.27"W	RJ035-RJ037
	Rio Vermelho II	22°43'18.36"S 42°34'50.64"W, 22°43'15.12"S 42°35'4.02"W	RJ038-RJ043
PJ Hybrids	CEMAFAUNA[e]	N/A	PJ035-PJ037
	Chácara Bom Jesus	9°26'52.53"S 40°33'29.25"W	PJ064-PJ070
	Chácara do Senhor dos Santos	9°24'33.76"S 40°30'47.06"W	PJ033
	Chácara Galo da Briga	9°20'47.85"S 40°25'18.30"W	PJ058-PJ063
	Recanto do Sossego	9°27'58.52"S 40°33'31.95"W	PJ030-PJ032
	Rio Verde	9°14'31.59"S 40°18'39.96"W	PJ038-PJ042
	Sítio Caranaíba	9°16'12.97"S 40°22'59.73"W	PJ050, PJ052-PJ056
	Sítio Picos	9°15'50.69"S 40°19'19.24"W	PJ048, PJ049, PJ051, PJ057
	Sítio Porto da Cruz	9°13'5.72"S 40°18'13.74"W	PJ043-PJ047

	Universidade do Estado da Bahia	9°25'16.63"S 40°28'58.73"W	PJ028, PJ029,PJ034
C. penicillata	Brasília Zoo, Brasília, DF	15°50'40.12"S 47°56'35.83"W	CPE016, CPE017
	Callitrichide Research Center [a]	N/A	CPE001-CPE008
	Clube Congresso, Brasília, DF	15°46'12.90"S 47°49'59.83"W	CPE012, CPE013
	Condominio Ouro Vermelho, Brasília, DF	15°52'28.65"S 47°46'14.94"W	CPE027, CPE028
	CONTAG, Brasília, DF	15°51'56.46"S 47°58'13.94"W	CPE020-CPE022
	Horto Florestal, MG	21° 7' 15.60"S 42° 22' 2.50"W	CPE009-CPE011
	IBAMA CETAS, Goiânia, Goiás[b]	16° 38' 5.55"S 49° 15'16.041"W	CPE039-CPE041
	Instituto Israel Pinheiro, Brasília, DF	15°48'11.03"S 47°47'57.49"W	CPE014, CPE015
	Jardim Botânico, Brasília, DF	15°51'40.15"S 47°49'43.47"W	CPE029-CPE035
	Parque dos Buritis, Goiânia, Goiás	16°40'55.22"S 49°15'43.08"W	CPE038
	Q19 Conjunto 6, Brasília, DF	15°45'1.15"S 47°50'34.10"W	CPE018, CPE019
	São Sebatião, Rua do Bosque, Brasília, DF	15°54'31.51"S 47°45'26.26"W	CPE024
	School Mistress House, Goiânia, Goiás	16°40'47.69"S 49°12'28.98"W	CPE036, CPE037
	SWPW Quadra 15, Conjunto 5, Brasília, DF	15°54'38.07"S 47°57'10.64"W	CPE025, CPE026
	Vila Weslyn, Brasília, DF	15°42'33.45"S 47°54'44.80"W	CPE023
C. jacchus	Callitrichide Research Center [a]	N/A	CJA013, CJA014
	IBAMA CETAS, Recife, PE[b]	N/A	CJA018-CJA041
	NEPRC[c]	N/A	CJA002-CJA011
	Parque Dois Irmãos, Recife, PE, Tapacurá Reserve, PE[d]	N/A	CJA043-CJA072

[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 Borstelmanna de Oliveira,
[e]Center for Management of Fauna of the Caatinga

Table S3.

PCR Multiplexes

Multiplex Set	**Locus Name**	**5'-Fluorescent Dye**	**Size Range**	**Reference**
1	caja1	6-FAM	363-391	Raveendran et al. (2008)
1	caja5	NED	233-250	Raveendran et al. (2008)
1	caja9	NED	165-200	Raveendran et al. (2008)
1	cj11	6-FAM	114-130	Nievergelt et al. (1998)
2	caja10	VIC	171-223	Raveendran et al. (2008)
2	caja11	PET	234-258	Raveendran et al. (2008)
2	caja13	6-FAM	342-370	Raveendran et al. (2008)
3	caja12	NED	212-266	Raveendran et al. (2008)
3	caja16	6-FAM	380-406	Raveendran et al. (2008)
3	caja18	PET	297-315	Raveendran et al. (2008)
3	cj14	6FAM	121-180	Nievergelt et al. (1998)
4	cj13	VIC	excluded	Nievergelt et al. (1998)
4	ham60	VIC	120-146	Katoh et al. (2009)
4	ham96	6-FAM	326-372	Katoh et al. (2009)
4	ham181	NED	194-228	Katoh et al. (2009)
5	caja14	6-FAM	198-230	Raveendran et al. (2008)
5	caja15	VIC	118-150	Raveendran et al. (2008)
5	caja17	6-FAM	352-426	Raveendran et al. (2008)
6	caja19	6-FAM	320-378	Raveendran et al. (2008)
6	ham41	6-FAM	excluded	Katoh et al. (2009)
6	ham141	VIC	287-242	Katoh et al. (2009)
6	ham55	PET	243-304	Katoh et al. (2009)
7	cj6	6-FAM	127-157	Nievergelt et al. (1998)
7	ham30	6-FAM	284-309	Katoh et al. (2009)
7	ham1	PET	175-201	Katoh et al. (2009)
8	ham3	6-FAM	74-111	Katoh et al. (2009)
8	ham6	NED	excluded	Katoh et al. (2009)
8	ham57	PET	230-244	Katoh et al. (2009)
8	lchu06	VIC	170-195	Galbusera and Gillemot (2008)
9	ham100	6FAM	220-248	Katoh et al. (2008)
9	ham116	VIC	273-298	Katoh et al. (2008)
9	ham146	6FAM	128-153	Katoh et al. (2008)
10	cj1	NED	117-177	Nievergelt et al. (1998)
10	ham47	PET	278-302	Katoh et al. (2009)

10	ham120	VIC	183-221	Katoh et al. (2009)
11	ham26	VIC	163-186	Katoh et al. (2009)
11	ham38	6-FAM	257-285	Katoh et al. (2008)
12	ham91	6-FAM	128-160	Katoh et al. (2009)
12	ham101	VIC	262-284	Katoh et al. (2009)
13	ham107	PET	262-289	Katoh et al. (2009)
13	ham150	6-FAM	154-170	Katoh et al. (2009)
14	ham8	PET	270-293	Katoh et al. (2009)
14	ham102	6-FAM	162-184	Katoh et al. (2009)
14	ham103	6-FAM	89-129	Katoh et al. (2009)
15	ham79	6-FAM	123-141	Katoh et al. (2008)
15	ham123	NED	149-173	Katoh et al. (2009)
15	ham184	VIC	169-204	Katoh et al. (2009)
N/A	caja6	NED	excluded	Nievergelt et al. (1998)
N/A	cj7	6-FAM	excluded	Raveendran et al. (2008)
N/A	cj15	VIC	excluded	Raveendran et al. (2008)

Note: Loci labeled as "excluded" were not included in a multiplex due to poor PCR amplification.

Table S4 A-D.

Locus-by-Locus Summary of Various Genetic Diversity Indices by Species and Hybrid Zone

A.

Locus	***C. penicillata***						
	N	***A***	**R**	**r**	**H_o**	**H_E**	**F_{IS}**
caja1	41	11	10.516	0.000	0.829	0.833	0.004
caja10	29	16	16.000	0.236	0.379	0.849	**0.553*** **
caja11	40	10	9.412	0.040	0.775	0.859	0.098
caja12	37	16	14.641	0.134	0.459	0.714	**0.356*** **
caja13	41	10	9.159	0.053	0.561	0.702	0.2*
caja14	40	11	10.504	0.012	0.800	0.836	0.043
caja15	41	10	9.246	0.032	0.732	0.805	0.092
caja16	36	9	8.540	0.166	0.500	0.799	**0.374*** **
caja17	39	17	15.841	0.049	0.795	0.920	0.136
caja18	37	8	7.947	0.007	0.811	0.803	-0.009
caja19	40	10	9.334	0.146	0.500	0.782	**0.361*** **
caja5	39	7	6.602	0.118	0.436	0.639	0.317
caja9	36	8	7.792	0.138	0.528	0.761	**0.307** **
cj1	39	15	14.325	0.061	0.769	0.914	0.158
cj11	41	5	4.415	0.203	0.293	0.631	**0.536*** **
cj14	39	13	12.070	0.088	0.564	0.756	**0.254* **
cj6	41	12	11.493	0.058	0.780	0.874	0.107
ham1	39	10	9.442	0.068	0.718	0.843	0.149
ham100	40	11	10.811	0.144	0.575	0.870	**0.339*** **
ham101	38	8	7.758	0.144	0.553	0.812	**0.32*** **
ham102	36	12	11.727	0.045	0.778	0.893	0.129
ham103	35	11	10.282	0.154	0.486	0.781	**0.378* **
ham107	38	11	10.351	0.100	0.553	0.722	**0.235*** **
ham116	39	10	9.291	0.154	0.385	0.671	**0.427*** **
ham120	40	13	11.537	0.058	0.600	0.695	0.137
ham123	29	6	6.000	0.161	0.517	0.828	**0.375* **
ham141	32	15	14.595	0.226	0.438	0.876	**0.5*** **
ham146	39	9	8.345	0.001	0.590	0.586	-0.006
ham150	40	7	6.780	0.000	0.725	0.744	0.026
ham181	40	12	11.280	0.058	0.750	0.873	0.141
ham184	35	15	14.425	0.014	0.857	0.891	0.038
ham26	30	7	6.966	0.155	0.400	0.692	**0.422** **
ham3	39	12	11.896	0.019	0.846	0.871	0.029
ham30	41	9	7.933	0.078	0.561	0.733	0.235
ham38	38	13	11.891	0.087	0.684	0.813	0.158
ham47	40	9	8.424	0.071	0.675	0.771	0.125
ham55	40	12	10.711	0.107	0.425	0.671	**0.367*** **

ham57	41	6	5.915	0.092	0.537	0.664	0.192
ham60	39	8	7.423	0.162	0.462	0.741	**0.377*****
ham79	35	9	8.945	0.068	0.686	0.768	0.107
ham8	31	13	12.803	0.082	0.677	0.879	**0.229***
ham91	41	13	11.154	0.040	0.780	0.830	0.060
ham96	34	13	12.622	0.034	0.794	0.854	0.07*
lchu06	40	16	14.982	0.106	0.700	0.920	**0.239*****

B.

Locus	***C. jacchus***						
	N	***A***	**R**	**r**	H_o	H_E	F_{IS}
caja1	50	9	8.489	0.101	0.620	0.827	0.250
caja10	59	12	9.803	0.004	0.814	0.848	0.041
caja11	59	6	4.215	0.000	0.220	0.205	-0.076
caja12	54	11	8.754	0.022	0.741	0.757	0.022
caja13	58	7	6.906	0.002	0.759	0.750	-0.011
caja14	60	7	6.210	0.076	0.500	0.645	0.225
caja15	58	8	7.471	0.032	0.776	0.841	0.078
caja16	52	6	5.875	0.081	0.423	0.568	**0.256****
caja17	60	11	9.164	0.002	0.583	0.620	0.059
caja18	53	5	4.508	0.113	0.491	0.687	0.286
caja19	52	10	8.032	0.067	0.596	0.735	0.189
caja5	53	3	2.997	0.072	0.491	0.564	0.131
caja9	55	7	6.378	0.005	0.673	0.721	0.067
cj1	57	8	6.706	0.000	0.544	0.546	0.003
cj11	48	3	2.978	0.029	0.542	0.518	-0.046
cj14	51	10	8.706	0.038	0.725	0.793	0.085
cj6	59	8	7.060	0.068	0.424	0.544	0.221
ham1	57	9	7.777	0.000	0.807	0.831	0.029
ham100	57	8	7.643	0.000	0.825	0.842	0.020
ham101	53	9	8.035	0.010	0.811	0.759	-0.068
ham102	55	5	4.731	0.056	0.582	0.624	0.068
ham103	55	12	9.619	0.013	0.745	0.776	0.039
ham107	59	10	7.550	0.018	0.729	0.716	-0.017
ham116	50	6	5.581	0.241	0.200	0.552	**0.637*****
ham120	56	10	7.830	0.071	0.339	0.431	0.213
ham123	57	12	10.162	0.025	0.719	0.787	0.086
ham141	54	10	8.260	0.000	0.870	0.800	-0.087
ham146	54	8	5.589	0.013	0.519	0.575	0.099
ham150	53	6	5.091	0.057	0.547	0.686	0.203

ham181	54	11	9.111	0.000	0.759	0.800	0.050
ham184	55	11	9.580	0.000	0.818	0.831	0.015
ham26	57	9	7.797	0.013	0.789	0.733	-0.078
ham3	59	9	7.801	0.039	0.627	0.680	0.077**
ham30	56	9	8.197	0.030	0.661	0.736	0.103
ham38	60	9	7.399	0.061	0.600	0.665	0.097
ham47	54	9	7.758	0.003	0.611	0.615	0.007
ham55	55	9	6.918	0.000	0.673	0.680	0.011
ham57	59	5	4.424	0.036	0.441	0.527	0.165
ham60	59	8	6.150	0.105	0.288	0.402	0.283
ham79	51	7	6.936	0.065	0.686	0.786	0.127*
ham8	53	8	6.188	0.005	0.509	0.538	0.053
ham91	61	11	8.396	0.029	0.656	0.695	0.056**
ham96	58	8	6.792	0.000	0.759	0.662	-0.146
lchu06	56	10	7.730	0.014	0.696	0.718	0.030
Total	55.568	8.386	7.120	0.037	0.618	0.673	0.082

C.

Locus	**PJ Zone**						
	N	***A***	**R**	**r**	**H_o**	**H_E**	**F_{IS}**
caja1	42	11	9.705	0.009	0.738	0.747	0.011
caja10	42	12	11.598	0.042	0.810	0.889	0.089***
caja11	42	9	8.256	0.000	0.786	0.747	-0.052
caja12	42	11	9.587	0.005	0.548	0.610	0.103
caja13	42	7	6.683	0.021	0.714	0.731	0.022
caja14	42	10	8.970	0.068	0.643	0.788	**0.184***
caja15	43	8	7.467	0.135	0.442	0.687	**0.357*****
caja16	37	7	6.686	0.013	0.622	0.592	-0.050
caja17	42	10	9.194	0.061	0.714	0.845	0.155
caja18	41	8	7.383	0.069	0.415	0.577	**0.282***
caja19	37	4	3.784	0.228	0.189	0.519	**0.635*****
caja5	42	5	4.682	0.131	0.429	0.612	**0.3****
caja9	41	9	8.241	0.036	0.659	0.775	0.150
cj1	41	8	7.097	0.149	0.390	0.653	**0.403***
cj11	40	4	3.720	0.135	0.275	0.471	0.416
cj14	40	13	11.421	0.026	0.725	0.742	0.022
cj6	40	5	4.889	0.050	0.425	0.529	0.197
ham1	40	10	9.284	0.035	0.675	0.754	0.105
ham100	37	9	8.522	0.036	0.757	0.800	0.054
ham101	41	8	7.518	0.040	0.659	0.764	0.138

ham102	41	8	7.677	0.028	0.756	0.804	0.059
ham103	29	9	9.000	0.288	0.276	0.797	**0.654*** **
ham107	40	9	8.349	0.097	0.525	0.677	0.224
ham116	35	6	5.827	0.260	0.171	0.609	**0.718*** **
ham120	40	9	8.165	0.000	0.675	0.662	-0.019
ham123	38	10	9.641	0.053	0.737	0.808	0.088
ham141	32	8	7.898	0.094	0.625	0.791	0.210
ham146	39	6	5.472	0.024	0.410	0.464	0.115
ham150	38	6	5.986	0.106	0.526	0.692	0.240
ham181	40	12	10.514	0.069	0.625	0.771	0.189
ham184	36	6	5.929	0.122	0.556	0.753	0.262
ham26	42	9	8.167	0.005	0.786	0.768	-0.023
ham3	38	9	8.459	0.052	0.684	0.787	0.131
ham30	40	8	7.614	0.004	0.875	0.794	-0.102
ham38	39	11	10.146	0.118	0.564	0.791	**0.287***
ham47	41	7	6.970	0.011	0.780	0.789	0.011
ham55	36	7	6.576	0.058	0.556	0.656	0.154
ham57	41	5	4.701	0.071	0.488	0.592	0.176
ham60	40	7	6.448	0.116	0.500	0.696	0.281
ham79	35	4	3.829	0.043	0.543	0.636	0.146
ham8	39	11	9.847	0.049	0.590	0.696	0.152
ham91	43	11	9.448	0.023	0.488	0.575	0.151*
ham96	36	9	8.345	0.009	0.750	0.767	0.022
lchu06	41	10	9.433	0.140	0.463	0.710	**0.347*** **

D.

Locus	**RJ Zone**						
	N	***A***	**R**	**r**	**H_o**	**H_E**	**F_{IS}**
caja1	43	9	8.004	0.000	0.581	0.666	0.126
caja10	35	7	6.991	0.165	0.486	0.808	**0.399*** **
caja11	44	6	5.995	0.024	0.727	0.786	0.075*
caja12	43	9	8.235	0.029	0.721	0.803	0.102
caja13	44	6	5.432	0.000	0.705	0.667	-0.057
caja14	43	8	7.330	0.000	0.791	0.751	-0.052
caja15	40	6	5.652	0.119	0.500	0.710	**0.295*** **
caja16	37	5	4.784	0.092	0.541	0.694	0.222
caja17	44	8	7.984	0.011	0.750	0.812	0.076
caja18	45	6	5.520	0.072	0.556	0.674	0.176
caja19	40	5	4.718	0.083	0.250	0.335	0.253
caja5	42	4	4.000	0.000	0.738	0.700	-0.055

caja9	42	3	2.690	0.121	0.214	0.361	0.407
cj1	43	5	4.865	0.020	0.628	0.681	0.078
cj11	41	3	2.998	0.000	0.415	0.396	-0.048
cj14	45	10	8.886	0.014	0.822	0.839	0.020
cj6	43	8	7.465	0.068	0.488	0.671	0.272
ham1	41	7	6.828	0.019	0.634	0.745	0.149
ham100	43	6	5.894	0.059	0.605	0.707	0.145
ham101	43	7	6.571	0.169	0.465	0.771	**0.397*****
ham102	36	7	6.734	0.030	0.694	0.779	0.109
ham103	34	6	6.000	0.165	0.441	0.706	0.375
Ham107	43	7	6.561	0.109	0.581	0.769	0.244
ham116	42	7	6.879	0.001	0.786	0.805	0.025
ham120	43	6	5.571	0.000	0.744	0.742	-0.003
ham123	39	5	4.740	0.107	0.436	0.656	**0.335*****
ham141	40	9	8.633	0.000	0.850	0.831	-0.022
ham146	43	6	5.245	0.013	0.605	0.578	-0.047
ham150	42	7	6.992	0.000	0.786	0.832	0.055
ham181	42	8	7.663	0.000	0.857	0.838	-0.023
ham184	36	7	6.769	0.007	0.722	0.716	-0.009
ham26	43	8	7.236	0.053	0.698	0.797	0.124
ham3	42	8	7.655	0.012	0.714	0.769	0.071
ham30	43	6	5.665	0.050	0.674	0.766	0.120
ham38	42	10	9.346	0.085	0.690	0.870	0.206
ham47	43	7	6.761	0.035	0.698	0.779	0.104
ham55	37	8	7.730	0.000	0.595	0.700	0.151
ham57	44	6	5.963	0.007	0.750	0.780	0.039
ham60	42	6	5.972	0.045	0.643	0.764	0.158
ham79	39	7	6.680	0.121	0.487	0.727	**0.33****
ham8	36	9	8.575	0.023	0.778	0.835	0.069
ham91	43	6	5.990	0.018	0.744	0.806	0.077
ham96	40	6	5.702	0.038	0.550	0.649	0.152
lchu06	42	7	6.879	0.036	0.738	0.802	0.080

Note: N is number of individuals sampled at a locus, A is the number of alleles at a locus, R is allelic richness, r is EM null allele frequency, H_o is observed heterozygousity, H_E is expected heterozygousity, F_{IS} is the inbreeding coefficient. F_{IS} values in bold indicate loci which were flagged by Microchecker for the possible presence of null alleles. F_{IS} values that are starred are significant for Hardy-Weinberg disequilibrium for various *P*-values as follows: * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

Table S5.

Allele Frequencies Uncorrected and Corrected for Presence of Null Alleles as Observed within Parental Species and Hybrid Zones at Each Locus

L	**Allele**	***C. penicillata***		***C. jacchus***		**PJ Zone**		**RJ Zone**	
		R	**C**	**R**	**C**	**R**	**C**	**R**	**C**
caja 1	**363**	0.012	0.012	-	-	0.012	0.012	-	-
	365	0.061	0.061	-	-	0.012	0.012	0.012	0.012
	367	0.073	0.073	-	-	0.036	0.036	0.547	0.546
	368	-	-	-	-	-	-	0.012	0.012
	369	-	-	-	-	0.071	0.069	-	-
	371	0.024	0.024	-	-	-	-	0.047	0.047
	373	0.341	0.341	0.030	0.021	0.060	0.060	0.012	0.012
	375	0.146	0.146	0.060	0.060	0.036	0.036	0.047	0.047
	377	0.122	0.122	0.090	0.083	-	-	-	-
	379	0.073	0.073	0.230	0.194	0.179	0.175	-	-
	381	0.085	0.085	0.260	0.237	0.452	0.449	0.081	0.081
	382	-	-	-	-	0.012	0.012	-	-
	383	0.037	0.037	0.060	0.060	0.119	0.119	0.163	0.163
	385	0.024	0.024	0.210	0.184	-	-	0.081	0.081
	387	-	-	0.050	0.050	-	-	-	-
	389	-	-	0.010	0.010	-	-	-	-
	391	-	-	-	-	0.012	0.012	-	-
	NULL	NA	-	NA	0.101	NA	0.009	NA	-
caja 5	**233**	0.038	0.038	-	-	-	-	-	-
	235	-	-	-	-	0.012	0.012	-	-
	239	0.013	0.013	0.443	0.417	0.357	0.314	0.417	0.417
	241	0.295	0.258	0.066	0.052	0.512	0.456	0.286	0.286
	243	0.526	0.465	0.491	0.459	0.071	0.041	0.083	0.083
	245	0.077	0.056	-	-	0.048	0.048	0.214	0.214
	247	0.026	0.026	-	-	-	-	-	-
	250	0.026	0.026	-	-	-	-	-	-
	NULL	NA	0.118	NA	0.072	NA	0.131	NA	-
caja 9	**165**	0.042	0.029	-	-	-	-	-	-
	168	0.333	0.278	-	-	-		-	-
	169	0.056	0.031	-	-	-	-	-	-

	178	0.097	0.097	-	-	0.024	0.024	-	-
	180	0.014	0.014	0.027	0.027	0.012	0.012	0.012	0.012
	182	0.347	0.315	0.455	0.452	0.268	0.012	0.774	0.687
	184	0.069	0.069	0.173	0.172	0.220	0.252	-	-
	186	0.042	0.029	-	-	0.012	0.213	-	-
	187	-	-	0.036	0.036	-		-	-
	189	-	-	-	-	0.061	0.061	-	-
	191	-	-	0.009	0.009	0.024	0.024	-	-
	193	-	-	0.182	0.180	0.329	0.316	0.214	0.180
	196	-	-	0.118	0.118	-		-	-
	200	-	-	-	-	0.049	0.049	-	-
	NULL	NA	0.138	NA	0.005	NA	0.036	NA	0.121
caja 10	**171**	-	-	0.102	0.101	-	-	-	-
	179	0.017	0.017	0.008	0.008	0.060	0.052	-	-
	181	0.052	0.036	0.008	0.008	0.036	0.036	-	-
	183	-	-	0.042	0.042	0.036	0.036	-	-
	185	0.034	0.034	0.203	0.202	0.036	0.036	0.257	0.214
	187	0.052	0.052	0.178	0.177	0.179	0.171	-	-
	189	0.017	0.017	0.237	0.237	0.202	0.199	-	-
	191	0.034	0.034	0.110	0.110	0.119	0.119	-	-
	193	-	-	0.068	0.068	0.048	0.048	0.286	0.226
	195	-	-	-	-	0.071	0.071	0.043	0.043
	196	0.034	0.018	0.008	0.008	-	-	-	-
	197	-	-	0.008	0.008	-	-	-	-
	198	0.362	0.249	-	-	-	-	-	-
	200	0.086	0.055	0.025	0.025	-	-	-	-
	201	-	-	-	-	0.119	0.109	-	-
	203	0.069	0.037	-	-	-	-	0.200	0.162
	205	0.034	0.034	-	-	-	-	0.114	0.103
	207	0.138	0.110	-	-	-	-	0.043	0.030
	209	0.017	0.017	-	-	0.012	0.012	-	-
	211	0.017	0.017	-	-	0.083	0.070	-	-
	213	0.017	0.017	-	-	-	-	-	-
	215	-	-	-	-	-	-	0.057	0.057
	223	0.017	0.017	-	-	-	-	-	-
	NULL	NA	0.236	NA	0.004	NA	0.042	NA	0.165
caja 11	**234**	-	-	0.008	0.008	0.036	0.036	-	-
	236	-	-	0.008	0.008	0.012	0.012	-	-
	238	0.163	0.158	0.890	0.890	0.381	0.381	0.170	0.170

	239	-	-	-	-	0.024	0.024	-	-
	240	-	-	0.068	0.068	-	-	0.193	0.184
	242	0.013	0.013	-	-	-	-	-	-
	244	0.125	0.115	0.017	0.017	0.060	0.060	-	-
	246	0.037	0.038	-	-	0.107	0.107	0.159	0.159
	248	0.150	0.146	0.008	0.008	0.310	0.310	0.352	0.342
	250	0.113	0.113	-	-	0.060	0.060	0.068	0.063
	251	-	-	-	-	0.012	0.012	-	-
	252	0.250	0.241	-	-	-	-	0.057	0.057
	254	0.037	0.038	-	-	-	-	-	-
	256	0.100	0.088	-	-	-	-	-	-
	258	0.013	0.013	-	-	-	-	-	-
	NULL	NA	0.040	NA	-	NA	-	NA	0.024
caja 12	**212**	-	-	0.009	0.009	-	-	-	-
	219	0.054	0.042	-	-	0.036	0.036	-	-
	221	-	-	0.324	0.318	0.131	0.129	-	-
	223	-	-	-	-	-	-	0.291	0.281
	225	0.014	0.014	0.009	0.009	0.012	0.012	-	-
	227	-	-	0.028	0.022	-	-	0.070	0.070
	228	-	-	-	-	-	-	0.012	0.012
	229	0.027	0.027	-	-	0.036	0.036	-	-
	231	0.014	0.014	0.315	0.308	0.607	0.604	0.163	0.163
	232	-	-	0.009	0.009	-	-	-	-
	233	0.527	0.451	0.000	-	-	-	0.047	0.047
	234	0.095	0.084	0.204	0.200	0.083	0.083	-	-
	235	0.027	0.027	-	-	-	-	-	-
	236	0.027	0.014	0.037	0.037	0.036	0.036	-	-
	238	0.027	0.027	0.037	0.037	0.024	0.024	-	-
	240	-	-	0.019	0.019	0.012	0.012	-	-
	241	0.014	0.014	-	-	-	-	-	-
	242	0.027	0.027	-	-	-	-	0.023	0.023
	244	-	-	-	-	-	-	0.279	0.269
	246	0.014	0.014	-	-	-	-	-	-
	250	0.014	0.014	-	-	-	-	0.012	0.012
	252	0.054	0.042	-	-	-	-	-	-
	254	0.027	0.027	-	-	-	-	0.105	0.096
	256	0.041	0.028	-	-	-	-	-	-
	257	-	-	0.009	0.009	-	-	-	-
	264	-	-	-	-	0.012	0.012	-	-
	266	-	-	-	-	0.012	0.012	-	-

	NULL	NA	0.134	NA	0.022	NA	0.005	NA	0.029
caja 13	**342**	0.024	0.024	-	-	-	-	-	-
	352	0.012	0.012	-	-	-	-	-	-
	354	0.012	0.012	-	-	0.083	0.079	-	-
	356	0.512	0.477	-	-	0.048	0.048	0.023	0.023
	358	0.024	0.014	0.043	0.043	-	-	-	-
	360	0.049	0.049	0.069	0.069	-	-	-	-
	362	0.024	0.024	0.103	0.103	0.107	0.107	0.023	0.023
	364	0.159	0.159	0.319	0.318	0.167	0.164	0.375	0.375
	366	0.098	0.091	0.362	0.361	0.464	0.456	0.148	0.148
	368	0.085	0.085	0.069	0.068	0.119	0.113	0.420	0.420
	370	-	-	0.034	0.034	0.012	0.012	0.011	0.011
	NULL	NA	0.053	NA	0.002	NA	0.021	NA	-
caja 14	**198**	-	-	0.175	0.167	0.345	0.320	0.047	0.047
	200	-	-	0.025	0.025	0.024	0.024	-	-
	204	0.062	0.063	0.008	0.008	0.048	0.048	-	-
	206	0.138	0.136	-	-	0.095	0.095	0.047	0.047
	208	0.125	0.125	0.550	0.506	0.107	0.093	0.267	0.267
	210	0.338	0.333	0.150	0.132	0.274	0.253	0.116	0.116
	212	0.025	0.025	0.025	0.025	0.012	0.012	0.105	0.105
	214	0.075	0.072	0.067	0.061	0.071	0.063	0.395	0.395
	216	-	-	-	-	0.012	0.012	0.012	0.012
	218	0.087	0.085	-	-	-	-	0.012	0.012
	220	-	-	-	-	0.012	0.012	-	-
	222	0.025	0.025	-	-	-	-	-	-
	226	0.025	0.025	-	-	-	-	-	-
	228	0.013	0.013	-	-	-	-	-	-
	230	0.087	0.088	-	-	-	-	-	-
	NULL	NA	0.012	NA	0.076	NA	0.068	NA	-
caja 15	**118**	0.024	0.024	-	-	-	-	-	-
	127	0.012	0.012	-	-	-	-	-	-
	133	0.024	0.024	-	-	-	-	-	-
	135	0.085	0.080	0.181	0.169	0.105	0.096	-	-
	137	0.317	0.307	0.233	0.231	0.523	0.449	0.263	0.250
	139	0.061	0.061	0.172	0.165	0.081	0.052	0.438	0.374
	141	-	-	-	-	-	-	0.025	0.013
	142	0.268	0.261	0.086	0.082	0.070	0.070	0.013	0.013
	144	0.073	0.073	0.009	0.009	0.012	0.012	-	-

	146	0.122	0.113	0.103	0.103	0.163	0.140	0.188	0.165
	148	0.012	0.012	0.172	0.165	0.023	0.023	0.075	0.065
	150	-	-	0.043	0.043	0.023	0.023	-	-
	NULL	NA	0.032	NA	0.032	NA	0.135	NA	0.119
caja 16	**380**	-	-	0.058	0.058	-	-	-	-
	381	-	-	0.038	0.038	-	-	0.081	0.071
	382	-	-	-	-	0.014	0.014	0.284	0.249
	383	-	-	0.144	0.128	0.176	0.176	-	-
	384	0.028	0.028	0.635	0.582	0.608	0.601	0.176	0.161
	389	0.028	0.014	0.029	0.029	0.041	0.035	0.000	-
	391	0.014	0.014	0.096	0.083	0.108	0.108	0.446	0.413
	393	0.097	0.075	-	-	0.027	0.027	-	-
	395	0.222	0.177	-	-	-	-	-	-
	397	0.167	0.137	-	-	0.027	0.027	-	-
	399	0.347	0.304	-	-	-	-	-	-
	401	0.014	0.014	-	-	-	-	-	-
	403	0.083	0.072	-	-	-	-	-	-
	406	-	-	-	-	-	-	0.014	0.014
	NULL	NA	0.166	NA	0.081	NA	0.013	NA	0.092
caja 17	**352**	0.038	0.038	-	-	-	-	-	-
	358	0.128	0.122	-	-	-	-	-	-
	360	0.013	0.013	-	-	-	-	0.091	0.089
	362	0.128	0.110	0.017	0.017	0.024	0.024	0.159	0.158
	364	-	-	0.092	0.091	0.083	0.076	-	-
	366	0.141	0.136	0.600	0.599	0.012	0.012	0.057	0.057
	368	0.051	0.042	0.033	0.033	0.190	0.176	0.102	0.100
	370	0.051	0.051	0.033	0.033	0.107	0.101	-	-
	372	0.051	0.051	0.083	0.083	0.262	0.246	-	-
	374	0.141	0.130	0.017	0.017	0.024	0.024	-	-
	376	0.077	0.077	0.083	0.083	0.119	0.113	0.045	0.045
	378	0.038	0.038	0.025	0.025	0.167	0.156	0.364	0.359
	380	0.038	0.038	-	-	-	-	-	-
	382	-	-	-	-	0.012	0.012	-	-
	386	-	-	-	-	-	-	0.102	0.102
	388	0.026	0.026	-	-	-	-	-	-
	396	0.013	0.013	-	-	-	-	-	-
	398	0.038	0.038	0.008	0.008	-	-	-	-
	400	-	-	0.008	0.008	-	-	-	-
	403	0.013	0.013	-	-	-	-	-	-

	418	-	-	-	-	-	-	0.080	0.080
	426	0.013	0.013	-	-	-	-	-	-
	NULL	NA	0.049	NA	0.002	NA	0.061	NA	0.011
caja 18	**297**	0.176	0.175	0.009	0.009	-	-	-	-
	299	0.108	0.107	0.038	0.038	0.012	0.012	0.267	0.246
	301	0.365	0.363	0.425	0.382	0.634	0.586	0.078	0.070
	303	0.068	0.065	0.255	0.211	0.061	0.061	0.489	0.457
	305	0.027	0.027	0.274	0.246	0.049	0.049	0.133	0.121
	307	0.041	0.041	-	-	0.134	0.114	0.022	0.022
	309	0.081	0.081	-	-	0.061	0.061	-	-
	311	0.135	0.135	-	-	0.012	0.012	0.011	0.011
	315	-	-	-	-	0.037	0.037	-	-
	NULL	NA	0.007	NA	0.113	NA	0.069	NA	0.072
caja 19	**320**	0.037	0.038	-	-	-	-	0.062	0.053
	322	0.062	0.041	0.067	0.067	0.189	0.137	0.062	0.063
	324	-	-	0.202	0.181	-	-	-	-
	344	-	-	0.010	0.010	-	-	-	-
	346	0.350	0.294	-	-	-	-	-	-
	348	0.287	0.239	-	-	-	-	-	-
	350	0.125	0.116	0.010	-	0.014	0.014	-	-
	352	0.037	0.038	-	-	0.135	0.090	0.812	0.749
	354	0.050	0.039	0.442	0.414	0.662	0.531	0.050	0.040
	356	-	-	0.019	0.019	-	-	-	-
	358	-	-	0.067	0.067	-	-	-	-
	365	-	-	0.163	0.145	-	-	-	-
	368	-	-	0.010	0.010	-	-	0.013	0.013
	372	0.013	0.013	-	-	-	-	-	-
	373	-	-	0.010	0.010	-	-	-	-
	376	0.013	0.013	-	-	-	-	-	-
	378	0.025	0.025	-	-	-	-	-	-
	NULL	NA	0.146	NA	0.067	NA	0.228	NA	0.083
cj1	**117**	0.090	0.082	0.640	0.640	0.524	0.446	0.430	0.422
	119	-	-	-	-	-	-	0.023	0.023
	125	0.090	0.082	-	-	-	-	-	-
	129	0.103	0.088	-	-	0.037	0.025	0.000	-
	132	0.128	0.122	-	-	0.012	0.012	0.326	0.318
	133	-	-	-	-	0.012	0.012	-	-
	134	0.064	0.064	-	-	-	-	-	-
	135	-	-	0.035	0.035	-	-	-	-

	136	0.013	0.013	-	-	-	-	-	-
	137	0.038	0.038	0.044	0.044	-	-	-	-
	139	0.064	0.055	0.211	0.211	0.268	0.219	0.035	0.035
	141	0.051	0.051	0.026	0.026	-	-	-	-
	143	0.013	0.013	-	-	-	-	-	-
	144	0.064	0.064	0.026	0.026	0.073	0.073	-	-
	146	0.192	0.177	0.009	0.009	0.061	0.051	-	-
	148	0.038	0.038	-	-	-	-	-	-
	149	0.026	0.026	-	-	-	-	-	-
	150	0.026	0.026	-	-	-	-	-	-
	152	-	-	-	-	0.012	0.012	0.186	0.182
	177	-	-	0.009	0.009	-	-	-	-
	NULL	NA	0.061	NA	-	NA	0.149	NA	0.020
cj6	**127**	0.061	0.043	-	-	-	-	0.035	0.035
	135	0.073	0.057	0.093	0.088	0.637	0.605	0.035	0.035
	137	0.024	0.024	0.042	0.036	0.037	0.038	0.384	0.355
	139	0.085	0.085	0.025	0.025	0.037	0.038	0.430	0.391
	141	0.232	0.223	0.102	0.092	0.025	0.025	0.035	0.035
	143	0.159	0.148	0.661	0.615	0.263	0.244	0.023	0.023
	145	0.183	0.178	0.051	0.051	-	-	0.012	0.012
	147	0.037	0.037	0.017	0.017	-	-	-	-
	149	0.073	0.073	0.008	0.008	-	-	-	-
	151	0.012	0.012	-	-	-	-	-	-
	153	0.037	0.037	-	-	-	-	0.047	0.047
	157	0.024	0.024	-	-	-	-	-	-
	NULL	NA	0.058	NA	0.068	NA	0.050	NA	0.068
cj11	**114**	-	-	-	-	0.013	0.013	-	-
	116	0.268	0.212	0.365	0.357	0.250	0.215	0.756	0.756
	118	0.183	0.137	0.594	0.579	0.688	0.598	0.061	0.061
	120	0.524	0.423	0.042	0.035	0.050	0.039	0.183	0.183
	124	0.012	0.012	-	-	-	-	-	-
	130	0.012	0.012	-	-	-	-	-	-
	NULL	NA	0.203	NA	0.029	NA	0.135	NA	-
cj14	**121**	0.474	0.426	-	-	0.037	0.038	-	-
	150	0.026	0.026	-	-	0.013	0.013	0.100	0.098
	152	-	-	0.010	0.010	-	-	0.156	0.156
	154	-	-	0.176	0.173	-	-	-	-
	156	0.013	0.013	0.353	0.340	0.312	0.303	0.211	0.210
	158	0.026	0.026	0.039	0.032	0.025	0.025	-	-
	160	0.077	0.077	0.157	0.154	0.400	0.393	0.167	0.165

	162	-	-	0.020	0.020	0.062	0.063	0.011	0.011
	164	0.038	0.038	0.039	0.039	0.037	0.038	0.033	0.033
	166	0.064	0.054	-	-	0.013	0.013	0.011	0.011
	168	0.038	0.027	-	-	0.013	0.013	0.056	0.052
	169	-	-	-	-	0.037	0.038	-	-
	170	0.090	0.072	0.176	0.164	-	-	0.244	0.240
	172	0.013	0.013	-	-	0.013	0.013	-	-
	174	0.077	0.077	0.020	0.020	0.025	0.015	0.011	0.011
	176	0.051	0.051	0.010	0.010	0.013	0.013	-	-
	180	0.013	0.013	-	-	-	-	-	-
	NULL	NA	0.088	NA	0.038	NA	0.026	NA	0.014
ham 1	**175**	-	-	-	-	-	-	0.293	0.283
	177	0.038	0.028	-	-	-	-	0.049	0.049
	181	0.038	0.038	-	-	0.013	0.013	-	-
	183	0.013	0.013	0.114	0.114	0.438	0.421	-	-
	185	-	-	-	-	0.037	0.029	0.110	0.110
	187	0.128	0.121	0.018	0.018	0.025	0.025	0.024	0.024
	188	-	-	-	-	0.013	0.013	-	-
	189	0.192	0.181	0.123	0.123	0.025	0.025	-	-
	191	0.269	0.251	0.193	0.193	0.200	0.190	0.110	0.110
	193	0.167	0.161	0.219	0.219	0.087	0.088	0.024	0.024
	195	0.103	0.087	0.237	0.237	0.062	0.063	0.390	0.382
	197	0.038	0.038	0.079	0.079	-	-	-	-
	199	0.013	0.013	0.009	0.009	0.100	0.100	-	-
	201	-	-	0.009	0.009	-	-	-	-
	NULL	NA	0.068	-	-	NA	0.035	NA	-
ham 3	**74**	-	-	-	-	0.013	0.013	-	-
	78	-	-	-	-	0.013	0.013	-	-
	84	-	-	0.017	0.017	-	-	-	-
	86	0.038	0.038	0.017	0.017	-	-	0.012	0.012
	88	-	-	-	-	0.079	0.079	0.107	0.105
	90	0.077	0.072	0.025	0.019	0.039	0.039	0.405	0.399
	92	0.115	0.115	0.042	0.042	0.053	0.053	0.036	0.036
	94	0.103	0.103	0.288	0.279	0.316	0.302	0.214	0.213
	96	0.295	0.290	0.483	0.465	0.289	0.279	0.095	0.093
	98	0.077	0.077	0.051	0.051	0.171	0.144	-	-
	100	0.077	0.072	0.068	0.063	-	-	-	-
	102	0.051	0.051	0.008	0.008	0.026	0.026	0.048	0.048
	104	0.051	0.045	-	-	-	-	-	-

	106	0.026	0.026	-	-	-	-	0.083	0.083
	108	0.051	0.051	-	-	-	-	-	-
	111	0.038	0.038	-	-	-	-	-	-
	NULL	NA	0.019	NA	0.039	NA	0.052	NA	0.012
ham 8	**270**	0.016	0.016	-	-	-	-	-	-
	272	0.016	0.016	0.009	0.009	0.013	0.013	0.167	0.164
	273	-	-	-	-	-	-	0.069	0.064
	274	0.194	0.161	-	-	0.013	0.013	0.083	0.083
	276	-	-	-	-	-	-	0.208	0.201
	280	0.032	0.032	-	-	-	-	-	-
	281	0.065	0.065	-	-	0.026	0.026	-	-
	282	0.016	0.016	0.113	0.112	0.090	0.090	-	-
	283	0.048	0.048	-	-	0.077	0.061	-	-
	284	-	-	0.085	0.085	0.077	0.077	0.153	0.153
	285	0.065	0.065	-	-	-	-	-	-
	286	0.065	0.052	0.660	0.657	0.526	0.498	0.264	0.258
	287	0.258	0.231	0.009	0.009	0.013	0.013	0.014	0.014
	288	-	-	0.104	0.103	-	-	-	-
	289	0.097	0.086	0.009	0.009	0.026	0.026	0.014	0.014
	290	-	-	0.009	0.009	0.128	0.123	0.028	0.028
	291	0.065	0.065	-	-	-	-	-	-
	292	-	-	-	-	0.013	0.013	-	-
	293	0.065	0.065	-	-	-	-	-	-
	NULL	NA	0.082	NA	0.005	NA	0.049	NA	0.023
ham 26	**163**	-	-	0.018	0.011	-	-	-	-
	165	0.033	0.033	0.430	0.426	0.012	0.012	0.267	0.243
	167	-	-	0.018	0.018	-	-	0.012	0.012
	168	-	-	-	-	0.071	0.070	-	-
	170	0.517	0.425	-	-	-	-	-	-
	172	0.100	0.100	-	-	-	-	0.023	0.023
	174	-	-	-	-	0.036	0.036	0.012	0.012
	176	0.017	0.017	0.026	0.026	0.024	0.024	0.198	0.189
	178	-	-	0.070	0.070	0.024	0.024	-	-
	180	0.183	0.161	0.193	0.192	0.298	0.297	0.256	0.249
	182	0.100	0.073	0.211	0.209	0.202	0.201	0.047	0.038
	184	0.050	0.035	0.026	0.026	0.321	0.320	0.186	0.182
	186	-	-	0.009	0.009	0.012	0.012	-	-
	NULL	NA	0.155	NA	0.013	NA	0.005	NA	0.053
ham	**284**	0.012	0.012	-	-	-	-	-	-

30									
	286	0.012	0.012	0.036	0.030	0.037	0.035	0.302	0.278
	288	0.183	0.165	0.018	0.018	0.175	0.175	0.047	0.038
	290	0.341	0.317	0.134	0.128	0.275	0.275	0.198	0.190
	292	0.354	0.317	0.045	0.045	0.025	0.025	0.302	0.293
	294	0.024	0.024	0.188	0.185	0.287	0.287	0.012	0.012
	296	0.037	0.037	0.062	0.063	-	-	0.140	0.140
	298	0.024	0.024	0.455	0.439	0.150	0.149	-	-
	300	0.012	0.012	0.054	0.054	0.013	0.013	-	-
	302	-	-	-	-	0.037	0.038	-	-
	309	-	-	0.009	0.009	-	-	-	-
	NULL	NA	0.078	NA	0.030	NA	0.004	NA	0.050
ham 38	**257**	0.026	0.026	-	-	-	-	-	-
	260	0.013	0.013	-	-	-	-	-	-
	264	0.092	0.083	-	-	-	-	0.143	0.129
	266	0.026	0.026	-	-	-	-	-	-
	268	0.382	0.360	-	-	0.026	0.014	-	-
	269	0.013	0.013	-	-	-	-	-	-
	270	0.105	0.105	0.067	0.055	0.244	0.224	0.155	0.148
	272	0.145	0.130	0.225	0.216	0.013	0.013	0.036	0.036
	274	0.013	0.013	0.525	0.497	0.372	0.317	-	-
	275	-	-	0.017	0.017	0.038	0.027	0.202	0.179
	276	-	-	0.008	0.008	-	-	0.012	0.012
	277	0.026	0.014	0.008	0.008	0.077	0.077	0.119	0.112
	278	-	-	0.017	0.009	0.013	0.013	0.012	0.012
	279	0.066	0.056	0.042	0.042	0.103	0.093	0.048	0.048
	281	0.079	0.059	0.092	0.087	0.051	0.040	0.107	0.092
	283	0.013	0.013	-	-	0.013	0.013	-	-
	285	-	-	-	-	0.051	0.051	0.167	0.148
	NULL	NA	0.087	NA	0.061	NA	0.118	NA	0.085
ham 47	**278**	-	-	0.009	0.009	-	-	-	-
	281	-	-	0.231	0.231	-	-	-	-
	283	0.050	0.050	0.028	0.028	0.037	0.037	0.151	0.140
	285	0.062	0.053	0.065	0.064	0.220	0.216	0.209	0.203
	287	0.062	0.053	0.037	0.037	0.159	0.159	0.337	0.323
	289	0.150	0.144	0.574	0.573	0.354	0.350	0.221	0.218
	291	0.400	0.380	0.019	0.019	0.122	0.122	0.035	0.035
	293	0.212	0.197	0.019	0.019	0.061	0.061	-	-
	295	0.037	0.027	0.019	0.019	0.049	0.045	0.023	0.023

	300	0.013	0.013	-	-	-	-	0.023	0.023
	302	0.013	0.013	-	-	-	-	-	-
	NULL	NA	0.071	NA	0.003	NA	0.011	NA	0.035
ham 55	**243**	0.550	0.472	-	-	-	-	-	-
	245	0.050	0.039	-	-	-	-	-	-
	255	0.163	0.155	0.018	0.018	-	-	-	-
	259	-	-	-	-	0.014	0.014	0.149	0.149
	261	0.013	0.013	-	-	-	-	-	-
	264	-	-	0.136	0.136	-	-	-	-
	266	-	-	0.009	0.009	-	-	0.027	0.027
	268	0.025	0.025	0.409	0.409	0.542	0.511	0.068	0.068
	270	0.062	0.063	0.018	0.018	0.194	0.184	0.041	0.041
	272	0.037	0.038	0.009	0.009	0.111	0.111	-	-
	274	0.037	0.026	0.373	0.373	0.028	0.028	0.514	0.514
	276	0.013	0.013	0.018	0.018	0.097	0.081	-	-
	277	-	-	-	-	0.014	0.014	-	-
	278	0.013	0.013	-	-	-	-	-	-
	287	-	-	0.009	0.009	-	-	-	-
	289	0.025	0.025	-	-	-	-	0.095	0.095
	297	0.013	0.013	-	-	-	-	-	-
	302	-	-	-	-	-	-	0.014	0.014
	304	-	-	-	-	-	-	0.095	0.095
	NULL	NA	0.107	NA	-	NA	0.058	NA	-
ham 57	**230**	0.061	0.061	-	-	-	-	0.352	0.350
	232	0.073	0.064	0.017	0.017	0.110	0.095	0.091	0.091
	234	0.524	0.481	0.322	0.309	0.585	0.547	0.227	0.226
	236	0.232	0.221	0.610	0.587	0.244	0.225	0.136	0.134
	238	-	-	0.034	0.034	0.012	0.012	0.159	0.158
	240	-	-	0.017	0.017	0.049	0.049	0.034	0.034
	242	0.085	0.058	-	-	-	-	-	-
	244	0.024	0.024	-	-	-	-	-	-
	NULL	NA	0.092	NA	0.036	NA	0.071	NA	0.007
ham 60	**120**	-	-	-	-	-	-	0.310	0.293
	122	0.013	0.013	-	-	-	-	-	-
	128	0.256	0.218	-	-	-	-	-	-
	130	0.423	0.362	0.008	0.008	0.013	0.013	0.190	0.178
	132	0.128	0.099	0.763	0.689	0.487	0.435	0.321	0.305
	134	0.013	0.013	0.034	0.026	0.013	0.013	-	-

	136	-	-	0.017	0.017	0.138	0.121	0.071	0.071
	138	0.077	0.043	0.017	0.009	-	-	0.036	0.036
	140	0.026	0.026	0.017	0.017	0.062	0.052	-	-
	142	0.064	0.064	0.136	0.119	0.225	0.198	0.071	0.071
	146	-	-	0.008	0.008	0.062	0.052	-	-
	NULL	NA	0.162	NA	0.105	NA	0.116	NA	0.045
ham 79	**123**	-	-	-	-	-	-	0.064	0.054
	125	0.071	0.051	0.176	0.172	0.300	0.283	-	-
	127	0.029	0.016	0.118	0.112	0.500	0.478	0.192	0.153
	129	0.057	0.057	0.373	0.349	0.186	0.181	0.103	0.103
	131	0.086	0.086	0.088	0.069	-	-	0.462	0.399
	133	0.443	0.418	0.167	0.162	0.014	0.014	0.013	0.013
	135	0.143	0.143	0.039	0.039	-	-	-	-
	137	0.071	0.071	0.039	0.031	-	-	0.141	0.133
	139	0.029	0.029	-	-	-	-	-	-
	141	0.071	0.062	-	-	-	-	0.026	0.026
	NULL	N/A	0.068	NA	0.065	NA	0.043	NA	0.121
ham 91	**128**	0.012	0.012	-	-	0.012	0.012	-	-
	134	0.012	0.012	-	-	-	-	0.047	0.047
	136	0.073	0.067	0.025	0.025	0.070	0.070	-	-
	138	0.305	0.297	0.008	0.008	0.012	0.012	0.302	0.295
	140	0.195	0.188	0.213	0.210	0.047	0.040	0.186	0.180
	142	0.122	0.112	0.492	0.477	0.640	0.623	0.198	0.196
	144	0.159	0.159	0.033	0.027	0.023	0.023	-	-
	146	0.012	0.012	0.139	0.139	0.116	0.116	-	-
	148	0.024	0.024	0.008	0.008	0.012	0.012	-	-
	150	0.012	0.012	0.049	0.045	0.023	0.023	-	-
	152	0.012	0.012	0.008	0.008	-	-	0.116	0.113
	154	0.012	0.012	0.008	0.008	0.035	0.035	0.151	0.151
	156	0.049	0.041	-	-	-	-	-	-
	160	-	-	0.016	0.016	0.012	0.012	-	-
	NULL	NA	0.040	NA	0.029	NA	0.023	NA	0.018
ham 96	**326**	0.221	0.221	-	-	-	-	-	-
	328	0.044	0.034	-	-	-	-	-	-
	330	-	-	0.052	0.052	0.264	0.263	0.013	0.013
	332	-	-	0.405	0.405	0.097	0.095	-	-
	334	0.029	0.029	0.414	0.414	0.361	0.358	0.037	0.038
	336	0.279	0.268	0.026	0.026	0.181	0.178	0.150	0.141

	338	0.029	0.029	0.009	0.009	-	-	0.537	0.515
	340	-	-	0.052	0.052	0.014	0.014	0.050	0.050
	342	0.044	0.044	0.034	0.034	0.014	0.014	0.212	0.206
	344	0.118	0.112	-	-	-	-	-	-
	346	0.059	0.059	0.009	0.009	0.028	0.028	-	-
	350	-	-	-	-	0.014	0.014	-	-
	352	-	-	-	-	0.028	0.028	-	-
	356	0.088	0.082	-	-	-	-	-	-
	362	0.015	0.015	-	-	-	-	-	-
	368	0.015	0.015	-	-	-	-	-	-
	370	0.029	0.029	-	-	-	-	-	-
	372	0.029	0.029	-	-	-	-	-	-
	NULL	NA	0.034	NA	-	NA	0.009	NA	0.038
ham 100	**220**	0.037	0.026	-	-	-	-	0.116	0.116
	222	0.025	0.025	-	-	-	-	-	-
	224	0.113	0.103	-	-	-	-	-	-
	226	0.100	0.090	-	-	0.027	0.027	0.477	0.453
	228	-	-	0.018	0.018	-	-	-	-
	230	0.100	0.070	0.202	0.202	0.162	0.154	0.023	0.023
	232	0.025	0.025	-	-	0.014	0.014	-	-
	234	0.050	0.050	-	-	0.054	0.054	-	-
	236	0.212	0.173	0.158	0.158	0.162	0.158	0.209	0.187
	238	0.237	0.192	0.167	0.167	0.014	0.014	0.116	0.104
	240	0.062	0.063	0.211	0.211	0.365	0.356	0.058	0.058
	242	-	-	0.070	0.070	-	-	-	-
	244	0.037	0.038	0.149	0.149	0.081	0.067	-	-
	246	-	-	0.026	0.026	-	-	-	-
	248	-	-	-	-	0.122	0.122	-	-
	NULL	NA	0.144	NA	-	NA	0.036	NA	0.059
ham 101	**262**	0.316	0.275	0.104	0.102	0.146	0.024	0.384	0.324
	268	0.105	0.095	0.236	0.234	0.293	0.104	0.198	0.158
	270	0.053	0.041	0.009	0.009	-	-	-	-
	272	0.237	0.198	-	-	0.024	0.276	-	-
	274	-	-	0.019	0.019	0.012	0.142	0.023	0.023
	276	0.158	0.149	0.019	0.019	0.037	0.340	0.105	0.105
	278	0.053	0.041	0.142	0.142	0.110	0.037	0.093	0.064
	280	0.066	0.043	0.396	0.393	0.354	0.024	0.186	0.145
	282	0.013	0.013	0.047	0.047	0.024	0.012	-	-
	284	-	-	0.028	0.024	-	-	0.012	0.012

	NULL	NA	0.144	NA	0.010	NA	0.040	NA	0.169
ham 102	**162**	0.028	0.028	-	-	0.098	0.093	0.028	0.028
	164	0.042	0.042	-	-	-	-	0.028	0.028
	166	0.014	0.014	-	-	0.037	0.037	0.250	0.242
	168	0.097	0.097	0.018	0.018	0.305	0.295	0.306	0.294
	170	0.069	0.061	0.418	0.400	0.049	0.049	-	-
	172	0.028	0.028	0.445	0.425	0.232	0.227	-	-
	174	0.153	0.136	0.082	0.064	0.207	0.205	0.236	0.230
	176	0.056	0.056	0.036	0.036	0.061	0.055	-	-
	178	0.208	0.200	-	-	0.012	0.012	-	-
	180	0.139	0.127	-	-	-	-	0.139	0.135
	182	0.083	0.083	-	-	-	-	0.014	0.014
	184	0.083	0.083	-	-	-	-	-	-
	NULL	NA	0.045	NA	0.056	NA	0.028	NA	0.030
ham 103	**89**	-	-	0.009	0.009	-	-	0.500	0.430
	91	0.100	0.077	-	-	0.103	0.056	0.059	0.046
	93	0.414	0.347	-	-	0.034	0.018	0.103	0.091
	97	0.014	0.014	-	-	-	-	-	-
	100	0.029	0.029	-	-	-	-	-	-
	104	-	-	0.018	0.018	-	-	-	-
	106	0.014	0.014	0.009	0.009	-	-	-	-
	108	-	-	0.018	0.018	-	-	-	-
	110	-	-	0.036	0.036	0.017	0.017	-	-
	112	0.171	0.152	0.082	0.082	-	-	-	-
	114	0.014	0.014	0.091	0.087	0.276	0.213	0.176	0.157
	116	0.043	0.043	0.400	0.395	0.086	0.055	0.088	0.064
	118	0.129	0.096	0.118	0.117	0.345	0.262	-	-
	120	0.057	0.045	0.200	0.198	0.103	0.056	0.074	0.048
	122	-	-	0.009	0.009	0.017	0.017	-	-
	124	0.014	0.014	-	-	0.017	0.017	-	-
	129	-	-	0.009	0.009	-	-	-	-
	NULL	NA	0.154	NA	0.013	NA	0.288	NA	0.165
ham 107	**262**	0.132	0.132	-	-	-	-	-	-
	264	0.500	0.451	-	-	-	-	-	-
	265	0.013	0.013	0.008	0.008	0.013	0.013	0.209	0.184
	266	0.105	0.077	-	-	0.013	0.013	0.372	0.340
	268	0.066	0.066	-	-	0.075	0.056	-	-
	269	0.053	0.042	0.017	0.017	0.050	0.050	-	-

	270	0.026	0.014	-	-	0.025	0.013	0.023	0.012
	274	0.013	0.013	0.008	0.008	-	-	-	-
	276	0.026	0.026	0.195	0.190	0.212	0.194	-	-
	278	-	-	0.042	0.038	-	-	0.012	0.012
	280	0.026	0.026	0.424	0.418	0.525	0.478	0.047	0.047
	282	-	-	0.263	0.260	0.050	0.050	0.151	0.137
	284	0.039	0.039	0.025	0.025	0.037	0.038	0.186	0.159
	286	-	-	0.008	0.008	-	-	-	-
	289	-	-	0.008	0.008	-	-	-	-
	NULL	NA	0.100	NA	0.018	NA	0.097	NA	0.109
ham 116	**273**	0.551	0.459	-	-	-	-	-	-
	275	0.051	0.040	0.030	0.020	0.057	0.044	0.274	0.273
	277	0.013	0.013	-	-	-	-	0.107	0.107
	282	0.026	0.026	-	-	-	-	-	-
	284	-	-	-	-	0.100	0.075	0.131	0.131
	286	0.051	0.040	0.640	0.509	0.600	0.437	0.155	0.154
	288	0.128	0.099	0.100	0.083	0.014	0.014	0.024	0.024
	290	0.115	0.106	0.190	0.127	0.057	0.057	0.274	0.273
	292	0.026	0.026	0.020	0.010	0.171	0.113	-	-
	294	-	-	0.020	0.010	-	-	-	-
	296	0.026	0.026	-	-	-	-	-	-
	298	0.013	0.013	-	-	-	-	0.036	0.036
	NULL	NA	0.154	NA	0.241	NA	0.260	NA	0.001
ham 120	**183**	0.050	0.030	-	-	-	-	-	-
	193	0.537	0.507	0.018	0.018	0.013	0.013	0.326	0.326
	195	-	-	0.062	0.056	-	-	-	-
	197	0.013	0.013	0.009	0.009	-	-	-	-
	199	0.013	0.013	-	-	-	-	0.012	0.012
	203	0.050	0.050	-	-	0.062	0.063	0.023	0.023
	205	0.013	0.013	0.009	0.009	-	-	-	-
	207	-	-	0.018	0.018	0.050	0.050	-	-
	209	0.013	0.013	0.009	0.009	-	-	-	-
	211	0.025	0.025	0.750	0.699	0.550	0.550	0.337	0.337
	213	0.013	0.013	0.045	0.045	0.163	0.163	-	-
	215	0.050	0.050	0.062	0.049	0.087	0.088	0.163	0.163
	217	0.075	0.067	0.018	0.018	0.050	0.050	0.140	0.140
	219	0.062	0.063	-	-	0.013	0.013	-	-
	221	0.087	0.088	-	-	0.013	0.013	-	-
	NULL	NA	0.058	NA	0.071	NA	-	NA	-

ham 123	**149**	-	-	0.035	0.035	0.039	0.039	-	-
	151	-	-	0.035	0.035	-	-	0.205	0.184
	155	-	-	0.018	0.018	-	-	-	-
	157	0.103	0.103	0.018	0.018	0.079	0.062	-	-
	159	0.224	0.180	0.377	0.368	0.316	0.299	0.013	0.013
	161	0.276	0.236	0.175	0.167	0.066	0.057	0.218	0.198
	163	0.172	0.136	0.193	0.189	0.276	0.273	0.513	0.446
	165	0.103	0.076	0.070	0.066	0.105	0.098	-	-
	166	-	-	0.009	0.009	-	-	-	-
	167	0.121	0.108	0.044	0.044	0.026	0.026	-	-
	169	-	-	0.018	0.018	0.013	0.013	0.051	0.051
	171	-	-	0.009	0.009	0.053	0.053	-	-
	173	-	-	-	-	0.026	0.026	-	-
	NULL	NA	0.161	NA	0.025	NA	0.053	NA	0.107
ham 141	**207**	-	-	0.019	0.019	0.031	0.017	-	-
	209	0.031	0.031	0.287	0.287	0.016	0.016	0.125	0.125
	211	0.062	0.048	0.185	0.185	0.250	0.229	0.312	0.312
	213	0.047	0.032	0.213	0.213	0.172	0.155	0.113	0.113
	215	0.016	0.016	0.037	0.037	0.344	0.314	0.037	0.038
	217	0.031	0.031	0.204	0.204	0.062	0.050	0.013	0.013
	219	0.031	0.016	0.028	0.028	0.078	0.078	-	-
	221	-	-	0.009	0.009	0.047	0.047	0.150	0.150
	222	0.156	0.106	0.009	0.009	-	-	-	-
	223	0.031	0.016	-	-	-	-	-	-
	224	0.297	0.235	-	-	-	-	0.075	0.075
	225	-	-	0.009	0.009	-	-	-	-
	226	0.094	0.080	-	-	-	-	-	-
	228	0.062	0.048	-	-	-	-	-	-
	230	0.094	0.067	-	-	-	-	-	-
	234	0.016	0.016	-	-	-	-	0.025	0.025
	236	0.016	0.016	-	-	-	-	0.150	0.150
	242	0.016	0.016	-	-	-	-	-	-
	NULL	NA	0.226	NA	-	NA	0.094	NA	-
ham 146	**128**	0.064	0.064	-	-	0.141	0.134	0.012	0.012
	130	0.115	0.115	-	-	-	-	-	-
	132	0.026	0.026	0.444	0.438	-	-	0.314	0.310
	134	0.628	0.627	0.481	0.475	0.718	0.701	0.570	0.564
	136	0.077	0.077	-	-	0.038	0.038	0.012	0.012

	138	-	-	0.028	0.028	-	-	-	-
	139	0.013	0.013	-	-	-	-	-	-
	140	-	-	-	-	0.013	0.013	0.023	0.023
	142	0.013	0.013	0.009	0.009	-	-	-	-
	145	0.038	0.038	0.009	0.009	-	-	-	-
	147	0.026	0.026	-	-	-	-	-	-
	149	-	-	0.009	0.009	-	-	-	-
	151	-	-	0.009	0.009	0.077	0.077	0.070	0.066
	153	-	-	0.009	0.009	0.013	0.013	-	-
	NULL	NA	0.001	NA	0.013	NA	0.024	NA	0.013
ham 150	**154**	0.062	0.063	-	-	0.079	0.079	0.286	0.286
	158	-	-	0.151	0.147	0.039	0.028	0.119	0.119
	160	0.025	0.025	0.009	0.009	-	-	-	-
	162	0.200	0.200	0.415	0.390	0.053	0.053	0.119	0.119
	164	0.338	0.337	0.349	0.322	0.500	0.454	0.107	0.107
	166	0.325	0.325	-	-	0.211	0.190	0.119	0.119
	168	0.025	0.025	0.066	0.066	-	-	0.202	0.202
	170	0.025	0.025	0.009	0.009	0.118	0.091	0.048	0.048
	NULL	NA	-	NA	0.057	NA	0.106	NA	-
ham 181	**194**	-	-	0.009	0.009	0.013	0.013	-	-
	196	0.025	0.025	0.296	0.296	0.100	0.100	0.107	0.107
	198	0.025	0.025	0.009	0.009	-	-	-	-
	200	0.013	0.013	0.028	0.028	0.037	0.027	0.155	0.155
	208	-	-	-	-	0.025	0.025	-	-
	210	-	-	0.028	0.028	0.087	0.080	-	-
	212	0.050	0.050	0.019	0.019	0.013	0.013	0.083	0.083
	214	0.212	0.203	0.259	0.259	0.263	0.240	0.036	0.036
	216	0.212	0.194	0.139	0.139	0.388	0.360	-	-
	218	0.087	0.088	0.176	0.176	0.013	0.013	0.226	0.226
	219	-	-	-	-	0.013	0.013	-	-
	220	0.100	0.093	-	-	0.037	0.038	0.143	0.143
	222	0.100	0.093	-	-	0.013	0.013	0.238	0.238
	224	0.125	0.119	0.028	0.028	-	-	0.012	0.012
	226	0.037	0.027	0.009	0.009	-	-	-	-
	228	0.013	0.013	-	-	-	-	-	-
	NULL	NA	0.058	NA	0.000	NA	0.069	NA	-
ham 184	**169**	0.043	0.043	0.027	0.027	-	-	0.139	0.136
	171	-	-	0.073	0.073	-	-	-	-

	173	0.057	0.057	0.018	0.018	-	-	0.069	0.069
	175	0.014	0.014	0.200	0.200	0.292	0.258	0.056	0.056
	177	0.057	0.052	0.273	0.273	0.361	0.332	0.458	0.456
	179	0.271	0.267	0.173	0.173	-	-	0.014	0.014
	180	0.086	0.086	0.155	0.155	0.028	0.015	0.236	0.234
	182	0.100	0.100	0.036	0.036	0.167	0.140	-	-
	184	0.057	0.052	0.009	0.009	0.125	0.106	0.028	0.028
	186	0.029	0.029	0.027	0.027	0.028	0.028	-	-
	188	0.086	0.086	-	-	-	-	-	-
	190	0.071	0.071	0.009	0.009	-	-	-	-
	192	0.029	0.029	-	-	-	-	-	-
	194	0.014	0.014	-	-	-	-	-	-
	196	0.071	0.071	-	-	-	-	-	-
	204	0.014	0.014	-	-	-	-	-	0.007
	NULL	NA	0.014	NA	-	NA	0.122	NA	-
lchu 06	**170**	0.175	0.153	-	-	-	-	-	-
	171	0.037	0.026	0.384	0.379	0.061	0.061	-	-
	174	-	-	0.009	0.009	0.024	0.024	-	-
	175	0.013	0.013	0.348	0.344	0.512	0.441	0.107	0.097
	176	0.100	0.082	-	-	-	-	0.107	0.107
	177	0.013	0.013	0.009	0.009	0.110	0.101	-	-
	178	0.037	0.026	0.018	0.018	0.122	0.095	0.190	0.180
	180	0.113	0.095	-	-	0.073	0.063	0.226	0.217
	182	0.013	0.013	0.027	0.027	0.012	0.012	0.024	0.024
	183	-	-	0.009	0.009	-	-	-	-
	184	0.025	0.025	-	-	0.037	0.025	0.310	0.303
	185	0.100	0.091	0.009	0.009	-	-	-	-
	186	0.100	0.082	0.116	0.114	0.024	0.013	-	-
	187	0.075	0.075	-	-	-	-	-	-
	188	0.050	0.050	0.071	0.069	-	-	0.036	0.036
	190	-	-	-	-	0.024	0.024	-	-
	191	0.075	0.075	-	-	-	-	-	-
	193	0.025	0.025	-	-	-	-	-	-
	195	0.050	0.050	-	-	-	-	-	-
	NULL	NA	0.106	NA	0.014	NA	0.140	NA	0.036

Note: L indicates locus column, R indicates column of uncorrected raw frequencies, and C indicates column of corrected frequencies. Cells labels as “NULL” indicate remaining amount of corrected allele frequencies that represents unobserved null alleles. Absence of an allele within a particular species or hybrid zone is indicated by a “-” symbol.

Table S6.

STRUCTURE Results for 10 Different Simulated Data Sets

Set	**q F1**	**F1 q range**	**F1 CI interval**	**q F2**	**F2 q range**	**F2 CI interval**	**q *C. jacchus* BC**	***C. jacchus* BC q range**	***C. jacchus* BC CI interval**	**q *C. penicillata* BC**	***C. penicillata* BC q range**	***C. jacchus* BC CI interval**
1	0.55	0.50-0.62	0.43-0.67	0.56	0.44-0.61	0.44-0.68	0.35	0.29-0.41	0.24-0.47	0.76	0.67-0.82	0.65-0.86
2	0.55	0.50-0.61	0.42-0.66	0.56	0.46-0.61	0.43-0.66	0.35	0.29-0.40	0.27-0.49	0.76	0.67-0.82	0.62-0.84
3	0.55	0.51-0.60	0.44-0.67	0.56	0.50-0.61	0.44-0.68	0.34	0.25-0.46	0.23-0.46	0.78	0.71-0.86	0.67-0.88
4	0.56	0.46-0.64	0.44-0.67	0.55	0.50-0.64	0.43-0.67	0.34	0.25-0.38	0.23-0.45	0.73	0.65-0.80	0.62-0.84
5	0.54	0.46-0.60	0.42-0.66	0.54	0.43-0.67	0.42-0.66	0.35	0.25-0.38	0.24-0.46	0.76	0.65-0.80	0.65-0.86
6	0.55	0.47-0.61	0.43-0.67	0.57	0.52-0.64	0.45-0.68	0.37	0.28-0.47	0.26-0.49	0.76	0.68-0.81	0.64-0.86
7	0.56	0.50-0.66	0.43-0.68	0.55	0.47-0.58	0.43-0.68	0.37	0.25-0.45	0.26-0.49	0.79	0.73-0.84	0.68-0.89
8	0.55	0.48-0.57	0.43-0.67	0.57	0.48-0.67	0.45-0.69	0.36	0.27-0.42	0.24-0.47	0.76	0.64-0.80	0.65-0.86
9	0.56	0.47-0.60	0.44-0.68	0.57	0.42-0.76	0.45-0.69	0.34	0.29-0.38	0.23-0.45	0.78	0.73-0.81	0.67-0.88
10	0.56	0.50-0.57	0.44-0.68	0.56	0.45-0.68	0.44-0.67	0.35	0.20-0.39	0.24-0.46	0.77	0.66-0.82	0.66-0.87

Note: q F1 is average *q*-coefficient of F1 simulated hybrids across replicate runs of each dataset, F1 *q* range is average *q*-coefficient range of F1 simulated hybrids across replicate runs of each dataset, F1 CI interval is the average 90% confidence interval of F1 simulated hybrids across replicate runs of each dataset. F2 represents simulated F2 hybrids, *C. jacchus* BC represents simulated hybrid offspring of a F1x pure *C. jacchus* backcross, and *C. penicillata* BC represented simulated hybrid offspring of a F1 x pure *C. penicillata* backcross. Columns for each simulated hybrid class follow same conventions as described for simulated F1 hybrids.

Table S7.

BAPS Results for 5 Different Simulated Data Sets

Set	**F1 q**	**F1 q range**	**F2 q**	**F2 q range**	***C. jacchus* BC q**	***C. jacchus* BC q range**	***C. penicillata* BC q**	***C. penicillata* BC q range**
1.00	0.51	0.45-0.56	0.48	0.40-0.53	0.24	0.18-0.28	0.74	0.62-1.00
2.00	0.49	0.46-0.56	0.45	0.33-0.52	0.26	0.21-0.28	0.76	0.65-0.84
3.00	0.49	0.43-0.60	0.52	0.45-0.61	0.24	0.15-0.28	0.74	0.68-0.83
4.00	0.51	0.45-0.55	0.52	0.35-0.68	0.24	0.12-0.29	0.77	0.70-0.81
5.00	0.49	0.43-0.55	0.50	0.42-057	0.24	0.20-0.27	0.75	0.62-1.00

Note: F1 *q* are average *q*-coefficient of F1 simulated hybrids across replicate runs of each dataset and F1 q range are average *q*-coefficient range of F1 simulated hybrids across replicate runs of each data set. F2 represents simulated F2 hybrids, *C. jacchus* BC represents simulated hybrid offspring of a F1x pure *C. jacchus* backcross, and *C. penicillata* BC represented simulated hybrid offspring of a F1 x pure *C. penicillata* backcross. Columns for each simulated hybrid class follow same conventions as described for simulated F1 hybrids.

Table S8.

Summary of Genetic Statistics for C. jacchus *and* C. *penicillata Separated by "Variant Sites" on Top and "All Sites" on the Bottom*

Variant Sites

Species	**N**	**Sites**	**% Polymorphic**	**P**	**H_o**	**HE**	**π**	**FIS**
C. jacchus	4.03	3638	19.49	0.933	0.1018	0.0799	0.0986	-1.4680E-03
C. penicillata	4.76	4525	100.00	0.709	0.4116	0.3727	0.4235	2.3116E-02

All Sites

Species	**N**	**Sites**	**% Polymorphic**	**P**	**H_o**	**HE**	**π**	**FIS**
C. jacchus	4.05	6441037	0.01	0.999	0.0001	4.5105E-05	5.5677E-05	-8.2916E-07
C. penicillata	5.08	7398171	0.06	0.999	2.5100E-04	2.2795E-04	2.5900E-04	1.4139E-05

Note: “Variant Sites” are those nucleotide positions which are polymorphic in at least one species, and “all sites” represent all Nucleotides across RAD tags regardless whether they represent a polymorphic site or not.
These classifications are taken from Catchen et al. (2013). “N” represents the average number of individuals genotypes at a locus, “sites” on top indicates nucleotide positions that may vary between C. jacchus and C. penicillata or within each species, “sites” on the bottom indicates all nucleotide sites regardless whether there are inter- and intraspecific differences or not, “% polymorphic” indicates the percentage of sites that are polymorphic within a species out of all variant sites on top or out of the entire data set on bottom, “P” indicates the average major allele frequency at each locus, “H_O” indicates average observed heterozyosity at each site, “H_E” indicates average expected heterozygosity at each site, “π” indicates per site nucleotide diversity, ”F_{IS}” indicates the average Wright’s inbreeding coefficient at each site.

APPENDIX B

PROJECT IACUC APPROVAL (PROTOCOL # 11-1150R AND AMENDMENTS)

Institutional Animal Care and Use Committee (IACUC)
Office of Research Integrity and Assurance
Arizona State University
Tempe, Arizona 85287-1103
Phone: (480) 965-2179 *FAX:* (480) 965-7772

Animal Protocol Review

ASU Protocol Number:	**11-1150R**
Protocol Title:	**Phylogenetics and Population History of Callitrichidae**
Principal Investigator:	**Anne Stone**
Date of Action:	**11/22/2010**

The animal protocol review was considered by the Committee and the following decisions were made:

☐ The original protocol was APPROVED as presented.
☒ The revised protocol was APPROVED as presented.
☐ The protocol was APPROVED with RESTRICTIONS or CHANGES as noted below. The project can only be pursued, subject to your acceptance of these restriction or changes. If you are not agreeable, contact the IACUC Chairperson immediately.
☐ The Committee requests CLARIFICATIONS or CHANGES in the protocol as described in the attached memorandum. The protocol will be considered when these issues are clarified and the revised protocol is submitted.
☐ The protocol was approved, subject to the approval of a WAIVER of provisions of NIH policy as noted below. Waivers require written approval from the granting agencies.
☐ The protocol was DISAPPROVED for reasons outlined in the attached memorandum.
☐ The Committee requests you to contact ____________________ to discuss this proposal.
☐ A copy of this correspondence has been sent to the Vice President for Research.
☐ Amendment was approved as presented.

RESTRICTIONS, CHANGES OR WAIVER REQUIREMENTS:

Approved # of Animals: 325 **Pain Level:** D **Species:** **Marmosets**
Approval Period: **11/22/2010 – 11/21/2013**

Signature: ______________________________ Date: 11/22/10
IACUC Chair or Designee

Original: Principal Investigator
Cc: IACUC Office
IACUC Chair

From:	Joanne Tetens
Sent:	Monday, November 22, 2010 2:01 PM
To:	Chantelle Miller; Stephen Massia
Subject:	RE: Stone 11-1150R

Excellent. I approve as written. Steve – are you OK with it?

Chantelle - Will you remove the yellow highlight?

J

From: Chantelle Miller
Sent: Monday, November 22, 2010 1:47 PM
To: Stephen Massia; Joanne Tetens
Subject: FW: Stone 11-1150R

Here is the final revision. If you approve, please let me know.

Thank you!

Chantelle Miller

From: Joanna Malukiewicz [mailto:jmalukie@asu.edu]
Sent: Monday, November 22, 2010 1:44 PM
To: Chantelle Miller
Subject: Re: Stone 11-1150R

Hi Chantelle,
Here you go!

IACUC Use Only	
Date: 11/8/2010	IACUC Protocol No.: 11-1150R

ANIMAL USE PROTOCOL
ARIZONA STATE UNIVERSITY INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
(revised March 2010)

Please read "Instructions for Completing the Animal Use Protocol" before completing. **Upon approval, this protocol will become a public record so please follow instructions carefully.**

PROJECT/PROGRAM TITLE: Phylogenetics and Population History of the Callitrichidae

SPECIES REQUESTED: (*Callimico goeldii*) Goeldi's monkey

(*Callithrix aurita*) buffy-tufted-ear marmoset

(*Callithrix flaviceps*) buffy-headed marmoset

(*Callithrix geoffroyi*) Geoffroy's tufted-ear marmoset

(*Callithrix jacchus*) common marmosets

(*Callithrix kuhlii*) Wied's black-tufted-ear marmoset

(*Callithrix penicillata*) black-tufted marmosets

(*Cebuella pygmaea*) or (*Callithrix pygmaea*) pygmy marmoset

(*Leontopithecus caissara*) black-faced lion tamarin

(*Leontopithecus chrysophygus)* black lion tamarin

(*Leontopithecus chrysomelas*) golden-headed lion tamarin

(*Leontopithecus rosalia*) golden lion tamarin

(*Mico acariensis*) Rio Acari marmoset

(*Mico argentatus/Callithrix argentata*) silvery marmoset

(*Mico chrysoleucus*) golden-white tassel-ear marmoset

(*Mico emiliae*) Snethlage's marmoset

(*Mico humeralifer*) black and white tassel-ear marmoset

(*Mico humilis/Callithrix humilis*) black-crowned dwarf marmoset

(*Mico intermedius*) aripuana marmoset

(*Mico leucippe*) golden-white bare-ear marmoset

(*Mico manicorensis*) manicore marmoset

(*Mico marcai*) Marca's marmoset

(*Mico mauesi*) Maues marmoset

(*Mico melanurus/ Callithrix melanura*) black-tailed marmoset

(*Mico nigriceps*) black-headed marmoset

(*Mico saterei*) Satere marmoset

(*Saguinus bicolor*) pied bare-face tamarin

(*Saguinus fuscicollis*) saddle-back tamarin

(*Saguinus fuscicollis avilapiresi*) Avila Pires' saddle-back tamarin

(*Saguinus fusciollis crandalli*) Crandall's saddle-back tamarin
(*Saguinus fuscicollis cruzlimai*) Cruz Lima's saddle-back tamarin
(*Saguinus fusciollis fuscus*) Lesson's saddle-back tamarin
(*Saguinus fuscicollis fuscicollis*) Spix saddle-back tamarin
(*Saguinus fuscicollis illigeri*) Illiger's saddle-back tamarin
(*Saguinus fuscicollis lagonotus*) Red-mantle saddle-back tamarin
(*Saguinus fuscicollis leucogenys*) Andean saddle-back tamarin
(*Saguinus fuscicollis melanoleucus*) white saddle-back tamarin
(*Saguinus fuscicollis nigrifrons*) Geoffroy's saddle-back tamarin
(*Saguinus fusciollis primitivus*) saddle-back tamarin
(*Saguinus fuscicollis weddelli*) Weddell's saddle-back tamarin
(*Saguinus graellsi*) Graell's black-mantle tamarin
(*Saguinus imperator imperator*) black-chinned emperor tamarin
(*Saguinus imerator subgrisescens*) bearded emperor tamarin
(*Saguinus inustus*) mottled-face tamarin
(*Saguinus labiatus*) white-lipped tamarin
(*Saguinus labiatus labiatus)* red-bellied tamarin
(*Saguinus labiatus rufiventer*) Gray's red-bellied tamarin
(*Saguinus labiatus thomasi)* Thomas' mustached tamarin
(*Saguinus leucopus*) silvery-brown bare-face tamarin
(*Saguinus martinsi martinsi*) Martin's bare-face tamarin
(*Saguinus midas*) golden-handed tamarin
(*Saguinus mystax*) moustached tamarin
(*Saguinus mystax mystax*) Spix's mustached tamarin
(*Saguinus mystax pileatus*) red-cap mustached tamarin
(*Saguinus mystax pluto*) white-rump mustched tamarin
(*Saguinus niger*) black-handed tamarin
(*Saguinus nigricollis nigricollis*) Spix's black mantle tamarin
(*Saguinus nigricollis hernandezi*) Hernandez-Camacho's black mantle tamarin
(*Saguinus tripartitus*) golden-mantle saddle back tamarin
(*Saguinus martinsi ochraceus*) Ochraceous bar-face tamarin
(*Saguinus oedipus*) cotton-top tamarin
(*Saguinus geoffroyi*) Geoffroy's tamarin
Callithrix jacchus (common marmoset) and *Callithrix penicillata* (black-tufted marmoset) hybrids

I. PERSONNEL INFORMATION

A. A single member of the university faculty and/or Principal Investigator (PI) is considered the responsible individual.

NAME:	Anne Stone	TITLE:	Professor
AFFILIATION:	School of Human Evolution and Social Change	Office Phone #	480 727 6310
Cell Phone #:		Dept. Phone #:	
Fax #		E-Mail:	acstone@asu.edu

B. Additional contact, if any, for IACUC business

NAME:	Joanna Malukiewicz	TITLE:	Graduate Student
AFFILIATION:	School of Life Sciences	Office Phone #	480 747 6594
Cell Phone #:	480 371 6599	Dept. Phone #:	
Fax #		E-Mail:	jmalukie@asu.edu

C. Protocol Type

Non-funded research

Grant / Contract (Also submit grant proposal with this protocol)

Granting Agency: Deadline:

Proposal Title:

Proposal Number:

Co-Investigator(s):

Teaching

Course Title, Schedule:

D. Protocol Status:

New

Renewal—Previous Protocol #:

Revision—Previous Protocol #:

E. List all persons involved in this protocol. The first person listed should be the PI.

Name	Title	Role in Protocol (What procedures will each person be doing?)	Species with which individual will have direct contact ("all" or list species)*	IACUC USE ONLY Training (mm/yy)
Anne Stone	Professor	PI- Project Over Site Only and no Animal Work	none	11/10 HSQ
Joanna Malukiewicz	Graduate Student	Most Animal Work. Exceptions to some aspects of Animal Work are described below	All	2/10 HSQ

For each individual, describe the individual's training and years experience with all listed species and procedures:
Anne Stone- general genetics laboratory experience with primate species, no direct experience conducting callitrichid field captures or sample collections. Project oversight only for this protocol – no animal procedures to be conducted by PI.

Joanna Malukiewicz- 1 month experience following *Saguinus midas* in Suriname, one time collection of skin samples from cadavers of *Leontopithecus rosalia, Callimico goeldii,* and *Saguinus midas.* Additional training will be provided by Drs. Boere, Ruiz-Miranda, and Borstellman de Oliveira on capture and handling of wild common and black-tufted marmosets (Callithrix jacchus and Callithrix penicillata) and their hybrids. Dr. Boere possesses a degree in veterinary medicine from the Federal University of Saint Mary and a doctoral degree in neuroscience and behavior from the University of Sao Paulo. He is currently a professor at the Federal University of Vicosa. He has published extensively on both wild and captive black-tufted marmosets, and his work includes captures and immobilization of these animals. Dr. Borstelmann de Oliveira possess a doctoral degree in animal behavior from the University of Sao Paulo, and is a professor at the Federal Rural University of Pernambuco. Her work and experience focus on the reproductive and feeding strategies of common marmosets, as well as their conservation genetics, and her published work includes capture methodologies for common marmosets. Dr. Ruiz-Miranda possesses a doctoral degree in animal behavior from the University of California Davis. He currently is a professor at the State University of North Fluminense Darcy Ribeiro. His work is involved in the conservation of marmosets and golden lion tamarins. He has conducted captures of exotic common and black-tufted marmosets introduced to the state of Rio de Janeiro.

* The answer provided in this column dictates which Level II species-specific IACUC training modules are required for each individual. An individual only needs to complete Level II certification for those species with which he or she will directly work.

Note: ASU requires that all personnel engaged in animal research or teaching be qualified through training or experience in order to conduct the work humanely. The IACUC requires the successful completion and renewal of Level I – The Humane Care and Use of Laboratory Animals as well as Level II species-specific training at least once every 3 years. A link to the individual training modules is available on the IACUC ASU homepage at: http://hazel.forest.net/latanet/client/asu/introduction.htm.

F. Have all personnel on this protocol completed the required IACUC Level I and Species-Specific Level II Training Modules as well as the Occupational Health and Safety Program Health Surveillance Questionnaire? The Training Modules and the Health Surveillance Questionnaire (HSQ) can be found at http://researchintegrity.asu.edu/iacuc/training/exams.htm

Yes. Proceed to section B. (Dr. Stone has completed Level I training only, as per Dr.Joanne Tetens because Dr. Stone will not be handling any animals for this project).
No. List the individuals who are not in compliance and identify their deficiencies with an "X" in the appropriate columns:

Name	**Training Modules**		**HSQ Clearance**
	Level I	**Level II**	

G. Describe any non-routine measures such as special vaccines or personal protective equipment that is required for animal and/ or human safety:

In Brazil, MMR vaccines are mandatory prior to handling of animals. Humans will be required to wear surgical facemasks, leather gloves, and latex gloves while handling captured monkeys in the hybrid zone between Bahia and Pernambuco. At the Rio de Janeiro sites, any researchers with a cold or other symptoms of illness are not allowed near marmosets. Facemasks will be worn during immobilization and sample collection from animals in Rio de Janeiro. At the Brasilia sites, facemasks are optional, vaccines for herpes A and B as well as rabies are strongly recommended. Any individuals with symptoms of herpes will not be allowed to capture animals in Brasilia. For collection of samples at zoos and research laboratories, facility personnel and veterinarians following each facility's individual safety protocols will collect samples.

H. Do you plan to use Department of Animal Care & Technologies (DACT) personnel and resources? If yes, for what facilities and procedures? (If this use is new or an expansion of previous use, please contact the DACT well in advance of need). No

II. PROJECT DESCRIPTION AND PROGRAM REQUIREMENTS

The University Animal Care and Use Committee (IACUC) is composed of both active animal users and lay persons. Regardless of background, each member has one vote, and it is therefore particularly important that the language of the application be understood by all. This applies to all sections of the application, but it is especially important that the goals and justifications of the proposed research be spelled out in the clearest possible terms. NOTE: Upon approval, this protocol will become a public record, so please do not disclose proprietary information such as home telephone number and address.

A. Please provide a brief (300 words or less) synopsis in **laymen terms** of proposed research.

Several unique primate characteristics such as twinning, cooperative breeding, and social suppression of ovulation define the Callitrichidae family (subfamily Callitrichinae). These unique characteristics have generated special interest in these primates over the last thirty years among field, laboratory, and biomedical researchers. However, many questions still remain about callitrichid evolutionary relationships and genetic diversity. Because these issues are intimately tied to the evolution of the characteristics that define the Callitrichidae family, it is the aim of this study to expand current knowledge on the population genetics, population histories, and phylogenetics of all callitrichd species. This part of the proposed research will focus on the following two questions about callitrichid evolution: (1) What are the species-level phylogenetic relationships between callitrichids, and (2) what is the genetic structure of callitrichid species?

The last two questions of the proposed research address the main topic of the dissertation of Joanna Malukiewicz- the role of hybridization in shaping the population history of *Callithrix,* specifically the two species of common and black-tufted marmosets (*Callithrix jacchus* and *Callithrix penicillata*) . Historically, a significant role for hybridization in animal evolution has been met with much skepticism from prominent biologists like Ernst Mayr. However, recent evolutionary studies of Old World monkeys and apes point to several ancient and modern episodes of genetic exchange between different animal species as a result of hybridization. Additionally, phenotypic, behavioral, and phylogenetic evidence suggest an important role for hybridization in the evolution of these two specific primates. Thus, this part of the proposed research will focus on the following two questions: (1) what are the patterns of divergence between callitrichid species, and (2) what are past and present levels of hybridization (i.e. successful mating between individuals from distinct populations) and genetic exchange between callitrichid species? We would like to extend knowledge of the role of hybridization in primate evolution by investigating this phenomenon in New World monkeys.

B. PLANNED USE OF ANIMALS. Begin with a clear statement of purpose and briefly provide background information and references to previous work. Include a clear description of the experimental design for all animal experiments planned and explain why the experiments must be performed. **It is critical that for each procedure you provide a detailed sequence of events that effectively describes what happens to the animals.** Flow charts, diagrams or tables are strongly recommended for complicated experimental designs. Please state how the research is expected to benefit the human community, the animal community, and/or society as a whole. (Details regarding surgical procedures and drug treatments are not necessary, as they will be addressed later.)

Callitrichid Phylogeny

The revision of New World primate systematics by Rylands et al. (2000) represents the current consensus on callitrichid taxonomy. However, callitrichid evolutionary relationships at the species and subspecies levels remain problematic and not fully resolved (Rylands et al., 2000). For the genus *Callithrix,* phylogenies based on molecular and morphological characteristics give conflicting views of species relationships (e.g., cranial morphology: Marroig et al., 2004; mtDNA: Tagliaro et al., 1997; pelage: Hershkovitz, 1968). Molecular phylogenies based on nuclear data (IRBP gene, Kelly and Mundy, 2001) and mtDNA (Perez-Sweeney et al., 2008) give conflicting views as to whether *Leontopithecus chrysopygus* (black lion tamarin) and *L. rosalia* (golden lio tamarin) are distinct species or not. Among tamarins (*Saguinus* spp.) a phylogeny based on ribosomal DNA could not resolve relationships between *S. niger* and *S. midas* (Arapripe et al., 2008). A phylogeny based on the mitochondrial gene ND1 could not resolve the evolutionary relationship between *S. leucopus* and *S. oedipus* (Tagiliaro et al., 2005). Because many callitrichids are endangered, the taxonomical classification of these primates carries important implications for their conservation. Therefore, the species level taxonomy of the Callitrichidae should be as clear as possible.

The above studies have employed single genetic markers to infer phylogenetic relationships among primate species. Nei and Kumar (2000) recommend using 10 or more genetic loci for accurate construction of phylogenetic trees among closely related taxa. Therefore, we will use 32 neutral autosomal loci described below (see IIC.3) to study callitrichid evolutionary relationships. Neutral genetic loci are especially useful for phylogenetic analysis because these markers are less likely than markers near genes to be subject to effects of homoplasy due to selection (Wildman, et al., 2009). As a result, the utilization of several independent autosomal loci free from effects of natural selection will allow us to infer finer grain phylogenetic relationships among callitrichids than previously possible.

The Callitrichidae family is of special interest to researchers due to the unique suite of characteristics possessed by these primates. Our research aims to address a number of basic questions that remain about callitrichid population histories, phylogenetics, and genetic diversity. An understanding of the underlining genetic diversity of callitrichids carries important implications for the population management of captive marmosets, tamarins, and Goeldi's monkey. Phylogenetic relationships and genetic diversity also carry important implication in the conservation of endangered organisms. As many callitrichid taxa are endangered, a detailed understanding of their evolutionary biology will aid in making sound policies for the management of threaten populations. The results of our research will allow other researchers to formulate and test new hypotheses about the evolution of unique callitrichid traits. Our data from neutral intergenic autosomal loci can be compared with results from phylogenetic and population genetic studies based on loci linked to genes. This comparison will allow for a better understanding of how neutral and selective forces shape the evolution of primate genomes.

Biological samples will be requested from captive populations of callitrichids from US and international zoos and research facilities. For international facility requests, collection protocol for any given facility will be submitted to IACUC for review prior to collection of samples. From each facility, frozen tissue samples available from callitrichid cadavers will be requested whenever such tissues are available. If frozen tissues are not available from a facility, the following types of samples from living animals will be requested to be collected opportunistically or during scheduled veterinary care: 1-5 mL whole blood per individual (based on standards set by each facility) collected into an EDTA Vacutainer, kept cold and shipped overnight on icepacks; 40-50 hairs with follicles from each individual plucked with sterilized forceps, placed into baggies, frozen immediately, and shipped on dry ice; 2-3 mm skin biopsies collected per individual, frozen immediately and shipped overnight on dry ice; cheek cells collected by gently swabbing the inside of the cheek for 60 seconds with an absorbent swab, immersing the swab in cheek cell lysis buffer stored in a tube, and shipping the tube overnight; fresh fecal samples frozen within and hour of voiding and shipped in dry ice. Sample collection will be completed by an individual facility's animal care staff and under that facility's collecting guidelines.

References

Araripe, J., Tagliaro, C. H., Rego, P. S., Sampaio, I., Ferrari, S. F., & Schneider, H. (2008). Molecular phylogenetics of large-bodied tamarins, *Saguinus* spp. (primates, Platyrrhini). *Zoologica Scripta, 37*(5), 461-467.
armosets and tamarins: Systematics, beahaviour, and ecology (pp. 95-129). New York: Oxford University Press.

Hershkovitz,.P. (1968). Metachromism or principle of evolutionary change in mammalian tegumentary colors. *Evolution, 22*(3), 556-&.

Marroig, G., Cropp, S., & Cheverud, J. M. (2004). Systematics and evolution of the jacchus group of marmosets (Platyrrhini). *American Journal of Physical Anthropology, 123*(1), 11-22.
Mayr, E. (1963). *Animal species and evolution.* Cambridge: The Belknap Press of Harvard University Press.

Nei, M., & Kumar, S. (2000). *Molecular evolution and phylogenetics.* New York: Oxford University Press.

Peng, Z., Elango, N., Wildman, D. E., & Yi, S. V. (2009). Primate phylogenomics: Developing numerous nuclear non-coding, non-repetitive markers for ecological and phylogenetic applications and analysis of evolutionary rate variation. *Bmc Genomics, 10*, 247.

Perez-Sweeney, B. M., Valladares-Padua, C., Martins, C. S., Morales, J. C., & Melnick, D. J. (2008). Examination of the taxonomy and diversification of *Leontopithecus* using the mitochondrial control region. *International Journal of Primatology, 29*(1), 245-263.

Rylands, A. B., Schneider, H., Langguth, A., Mittermeir, R. A., Groves, C. P., & Rodriguez-Luna, E. (2000). An assessment of the diversity of the New World primates. *Neotropical Primates, 8*, 61-93.

Tagliaro, C. H., Schneider, M. P. C., Schneider, H., Sampaio, I. C., & Stanhope, M. J. (1997).

Marmoset phylogenetics, conservation perspectives, and evolution of the mtDNA control region. *Mol. Biol. Evol., 14*, 674.

Tagliaro, C. H., Schneider, H., Sampaio, I., Schneider, M. P. C., Vallinoto, M., & Stanhope, M. (2005). Molecular phylogeny of the genus *Saguinus* (Platyrrhini, primates) based on the ND1 mitochondrial gene and implications for conservation. *Genetics and Molecular Biology, 28*(1), 46-53.

Thalmann, O. H., Fischer, A. H., Lankester, F. H., Paabo, S. H., & Vigilant, L. H. (2007). The complex evolutionary history of gorillas: insights from genomic data. *Molecular Biology and Evolution, 24*(1), 146-158.

Wildman, D. E., Jameson, N. M., Opazo, J. C., & Yi, S. V. (2009). A fully resolved genus level phylogeny of neotropical primates (Platyrrhini). *Molecular Phylogenetics and Evolution, 53*(3), 694-702.

Marmoset Hybridization

To date, the identification of hybrids between different marmoset species has been based on the identification of individuals that are phenotypic intermediates between the two parental species (common marmoset-white and bushy ear tufts; black-tufted marmoset-black and low-sloping ear tufts; hybrids-mixture of parental phenotypes for ear tufts). In studying hybrid zones, the addition of molecular data will allow for the quantification of ancestry from each parental species that a hybrid may possess. Molecular data will also allow for the assessment of the amount and direction of genetic introgression (penetrence of genetic material from one species into the other) occurring within the two hybrid zones of interest for this study. Additionally, molecular data from DNA collected away from hybrid zones in non-sympatric areas of each species' natal range can uncover ancient episodes of hybridization between common and black-tufted marmosets. Such analyses can be accomplished by using the observed molecular data to make evolutionary inference about hybridization in terms of the past and present population history of a species.

Samples from wild marmosets will be collected at four localities: black-tufted marmosets at Jardim Botanico de Brasilia, Brasilia, Federal District, Brazil; wild common marmosets from the NE state of Pernambuco; common/hybrid/black-tufted marmosets at a natural hybrid zone near of the city of Petrolina, state of Pernamubuco, and Juazeiro, state of Bahia; and exotic common and black tufted marmosets and their hybrids in Rio Bonito and Silva Jardim municipalities, state of Rio de Janeiro, Brazil. Joanna Malukiewicz will be directly involved in captures of all wild common marmosets, black-tufted marmosets, and their hybrids, as indicated below.

In Jardim Botanico de Brasilia, tissues will be sampled from black-tufted marmosets according to the following sequence of events, under the guidance of Dr. Vanner Boere:

1. Animal captures are generally done between 6 AM and 9 AM, and animals will not kept overnight for recuperation. Instead, all processing of animals will be done the same day along with their release.
2. Animal traps will be set with banana as bait for marmosets in areas frequented by the monkeys such as feeding trees.
3. When an animal enters the trap, the door to the trap will be closed manually with a nylon cord.
4. Traps containing the caught animal will be covered with a dark cloth and moved 300-500m from the site of capture.
5. With the handler wearing gloves resistant to animal bites, the marmoset will be removed from the cage and injected with ketamine (20mg/kg) into the intramuscular region of the thigh. The sole use of ketamine for immobilization has not resulted in any animal mortality, and ketamine has proven sufficient for all procedures carried out while an animal is sedated. Atipamezole is not readily available in Brazil for veterinary use as a reversal agent for dexmedetomidine. Therefore Dr. Boere does not use ketamine/dexmedetomdine with atipamezole reversal during animal captures.
6. The animal will be placed back into the cage, which remains covered with the cloth, for the ketamine to take effect.
7. Once the animal is immobilized, it will be removed from the cage and placed on a field table.
8. The animal will be examined, identified, photographed, and weighed. A record of each

capture will be kept that notes the species (or hybrid), phenotype of ears, method of capture, sex of animal, fur description, scars, weight, age, and inventory of biological samples taken.

9. Every 5 minutes, the temperature and vital signs of the animal will be checked by a veterinarian while the animal is immobilized. Joanna Malukiewicz will keep a running record of animals' vitals during processing.
10. 40-50 hairs with root will be plucked with flame-sterilized forceps from the animal's tail. Collected hair will be placed in envelopes containing silica gel for storage.
11. Using a sterilized skin biopsy punch, a 3mm diameter biopsy will be taken from the ear of the immobilized animal. Prior to collection, the collection site will be wiped with povidone iodine and an alcohol-prep pad. The collection of skin will made in the earlobe, in a place chosen with the aid of a flashlight to avoid blood vessels, to reduce bleeding. To collect the biopsy, the punch will be simultaneously twisted and pressed into the animal's ear. The tissue sample will be then lifted with a pair of sterile forceps and cut with sterile scissors. The sample will be preserved in 25% (w/v) NaCl saturated DMSO. After collection, the area of the ear will be compressed with sterile clean gauze to stop bleeding.
12. Any fecal material voided by the animal during processing will be collected by Joanna Malukiewicz and stored for long term in RNAlater.
13. After collection of all biological tissues, the animal will be placed in a clean pet-carrier. The pet-carrier will be placed under the field table and covered with a dark cloth. Animals are watched for hypothermia and shock. Animals are not given water or food until complete recovery from sedation.
14. Once the animal is recovered from the effects of ketamine, it will be transported back in the pet carrier to the original site of capture and released. If an animal experiences shock and reduction of vital signs, a procedure of resuscitation is followed using epinephrine, hydrocortisone, saline infusion and heating.
15. The day following captures, animals will be observed for a few hours to check whether the animals are all right. Dr. Boere has not lost any animals during field captures.
16. If captured animals are brought into a laboratory for sample collection instead of the field, sedation and collection procedures are similar as in the field. Animals are also not maintained overnight in a laboratory, except in the rare event that the animal may show signs of a physical anomaly. If an animal stays overnight in a laboratory, it will receive fruit and water. After an animal is recuperated from any procedures in the laboratory it is returned to its original site of capture.

At the Rio de Janeiro sites, samples will be collected with the following procedure under the guidance of Dr. Carlos Ruiz-Miranda. The graduate student associated with this protocol will not perform immobilization steps of animals:

1. Animals will be captured in family groups using traps baited with banana. Traps will be covered with a dark cloth to calm the animals, and the traps will be carried by researchers to a car and driven to a field laboratory. The laboratory is about 20 km away from the field sites.
2. At the laboratory, animals will be individually removed from traps and placed into a squeeze trap. The animal will be guided to the back of the squeeze trap. Enough force will be applied in the squeeze trap to the animal so that it cannot physically struggle. At this point the animal will be injected with ketamine into the intramuscular region of the thigh. The ketamine dose depends on what would be done. For simple procedures, such as collecting hair or putting a radio collar, it is 10mg/kg. A higher dose is used (~20 mg/kg) if fully processing the animal (e.g., body measurements, blood collection, hair collection, tattooing and dye marks) because that can take up to 30 minutes. Animals have awakened with 10 mg/Kg in the middle of the processing.
3. The procedure of ketamine/dexmedetomidine with atipamezole reversal is avoided because this requires another injection, and the risks do not warrant the benefits (as per Dr. Carlos Ruiz-Miranda). The immobilized animals are kept confined until they fully awaken, usually an overnight period or at least 6 hours.

4. Once the animal is sedated, a researcher wearing gloves will take the animal out of the squeeze cage. The animal will be weighed, clinically examined, and tattooed. Animals are tattooed on the inside of the right thigh after shaving off the hair, and tattooing is performed with use of a commercial animal tattoo pen. Prior to collection, the collection site will be wiped with povidone iodine and an alcohol-prep pad. A record of each capture will be kept that notes the species (or hybrid), phenotype of ears, method of capture, sex of animal, fur description, scars, weight, age, and inventory of biological samples taken.
5. 40-50 hairs with root will be plucked with flame-sterilized forceps from the animal's tail. Collected hair will be placed in envelopes containing silica gel for storage.
6. Using a sterilize skin biopsy punch, a 3mm diameter biopsy will be taken from the ear of the immobilized animal. Prior to collection, the collection site will be wiped with povidone iodine and an alcohol-prep pad. The collection of skin will made in the earlobe, in a place chosen with the aid of a flashlight to avoid blood vessels, to reduce bleeding. To collect the biopsy, the punch will be simultaneously twisted and pressed into the animal's ear. The tissue sample will be then lifted with a pair of sterile forces and cut with sterile scissors. The sample will be preserved in 25% (w/v) NaCl saturated DMSO. After collection, the area of the ear will be compressed with sterile clean gauze to stop bleeding.
7. Any fecal material voided by the animal during processing will be collected by Joanna Malukiewicz and stored for long term in RNAlater.
8. The laboratory is kept heated at 28C, and if an animal suffers from hypothermia, it is made more comfortable by being placed into a "nest" of materials such as towels to keep it warm. Any animals that look sick or weak are not processed or immobilized. Hair may be collected from such animals but no anesthesia is given to them.
9. The animal will be placed back into the original trap used for capture, which at this point will be clean. Banana will be placed into the trap. Animals will be allowed to recover over night in the laboratory. The animals are kept in individual cages, but animals from the same group are placed adjacently to each other. Cages are all covered with cloth, and marmosets are monitored during their waking from ketamine. After that researchers do not come into the room because wild marmosets get too agitated when people are walking by and may injure themselves by hitting the sides of the cages as they try to escape. In 11 years there has been no mortality during overnight stays at the field laboratory.
10. Animals will be brought back by car to the original site of capture and released.

At the Bahia and Pernambuco sites, samples will be collected using the following procedure under the guidance of Dr. Maria Borstellman de Oliveira. The graduate student associated with this protocol will partake in all steps listed below at Bahia and Pernambuco sites:

1. A site of capture will be chosen to avoid overlap between territories of two or more marmoset social groups. Additionally, captures will avoid trapping of females near the end of their pregnancies or groups that contain newborn infants.
2. Once a group is chosen for capture, the next step will be to gradually habituate the group to the presence of traps and researchers for unhabituated groups. Progress towards habitation will be noted with daily notes that record the phase of habitation, time animals are exposed to bait/traps without bait/traps with bait/researchers and for how long, distance between animals and researchers, time contact ended, animals' behavior, and how contact ended. Platforms that will hold traps will be first put up at the capture site, then traps will be put on the platforms, then bait (usually a fruit such as pineapple that attracts the monkeys), and then finally the researchers will be present around the traps. When the capture phase begins, researchers will all wear black cloths over their faces to reduce stress on the animals. Care will be taken to only capture one animal per cage. If two animals are caught in the same trap, another trap will be brought over to which the second animal is transferred.
3. Capture cages are modified metal Tomahawk models, with a wooden box attached to the bottom of the cage. The fruit bait will be placed in between the bottom of the cage and wooden basket, and also surrounded by a screen to avoid being eating from the outside by un-trapped monkeys. Cages will be placed on platforms located in trees. Generally, once the monkeys get habituated to the presence of the bait, traps, and researchers, they will begin to enter the baited cages and can be caught.

4. Once caught, animals will be moved ~100m away from site of capture. Handlers of the animals will wear leather gloves to protect themselves against animal bites. A record of each capture will be kept that notes the species (or hybrid), phenotype of ears, method of capture, sex of animal, fur description, scars, weight, age, and inventory of biological samples taken.
5. Animals will be immobilized intramuscularly with ketamine (10 mg/kg). To administer the anesthetic, one of the animal's legs will be gently pulled out through a space at the bottom of the cage. If this is not possible, the animal will be put into a black bag, and the leg will be exposed for administration of ketamine. Animals are given a general clinical exam and morphometric measures are taken on the animals.
6. Because animals will only have to be captured once, Dr. Borstelmann de Oliveira has advised that we do not tattoo any captured animals for identification. Therefore animals will not be marked with any sort of methods for identification (including tattoos, collars, or picric acid).
7. Finally, Joanna Malukiewicz will take epithelial tissue samples from captured monkeys in the same manner as described above. 40-50 hairs with root will be plucked with flame-sterilized forceps from the animal's tail. Collected hair will be placed in envelopes containing silica gel for storage. Using a sterilize skin biopsy punch, a 3mm diameter biopsy will be taken from the ear of the immobilized animal. Prior to collection, the collection site will be wiped with povidone iodine and an alcohol-prep pad. The collection of skin will made in the earlobe, in a place chosen with the aid of a flashlight to avoid blood vessels, to reduce bleeding. To collect the biopsy, the punch will be simultaneously twisted and pressed into the animal's ear. The tissue sample will be then lifted with a pair of sterile forceps and cut with sterile scissors. The sample will be preserved in 25% (w/v) NaCl saturated DMSO. After collection, the area of the ear will be compressed with sterile clean gauze to stop bleeding.
8. Any fecal material voided by the animal during processing will be collected by the graduate student associated with this protocol and stored for long term in RNA later.
9. Whenever possible, marmoset captures and releases are done on the same day. If a group is captured in the morning, there is time to process the animals and release the group by evening. In case captures take place late into the afternoon, animals are processed and samples are collected at night. Animals are not supplied with water or food during the night. In case an animal has to remain in captivity within a cage for over 12 hours, a preparation of gelatin and honey (made without flavor or color) is supplied to the animals after they are processed. The animals lick this preparation while they are still under the influence of ketamine. In the experience of Dr. Borstelmann de Oliveira, this preparation is sufficient to nourish and hydrate the recovering animals, and she does not offer extra water or food to the animals.

The findings of this study will expand current knowledge about marmoset population history and population genetics. This study will take a multifaceted look at hybridization in terms of time and place. In turn, data from this study will facilitate the formulation of new hypotheses about the role played by hybridization during the evolution of human and non-human primates. The broader impacts of this study are defined by conservation implications, as well as opportunities that will be created for international collaboration and outreach. The Brazilian project collaborators include a number of well-established marmoset and tamarin researchers such as Drs. Carlos Ruiz-Miranda, Adriana Grativol, Maria Adelia Borstelmann de Oliveira, and Dr. Vanner Boere. At least one ASU undergraduate and 2 Brazilian students will be trained in molecular methods for this project to specifically work within the wet laboratory setting. Finally, the graduate student associated with this protocol will develop scientific inquiry lessons based on the proposed research for students at Sandra Day O'Connor High School, Phoenix, AZ (where the graduate student has previously worked as a National Science Foundation GK-12 Teaching Fellow) with the aim of piquing the scientific interest of younger learners. From the perspective of conservation, the results of this project carry important implications for population management of black-tufted, common marmosets, and one of Brazil's most highly endangered primates, the golden lion tamarin. Exotic marmosets in Rio de Janeiro are thought to be encroaching on the habitats of golden lion tamarins. Hybridization data are needed for Rio de Janeiro's exotic marmosets to determine how to manage these exotic (and possibly invasive) marmosets in places where they pose conservation threats to golden lion tamarins.

C. RATIONALE FOR INVOLVING ANIMALS AND THE APPROPRIATENESS OF THE **SPECIES AND NUMBER** USED. Keeping in mind the principles of the "3 R's" (Refinement, Reduction, and Replacement answer the following:

1. Why must live vertebrates be used in this study?

Callitrichid Phylogeny

In some cases, facilities have biological tissues available from deceased animals. However, these tissues are not available from all facilities. Whenever possible, tissues from deceased animals are available from zoos or research facilities, these tissues will be requested instead of tissues for living animals. If a facility has no available tissues from deceased animals, we will request tissues collected opportunistically from live animals or those live animals undergoing scheduled veterinary care.

Marmoset Hybridization

The sampling scheme for the proposed study calls for sampling of DNA from unrelated individuals both within and outside of marmoset hybrid zones. Within the hybrid zones, to Joanna Malukiewicz's knowledge, no marmoset tissue samples have been previously collected or are available from deceased individuals.

For black-tufted marmosets, this is a relatively little studied species within Brazil. As a result, biological samples from deceased black-tufted marmosets are limited. On the other hand, the common marmoset is a relatively well-studied species. Biological samples previously collected from wild marmosets within their natal range may be available from the graduate student's project collaborator Dr. Maria Adélia Borstelmann de Oliveira. In that case, the Joanna Malukiewicz will incorporate previously collected samples into the proposed research to reduce the number of common marmosets to be further sampled.

2. Why are you using the requested species rather than other species?

Callitrichid Phylogeny

The aim of this part of the proposed research is to construct the most complete species-level phylogeny of the family Callitrichidae possible following the classification of Rylands et al. (2000). Therefore, the requested species to be included in this study represent all of the species classified by Rylands et al. (2000) as callitrichids.

References

Rylands, A. B., Schneider, H., Langguth, A., Mittermeir, R. A., Groves, C. P., & Rodriguez-Luna, E. (2000). An assessment of the diversity of the New World primates. *Neotropical Primates, 8*, 61-93.

Marmoset Hybridization

These two species are specific to the two hybrid zones of interest in the proposed research.

3. What is the rationale supporting the numbers of animals proposed? Typically, a power analysis should be performed to support the proposed sample sizes. A table depicting the number of animals to be used is required.

Callitrichid Phylogeny

For each species, with the exception of black-tufted marmosets and common marmosets, we are requesting 5 representative tissue samples. At least one tissue sample from each species of interest will allow us to conduct interspecific phylogenetic comparisons. The other samples will allow us to gauge intraspecific variability. We are requesting 10 samples each for common and black-tufted marmosets because Joanna Malukiewicz will use these samples to optimize and test amplification of various genetic loci used in her dissertation. These samples will also allow her to initially gauge how variable these markers are among unrelated individuals within each species.

1. (*Callimico goeldii*) Goeldi's monkey	5
2. (*Callithrix aurita*) buffy-tufted-ear marmoset	5
3. (*Callithrix flaviceps*) buffy-headed marmoset	5
4. (*Callithrix geoffroyi*) Geoffroy's tufted-ear marmoset	5
5. (*Callithrix jacchus*) common marmoset	10
6. (*Callithrix kuhlii*) Wied's black-tufted-ear marmoset	5
7. (*Callithrix penicillata*) black-tufted marmoset	10
8. (*Cebuella pygmaea*) or (*Callithrix pygmaea*) pygmy marmoset	5
9. (*Leontopithecus caissara*) black-faced lion tamarin	5
10. (*Leontopithecus chrysophygus)* black lion tamarin	5
11. (*Leontopithecus chrysomelas*) golden-headed lion tamarin	5
12. (*Leontopithecus rosalia*) golden lion tamarin	5
13. (*Mico acariensis*) Rio Acari marmoset	5
14. (*Mico argentatus/Callithrix argentata*) silvery marmoset	5
15. (*Mico chrysoleucus*) golden-white tassel-ear marmoset	5
16. (*Mico emiliae*) Snethlage's marmoset	5
17. (*Mico humeralifer*) black and white tassel-ear marmoset	5
18. (*Mico humilis/Callithrix humilis*) black-crowned dwarf marmoset	5
19. (*Mico intermedius*) aripuana marmoset	5
20. (*Mico leucippe*) golden-white bare-ear marmoset	5
21. (*Mico manicorensis*) manicore marmoset	5
22. (*Mico marcai*) Marca's marmoset	5
23. (*Mico mauesi*) Maues marmoset	5
24. (*Mico melanurus/ Callithrix melanura*) black-tailed marmoset	5
25. (*Mico nigriceps*) black-headed marmoset	5
26. (*Mico rondi)* Rodon's marmoset	5
27. (*Mico saterei*) Satere marmoset	5
28. (*Saguinus bicolor*) pied bare-face tamarin	5
29. (*Saguinus fuscicollis*) saddle-back tamarin	5
30. (*Saguinus fuscicollis avilapiresi*) Avila Pires' saddle-back tamarin	5
31. *(Saguinus fusciollis crandalli*) Crandall's saddle-back tamarin	5
32. (*Saguinus fuscicollis cruzlimai*) Cruz Lima's saddle-back tamarin	5
33. (*Saguinus fusciollis fuscus*) Lesson's saddle-back tamarin	5
34. (*Saguinus fuscicollis fuscicollis*) Spix saddle-back tamarin	5
35. (*Saguinus fuscicollis illigeri*) Illiger's saddle-back tamarin	5
36. (*Saguinus fuscicollis lagonotus*) Red-mantle saddle-back tamarin	5

37. (*Saguinus fuscicollis leucogenys*) Andean saddle-back tamarin	5
38. (*Saguinus fuscicollis melanoleucus*) white saddle-back tamarin	5
39. (*Saguinus fuscicollis nigrifrons*) Geoffroy's saddle-back tamarin	5
40. (*Saguinus fusciollis primitivus*) saddle-back tamarin	5
41. (*Saguinus fuscicollis weddelli*) Weddell's saddle-back tamarin	5
42. (*Saguinus graellsi*) Graell's black-mantle tamarin	5
43. (*Saguinus imperator imperator*) black-chinned emperor tamarin	5
44. (*Saguinus imerator subgrisescens*) bearded emperor tamarin	5
45. (*Saguinus inustus*) mottled-face tamarin	5
46. (*Saguinus labiatus*) white-lipped tamarin	5
47. (*Saguinus labiatus labiatus)* red-bellied tamarin	5
48. (*Saguinus labiatus rufiventer*) Gray's red-bellied tamarin	5
49. (*Saguinus labiatus thomasi)* Thomas' mustached tamarin	5
50. (*Saguinus leucopus*) silvery-brown bare-face tamarin	5
51. (*Saguinus martinsi martinsi*) Martin's bare-face tamarin	5
52. (*Saguinus midas*) golden-handed tamarin	5
53. (*Saguinus mystax*) moustached tamarin	5
54. (*Saguinus mystax mystax*) Spix's mustached tamarin	5
55. (*Saguinus mystax pileatus*) red-cap mustached tamarin	5
56. (*Saguinus mystax pluto*) white-rump mustched tamarin	5
57. (*Saguinus niger*) black-handed tamarin	5
58. (*Saguinus nigricollis nigricollis*) Spix's black mantle tamarin	5
59. (*Saguinus nigricollis hernandezi*) Hernandez-Camacho's black mantle tamarin	5
60. (*Saguinus tripartitus*) golden-mantle saddle back tamarin	5
61. (*Saguinus martinsi ochraceus*) Ochraceous bar-face tamarin	5
62. (*Saguinus oedipus*) cotton-top tamarin	5
63. (*Saguinus geoffroyi*) Geoffroy's tamarin	5

Marmoset Hybridization

The graduate student will need to collect tissues from 100 individuals in each hybrid zones. These totals are higher than the minimum suggested by the power analysis described below. Extra individuals will be sampled to accommodate for individuals with ambiguous genotypes that may not be included in genetic analyses. Also, the actual number of hybrid individuals found within each hybrid zone is unknown. It will be assumed that 1/3 of animals sampled will be hybrids and 2/3 purebreds. That will be approximately 34 hybrids, 33 sampled common marmosets, and 33 black-tufted marmosets sampled within each zone. These intra-category caps will ensure that enough animals are sampled from each category to give this study appropriate power for evolutionary inference.

The graduate student conducted a power analysis to determine a sampling strategy within marmoset hybrid zones that will be informative enough to differentiate between hybrid and pure individuals. Further, gathered data should allow for differentiation between different classes of hybrids (first generation, second generation, and backcrosses). The analysis indicated that at least 50 total individuals should be sampled within the hybrid zone and genotyped at 48 independent microsatellite loci to meet the above goals (see Table 1). The level of hybridization in the analysis was assumed to be 10% as a conservative estimate below levels reported by published primate hybridization studies (e.g. Alberts and Altmann, 2001; Cortes-Ortiz et al., 2007). The analysis was conducted by using the MS coalescent simulation software (Hudson, 2002) to generate gene genealogies of two populations recently diverged from their most common recent ancestor without subsequent gene flow. Gene genealogies were simulated for 50 diploid individuals at 48 independent genetic loci. The MARKSIMS program (Hassal and Payseur, 2010) was run in conjunction with MS to simulate evolution of each independent as a microsatellite marker evolving under a stepwise mutation model with theta (4Neu)= 16.36.

HYBRIDLAB 1.0 (Nielsen et al., 2006) was used to simulate 1000 possible genotypes of pure and hybrid individuals found in a zone of recent secondary contact between two previously isolated populations. Genotypes were combined in sets of 12, 24, 36, and 48 microsatellite loci. Then STURCTURE (Pritchard et al., 2000) input files were created from these genotype combinations to simulate sampling within a hybrid zone for each possible combination of the following scheme: (1) 40, 50, 60, 70, 80, 90, and 100 individuals (45% pure common, 45% pure black-tufted, and 10% hybrids (F1, F2, or backcross individuals within the sample), and (2) 12, 24, 36, and 48 microsatellite loci. A hybrid was assumed to have a STRUCTURE q value between .1 and .9 (proportion ancestry from a given species). The ability of each sampling scheme to differentiate between hybrid and pure individuals was tested in STRUCTURE using the criteria of Vaha and Primmer (2006): the power of each data set was "the proportion of individuals in a group that were correctly identified as hybrid or pure." Parameters in STRUCTURE were set to the admixture model (alpha was set to be inferred with an initial value of 1.0), and the correlated allele frequencies model (lamda set to 1.0). For each STRUCTURE run, 50,000 Markov Chain Monte Carlo (MCMC) iterations were preceded by a 10,000 MCMC iteration burn-in period. Runs for each data set were replicated 5 times, and power calculations averaged over the 5 runs. Please see Table 2 for paramaters used in simulations for this part of the power analysis.

The graduate student utilized SIMDIV (genfaculty.rutgers.edu/hey/software#SIMDIV) to generate simulated NAIL data (molecular markers which are found outside of gene regions and thought to evolve without the effects of natural selection) using models of marmoset population divergence under both isolation and moderate migration. Preliminary IMa (Hey and Nielsen, 2007) runs of the SIMDIV data suggested that at least 30 individuals from each species should be sampled along with 32 NAILs (see Table 1). We are requesting the capture of 50 animals from each species to be sampled from non-hybridizing populations. Extra individuals will be sampled to accommodate for individuals with ambiguous genotypes that may not be included in genetic analyses. This data set allows for population parameters to be more accurately estimated via proper mixing and convergence of IMa software's coupled Markov Chain Monte Carlo runs. IMa software will be used to analyze DNA obtained from common marmosets and black-tufted marmoset populations that are non-hybridizing and non-sympatric. These analyses will be

Population Model Assumptions for Hybrid Zone and IMa Power Analysis
(1) Constant and equal population sizes in diverged and ancestral population (2) Equal male and female effective population sizes (3) A marmoset generation time of 1.5 years (after Tardif et al., 2003) (4) Rough estimate of population divergence time of 0.5 million years ago (after *C.jacchus* x *C. geoffroyi* divergence time, Barroso et al., 1997)

specifically used to make inferences about past hybridization between the two marmoset species of interest. Please see Table 2 for parameters used in simulations for this part of the power analysis.

Table 1. Total number of samples to be collected at each locality

Location	Total No. Animals to Be Sampled
Bahia-Pernambuco Hybrid Zone	100
Brasilia	50
Pernambuco	50
Rio de Janeiro Hybrid Zone	100

Table 2. Assumption and Parameter Summary used in Power Analyses

Hybrid Zone Power Analysis Model Parameters
(1) Ne – an effective size of 84,000 (2x female catarrhine/platyrrhine average effective population size from mtDNA (mitochondrial DNA) calculated by Piganeu and Eyre-Waler (2009) (2) u- a mutation rate per locus per generation of 4.87 x 10^{-5} (Sainudiin et al., 2004) (3) divergence time of 0.99 units of 4Ne generations.
IMa Power Analysis
(1) Ne – an effective size of 84,000 (2x female catarrhine/platyrrhine average effective population size from mtDNA (mitochondrial DNA) calculated by Piganeu and Eyre-Waler (2009) (2) u- a mutation rate per 2 x 10 ^-9 per base pair per year (based on percent divergence and time of separation of *C.jacchus* x *C. geoffroyi,* Barroso et al., 1997) (3) Rough estimate of population divergence time of 0.5 million years ago (after *C.jacchus* x *C. geoffroyi* divergence time, Barroso et al., 1997)

References:
Alberts, S. C., & Altmann, J. (2001). Immigration and hybridization patterns of yellow and anubis baboons in and around Amboseli, Kenya. *American Journal of Primatology, 53*(4), 139-154.
Cortes-Ortiz, L., Duda, T. F., Jr., Canales-Espinosa, D., Garcia-Orduna, F., Rodriguez-Luna, E., & Berminghamt, E. (2007). Hybridization in large-bodied New World primates. *Genetics, 176*(4), 2421-2425.
Haasl, R. J., & Payseur, B. A. (2010). Multi-locus inference of population structure: A comparison between single nucleotide polymorphisms and microsatellites. *Heredity*, advanced access.
Hudson, R. R. (2002). Generating samples under a Wright-Fisher neutral model of genetic variation. *Bioinformatics*, 18(2), 337-338.
Hey, J., & Nielsen, R. (2007). Integration within the Felsenstein equation for improved Markov chain Monte Carlo methods in population genetics. *Proceedings of the National Academy of Sciences, 104(8),* 2785-2790.
Nielsen, E. E. G., Bach, L. A., & Kotlicki, P. (2006). HYBRIDLAB (version 1.0): A program for generating simulated hybrids from population samples. *Molecular Ecology Notes*, 6(4), 971-973.
Pritchard, J.K., Stephens, M., & Donnelly, P. (2000) Inference of population structure using multilocus genotype data. *Genetics, 155,* 945-959.

4. What refinements, if any, have been made to reduce the number of animals used and the potential detrimental effects on the study animals?
The power analysis above was conducted to determine what will be the minimum number of individuals necessary for sampling to provide a dataset powerful enough to conduct this project. The graduate student will be following collaborators' established protocols for capture of animals. These protocols include steps to minimize animal stress as discussed in answers to question B.

5. If the procedures will cause pain or distress to the animals, provide a written narrative of the methods used to determine whether or not alternatives exist to these procedures.

Marmoset Hybridization

The graduate student is working with established Brazilian primatologists and veterinarians (Drs. Maria Adélia Borstelmann de Oliveira, Carlos Ruiz-Miranda, and Vanner Boere) as collaborators in the capture of wild marmosets. The methodology used at each locality to capture marmosets is determined by which collaborator will aid the graduate student at a given field site. Each collaborator has a number of years of experience in the capture of wild marmosets. For example, Dr. Ruiz-Miranda uses a capture protocol that was designed by veterinarians at the National Zoo, Washington, DC, over twenty years ago and has not lost a single animal (personal communication, C. Ruiz-Miranda). Dr. Boere is a veterinarian by training, and Dr. Borstelmann de Oliveira is a primatologist with over a decade of field experience.

In terms of biological tissue collection, several tissue types were considered in terms of invasiveness and goals of the project. Marmosets and other callitrichid species are chimeric (i.e. an individual can possess tissues originating from another individual). Chimerism results from frequent twinning in callitrichids, and the exchange of stem cells *in utero* between twins (Benirshke et al., 1962). Ross et al. (2007) found that levels of chimerism in the species *Callithrix kuhili* vary in different tissue types. Feces was 9.09% chimeric, skin from deceased animals was 5.6% chimeric, hair from deceased animals was 17.1% chimeric, and hair from live animals was 26% chimeric. The data of Ross et al. (2007) suggest that ease of genotyping marmosets at various genetic autosomal loci will depend on what type of tissue from which DNA was obtained. Generally, more chimeric tissue would make it harder to distinguish "self" from "non-self" genotypes.

Feces would be the least invasive, low chimeric tissue type from which to obtain DNA. However, fecal samples are difficult to obtain from wild marmosets (Carlos Ruiz Miranda, personal communication) without capture. Because marmosets will be captured and immobilized in the wild, saliva collection can be another minimally invasive option. However, Ross et al. (2007) found that this tissue type was 51.6% chimeric. Epithelial tissue is arguable the next type of minimally invasive, low-chimeric tissue type that can be collected from immobilized marmosets. The graduate student's preliminary data from DNA extracted from the skin vs. blood tissue of captive common marmosets indicates that animals' "self" vs. "non-self" genotype is more easily determined from skin DNA samples. Hair will also be collected from immobilized animals by plucking 40-50 hairs with follicles from the tip of each captured animal's tail. Fecal samples will be collected from animals if the animal voids feces during processing. Skin is the least chimeric, minimally invasive tissue that will be collected along with fecal and hair samples.

References:
Benirshke, K., Anderson J.M., Brownhill, L.E. (1962) Marrow chimerism in marmosets. *Science, 138,* 384-393.
Ross, C. N., French, J. A., & Orti, G. (2007). Germ-line chimerism and paternal care in marmosets (Callithrix kuhlii). *Proceedings of the National Academy of Sciences of the United States of America, 104*(15), 6278-6282.

III. CONTROLLED SUBSTANCES

A. Does this protocol involve the use of DEA-listed control substances (e.g., ketamine, pentobarbital, androgens, diazepam, buprenorphine)?

No. Proceed to section **IV**.

Yes. List all controlled substances:

ketamine originating in the US

IV. EMERGENCY CONTACT

A. Who should be contacted in case of an animal emergency? **Note: This information will be redacted if this protocol is requested as a public document.**

Name: The graduate student will be conducting collections in the field, with experts who handle wild marmosets on a regular basis. These individuals will be available on site if there is an emergency during marmoset captures.

Home Phone #
Office Phone #
Cell Phone #:

V. DUPLICATION AND ALTERNATIVES

The Animal Welfare Act requires that you document your justifications with data from **two** or more sources. One source **must** be a set of searches of a relevant database: name the database searched, the terms searched, when it was searched and the frequency of searches. The second source can be a set of searches of a second relevant database, or consultation with a laboratory animal science veterinarian, or courses/meetings/consultations with qualified personnel. Sufficient documentation, such as the consultant's name and qualifications and the date and content of the consult, should be provided to the IACUC to demonstrate the expert's knowledge of the availability of alternatives in the specific field of study. Consultation with the university attending veterinarian **must** be performed if the animals are expected to experience pain or distress. Examples include PUBMED, Web of Science. **[**Note: USDA Animal Welfare Information Center provides an in depth listing of database resources on their website at http://awic.nal.usda.gov/nal_display/index.php?info_center=3&tax_level=1&tax_subject=184.**]**

A. Provide the following details for the most recent literature search used to explore for duplicative research. (The literature search documents that the research will not unnecessarily duplicate previous research). **Teaching protocols do not need to conduct this search.**

Date that search was conducted (Must be within 60 days of the IACUC review date): 10/15/2010
Database used: Primate Lit
Publication years covered by the search: 1971-2010

Keywords used: *Callithrix* population genetics; marmoset evolution; marmoset phylogeny; callitrichid phylogeny

B. Provide the following details for the most recent literature search used to explore for alternatives to animal use and alternatives to painful procedures. Alternatives should be considered for any aspect of the protocol that may cause more than momentary or slight pain or distress to the animal. Alternatives to be considered include those that would: 1) refine the procedure to minimize discomfort that the animal(s) may experience; 2) reduce the number of animals used overall; or 3) replace animals with non-animal alternatives (e.g., computer models or tissue culture). **All protocols (both research and teaching) MUST conduct this search.**

Date that search was conducted (Must be within 60 days of the IACUC review date): 10/15/2010
Database used: *Pubmed*
Publication years covered by the search: 1978- 2004
Keywords used: marmoset sample collection; marmoset fecal; marmoset capture

C. Results of search for alternatives: Please comment on the application(s) of any identified alternatives, including how these alternatives may be or may not be incorporated to modify a procedure to either lessen or eliminate potential pain and distress. You must include sufficient information for the IACUC to determine that a reasonable, good faith effort was made to determine the availability of alternatives. If the search identified any alternative methods (ones that could be used to accomplish the goals of the animal use proposal), you must clearly explain and justify why this alternative cannot be used.

The search for "marmoset sample collection" brought up two articles. One of the articles by Schultz-Darken (2003) describes methods of common marmoset sample collection for biomedical use. For example, this article discusses restraint of captive marmosets for sample collection (blood) using a polyvinylchloride cylinder to avoid sedation of the animal. However, given that sample collection for this project will be in the wild, collection of animals with out sedation would probably be highly stressful for animals given that they will be handled by strangers in an area outside of their familiar surroundings. Fecal samples would be an alternative tissue type that would be less invasive to collect then epithelial samples from marmosets. However, this tissue type was shown to be nearly twice as chimeric as skin in *C. kuhli* by Ross et al. (2008). Additionally, fecal samples are difficult to collect in the wild given the small size of the fecal pellets without capturing animals (personal communication, Carlos Ruiz-Miranda). The search in PubMed on marmoset fecal samples recovered a recent publication on which Dr. Ruiz-Miranda is a co-author, and indeed fecal samples required the capture of marmosets. Therefore fecal samples will be collected in addition to skin and hair if an animal voids during processing.

Numerous articles were found for the search for "marmoset capture" that discussed the handling of captive marmosets or training captive common marmosets to urinate. For example, Tardif et al. (2006) discuss dietary changes for New World monkeys being switched between different research facilities and training monkeys to change within the same facility. Because the proposed project will collect samples one time and in the wild, many of the suggestions given by Tardif et al. (2006) would be difficult to apply outside of captivity. Other articles resulting from this search addressed diagnosis of diseases in marmosets, stress, and immunological topics

Williams et al. (2008) introduced a device, the Primaport, for the transportation of small primates for routine veterinary and laboratory procedures that also minimizes capture stress on the animals. The device is shaped as an upright cylinder with a vented dome-shaped lid, and a snap-on base. The device is initially placed in the house cage of laboratory primates, and primates are trained to enter the device through positive reinforcement with food rewards. While this device is very suitable to use for safer handling of marmosets within a laboratory environment, it will be difficult to use this device in the field. If used with a captive animal, the animal enters the device and a researcher removes the device and closes it off by snapping on the bottom base. This method of capture will not work in the field because unlike a conventional trap, the animal does not become sealed in the device upon entering it. If a field research has to close off the device once the monkey enters it, it is very likely that the animal will have enough time to escape prior to being caught. Additionally, the shape of the device is designed for short-term transport of the animals. In the field, animals will need time to recover after sedation, and the rectangular shape of a cage will give more room and comfort to the recovering animal than an upright cylinder.

References

Ross, C. N., French, J. A., & Orti, G. (2007). Germ-line chimerism and paternal care in marmosets (Callithrix kuhlii). *Proceedings of the National Academy of Sciences of the UnitedStates of America,* 104(15), 6278-6282.

Schultz-Darken, N.J. (2003) Sample Collection and Restraint Techniques used for Common Marmosets (*Callithrix jacchus*). *Comparative Medicine,* 53(4) 360-363.

Williams, P.T., Poole, M.J., Katos, A.M., Hilmans, C.J. (2008) A new device for the capture and transport of small nonhuman primates in scientific research. *Lab animal,* 37(3), 116-119.

Tardif S, Bales K, Williams L, Moeller EL, Abbott D, Schultz-Darken N, Mendoza S, Mason W, Bourgeois S, Ruiz J (2006) Preparing New World monkeys for laboratory research. *ILAR J.,* 47(4), 307-315.

D. Describe any other procedures (e.g., participation in meetings, review of journals) that are used to explore and evaluate alternatives: The graduate student interviewed the three collaborators that will be aiding her in the capture of marmosets and sample collection in the wild. Each collaborator was asked to describe in detail their respective methods for capturing, immobilization, handling, sample collection, and release of wild marmosets. Another resource which has been particularly helpful is a book entitled "Field and Laboratory Methods in Primatology" and edited by JM Setchell and DJ Curtis. The graduate student has reviewed this book to understand the steps taken to habituate wild primates, how to trap marmosets, monkey sedation, handling of animals which sedated, and collection of biological specimens from sedated monkeys. Many suggestions given in this book match the methodology used by the graduate student's collaborators in capture of marmosets. For example, this book recommends usage of Tomahawk traps for marmoset capture. Habituation of monkeys is described as a step-by-step process where animals are first exposed to traps, then baited traps, then to the presence of researchers. Additionally, marmoset recovery after sedation is recommended in a quiet, dark environment in the cage used for capture. These suggestions are the same guidelines that collaborators of this project follow based on the interviews the graduate student held with them.

E. Does this research replicate previous work?

No. Proceed to section **VI.**

Yes. Explain why the replication is necessary:

Not applicable. This is a teaching protocol.

VI. CATEGORY OF PAIN OR DISTRESS

The USDA Regulations define a "painful or distressful procedure" as "any procedure that would reasonably be expected to cause more than slight or momentary pain or distress in a human being to which that procedure was applied; that is, pain in excess of that caused by injections or other minor procedures." Using the table below, list all species of live vertebrate animals to be used in the proposed study and indicate the number of animals to be used under the appropriate USDA category. For an animal undergoing multiple procedures, list the animal under the highest level of pain expected for that animal.

Species	Number per USDA Category*				Total number of animals requested
	B	C	D	E	

Callithrix jacchus			126		126
Callithrix penicillata			126		126
Hybrids			68		68
Mico rondoni			5		5

	B	C	D	E	Total
(*Callimico goeldii*) Goeldi's monkey			5		5
(*Callithrix aurita*) buffy-tufted-ear marmoset			5		5
(*Callithrix flaviceps*) buffy-headed marmoset			5		5
(*Callithrix geoffroyi*) Geoffroy's tufted-ear marmoset			5		5
(*Callithrix kuhlii*) Wied's black-tufted-ear marmoset			5		5
(*Cebuella pygmaea*) or (*Callithrix pygmaea*) pygmy marmoset			5		5
(*Leontopithecus caissara*) black-faced lion tamarin			5		5
(*Leontopithecus chrysophygus)* black lion tamarin			5		5
(*Leontopithecus chrysomelas*) golden-headed lion tamarin			5		5
(*Leontopithecus rosalia*) golden lion tamarin			5		5
(*Mico acariensis*) Rio Acari marmoset			5		5
(*Mico argentatus/Callithrix argentata*) silvery marmoset			5		5
(*Mico chrysoleucus*) golden-white tassel-ear marmoset			5		5
(*Mico emiliae*) Snethlage's marmoset			5		5
(*Mico humeralifer*) black and white tassel-ear marmoset			5		5
(*Mico humilis/Callithrix humilis*) black-crowned dwarf marmoset			5		5
(*Mico intermedius*) aripuana marmoset			5		5
(*Mico leucippe*) golden-white bare-ear marmoset			5		5
(*Mico manicorensis*) manicore marmoset			5		5
(*Mico marcai*) Marca's marmoset			5		5
(*Mico mauesi*) Maues marmoset			5		5
(*Mico melanurus/ Callithrix melanura*) black-tailed marmoset			5		5
(*Mico nigriceps*) black-headed marmoset			5		5
(*Mico saterei*) Satere marmoset			5		5
(*Saguinus bicolor*) pied bare-face tamarin			5		5
(*Saguinus fuscicollis*) saddle-back tamarin			5		5
(*Saguinus fuscicollis avilapiresi*) Avila Pires' saddle-back tamarin			5		5
(Saguinus fusciollis crandalli) Crandall's saddle-back tamarin			5		5
(*Saguinus fuscicollis cruzlimai*) Cruz Lima's saddle-back tamarin			5		5
(*Saguinus fusciollis fuscus*) Lesson's saddle-back tamarin			5		5

(*Saguinus fuscicollis fuscicollis*) Spix saddle-back tamarin			5		5
(*Saguinus fuscicollis illigeri*) Illiger's saddle-back tamarin			5		5
(*Saguinus fuscicollis lagonotus*) Red-mantle saddle-back tamarin			5		5
(*Saguinus fuscicollis leucogenys*) Andean saddle-back tamarin			5		5
(*Saguinus fuscicollis melanoleucus*) white saddle-back tamarin			5		5
(*Saguinus fuscicollis nigrifrons*) Geoffroy's saddle-back tamarin			5		5
(*Saguinus fusciollis primitivus*) saddle-back tamarin			5		5
(*Saguinus fuscicollis weddelli*) Weddell's saddle-back tamarin			5		5
(*Saguinus graellsi*) Graell's black-mantle tamarin			5		5
(*Saguinus imperator imperator*) black-chinned emperor tamarin			5		5
(*Saguinus imerator subgrisescens*) bearded emperor tamarin			5		5
(*Saguinus inustus*) mottled-face tamarin			5		5
(*Saguinus labiatus*) white-lipped tamarin			5		5
(*Saguinus labiatus labiatus)* red-bellied tamarin			5		5
(*Saguinus labiatus rufiventer*) Gray's red-bellied tamarin			5		5
(*Saguinus labiatus thomasi)* Thomas' mustached tamarin			5		5
(*Saguinus leucopus*) silvery-brown bare-face tamarin			5		5
(*Saguinus martinsi martinsi*) Martin's bare-face tamarin			5		5
(*Saguinus midas*) golden-handed tamarin			5		5
(*Saguinus mystax*) moustached tamarin			5		5
(*Saguinus mystax mystax*) Spix's mustached tamarin			5		5
(*Saguinus mystax pileatus*) red-cap mustached tamarin			5		5
(*Saguinus mystax pluto*) white-rump mustched tamarin			5		5
(*Saguinus niger*) black-handed tamarin			5		5
(*Saguinus nigricollis nigricollis*) Spix's black mantle tamarin			5		5
(*Saguinus nigricollis hernandezi*) Hernandez-Camacho's black mantle tamarin			5		5
(*Saguinus tripartitus*) golden-mantle saddle back tamarin			5		5
(*Saguinus martinsi ochraceus*) Ochraceous bar-face tamarin			5		5
(*Saguinus oedipus*) cotton-top tamarin			5		5
(*Saguinus geoffroyi*) Geoffroy's tamarin			5		5

*USDA PAIN CATEGORIES: (see http://researchintegrity.asu.edu/iacuc/apply/USDApaincategories-examples.doc for a more complete description of the below categories)

Classification B: Includes animals that are used solely for breeding (e.g., to produce experimental animals or to maintain experimental lines).

Classification C: Includes the use of animals in procedures involving no pain or distress (e.g., non-invasive parenteral drug delivery, peripheral blood collection, euthanasia, short-term manual or chemical restraint, and tumor propagation or toe-clipping performed according to ASU guidelines).

Classification D: Alleviated pain. Animals used in procedures that could cause pain or distress but appropriate anesthetic, analgesic, or tranquilizing drugs are used (e.g., surgery, periorbital blood collection, perfusion, or administration of irritating chemicals.

Classification E: Unalleviated pain. Includes the use of animals in procedures that involve pain or distress but the use of appropriate anesthetic or analgesic would have an adverse effect (e.g., negative conditioning, unrelieved post-surgical pain, death without euthanasia).

VII. ASSURANCE:

The information contained herein is accurate to the best of my knowledge. I have carefully compared the proposed work with the current state of knowledge in this field by reviewing the literature and it is my professional opinion that the proposed work meets high standards of scientific merit. If the study involves pain and distress to the animal, whether or not it is relieved by anesthetics or analgesics, I have (1) reviewed the literature related to this work and have found no significant studies which could make this protocol unnecessarily duplicative, and (2) considered alternatives to animal use and found none available, as described above. Procedures involving animals will be carried out humanely and all procedures will be performed by or under the direction of trained or experienced persons. Any revisions to animal care and use in this project will be promptly forwarded to the Institutional Animal Care and Use Committee for review. Revised protocols will not be used until Committee clearance is received. The use of alternatives to animal models has been considered and found to be unacceptable at this time.

The principal investigator, by signing below, and the IACUC recognize that other medications may be given to the animals for veterinary care purposes (including humane euthanasia of animals in pain that cannot be controlled, as determined by the University Veterinarian or an euthanasia-certified principal investigator).

Principal Investigator – Please Print ______ Date ______

Principal Investigator Signature ______ Date ______

***Department Chair ______ Date ______

***College Dean ______ Date ______

****ASU Polytechnic requires these signatures.**

NOTE: Principal investigators are requested to attach a two-page biosketch reflecting their most recent pertinent experience. **Also include a current curriculum vitae for all senior participants.**

DETAILED USE OF ANIMALS

This section must be completed for each species used.

Link to additional Detailed use of Animals form:

Common Name: next to each species is listed the IUCN (International Union for Conservation of Nature) Red List v 3.1 Designation

(*Callimico goeldii*) Goeldi's monkey	vulnerable
(*Callithrix aurita*) buffy-tufted-ear marmoset	vulnerable
(*Callithrix flaviceps*) buffy-headed marmoset	endangered
(*Callithrix geoffroyi*) Geoffroy's tufted-ear marmoset	Least concern
(*Callithrix kuhlii*) Wied's black-tufted-ear marmoset	Near threatened
(*Cebuella pygmaea*) or (*Callithrix pygmaea*) pygmy marmoset	Least concern
(*Leontopithecus caissara*) black-faced lion tamarin	Critically endangered
(*Leontopithecus chrysophygus)* black lion tamarin	endangered
(*Leontopithecus chrysomelas*) golden-headed lion tamarin	endangered
(*Leontopithecus rosalia*) golden lion tamarin	endangered
(*Mico acariensis*) Rio Acari marmoset	Data no available
(*Mico argentatus/Callithrix argentata*) silvery marmoset	Least concern
(*Mico chrysoleucus*) golden-white tassel-ear marmoset	Data not available
(*Mico emiliae*) Snethlage's marmoset	Data not available
(*Mico humeralifer*) black and white tassel-ear marmoset	Data not available
(*Mico humilis/Callithrix humilis*) black-crowned dwarf marmoset	vulnerable
(*Mico intermedius*) aripuana marmoset	Least concern
(*Mico leucippe*) golden-white bare-ear marmoset	vulnerable
(*Mico manicorensis*) manicore marmoset	Least concern
(*Mico marcai*) Marca's marmoset	Data not available
(*Mico mauesi*) Maues marmoset	Least concern
(*Mico melanurus/ Callithrix melanura*) black-tailed marmoset	Least concern
(*Mico nigriceps*) black-headed marmoset	Data not available
(*Mico rondoni)* Rondon's marmoset	Data not available
(*Mico saterei*) Satere marmoset	Least concern
(*Saguinus bicolor*) pied bare-face tamarin	endangered
(*Saguinus fuscicollis*) saddle-back tamarin	Least concern
(*Saguinus fuscicollis avilapiresi*) Avila Pires' saddle-back tamarin	Least concern
(*Saguinus fusciollis crandalli*) Crandall's saddle-back tamarin	Least concern
(*Saguinus fuscicollis cruzlimai*) Cruz Lima's saddle-back tamarin	Least concern

(*Saguinus fusciollis fuscus*) Lesson's saddle-back tamarin	Least concern
(*Saguinus fuscicollis fuscicollis*) Spix saddle-back tamarin	Least concern
(*Saguinus fuscicollis illigeri*) Illiger's saddle-back tamarin	Least concern
(*Saguinus fuscicollis lagonotus*) Red-mantle saddle-back tamarin	Least concern
(*Saguinus fuscicollis leucogenys*) Andean saddle-back tamarin	Least concern
(*Saguinus fuscicollis melanoleucus*) white saddle-back tamarin	Least concern
(*Saguinus fuscicollis nigrifrons*) Geoffroy's saddle-back tamarin	Least concern
(*Saguinus fusciollis primitivus*) saddle-back tamarin	Least concern
(*Saguinus fuscicollis weddelli*) Weddell's saddle-back tamarin	Least concern
(*Saguinus graellsi*) Graell's black-mantle tamarin	Data not available
(*Saguinus imperator imperator*) black-chinned emperor tamarin	Least concern
(*Saguinus imerator subgrisescens*) bearded emperor tamarin	Least concern
(*Saguinus inustus*) mottled-face tamarin	Least concern
(*Saguinus labiatus*) white-lipped tamarin	Least concern
(*Saguinus labiatus labiatus)* red-bellied tamarin	Least concern
(*Saguinus labiatus rufiventer*) Gray's red-bellied tamarin	Least concern
(*Saguinus labiatus thomasi)* Thomas' mustached tamarin	Least concern
(*Saguinus leucopus*) silvery-brown bare-face tamarin	endangered
(*Saguinus martinsi martinsi*) Martin's bare-face tamarin	Least concern
(*Saguinus midas*) golden-handed tamarin	Least concern
(*Saguinus mystax*) moustached tamarin	Least concern
(*Saguinus mystax mystax*) Spix's mustached tamarin	Least concern
(*Saguinus mystax pileatus*) red-cap mustached tamarin	Least concern
(*Saguinus mystax pluto*) white-rump mustched tamarin	Least concern
(*Saguinus niger*) black-handed tamarin	vulnerable
(*Saguinus nigricollis nigricollis*) Spix's black mantle tamarin	Least concern
(*Saguinus nigricollis hernandezi*) Hernandez-Camacho's black mantle tamarin	Least concern
(*Saguinus tripartitus*) golden-mantle saddle back tamarin	Near threatened
(*Saguinus martinsi ochraceus*) Ochraceous bar-face tamarin	Data not available
(*Saguinus oedipus*) cotton-top tamarin	Critically endangered

(*Saguinus geoffroyi*) Geoffroy's tamarin	Least concern
Callithrix jacchus (common marmoset) and *Callithrix penicillata* (black-tufted marmoset) hybrids	Least concern for both species

Scientific Name:

I. **ANIMAL INFORMATION**

A. Is this a threatened or endangered species?

No. Proceed to section I. B.

Yes. Describe why this work must be done on this species and why the project will not have a significant negative impact on the species: The phylogeny of the family Callitrichidae is currently unresolved at the genus level for many of its members. Also many members of this family are endangered as can be see from the list above. Resolving the phylogentic relationships of callitrichids would allow us to understand their diversity and aid in resolving their evolutionary relationships. For endangered species, knowing their evolutionary history is often of great benefit in making conservation decisions for their protection. Samples from all species, with the exception of *C. jacchus* and *C. penicillata*, will be obtained whenever possible from pre-existing samples, non-invasively collected samples, or sampled collected opportunistically or under scheduled veterinary care. With the exception of *C. jacchus* and *C. penicillata*, Joanna Malukiewicz will not be capturing any additional species with the frame of this project. These measures will minimize any direct impact that procurement of samples may have of threatened callitrichids.

B. Maximum # of animals to be used

Per Year: Depends on availability of zoo Tissues samples and number of Animals captured in Brazil

Entire three years of protocol: 625

C. Sex: male and female Age or Weight Range: 120- 900 g

D. Source (e.g., commercial versus other, donated, captured from wild): wild, captive

E. Please LIST all labs and/or rooms **outside of the ASU centralized vivaria** where you intend to keep or use live animals in connection with the animal use covered under this protocol. This list is for IACUC information to assure each location is inspected semi-annually. **Listing rooms here does not assure approval of this space for use.**

Building	Room #	Max Length of Stay	Method of Transport	Purpose

II. **MAJOR CATEGORIES OF USE**

A. Will animals be immunized for antibody production?

No. Proceed to section II. B.

Yes. Complete the following table.

Injection:

Volume of injectate	Adjuvant	Route	Min. Frequency	Max. # of injections

I.

Collection: If terminal, check here Otherwise complete the following.

Route	Max. Volume	Min. Frequency	Max. # of collections

B. Will tissues or blood be harvested (other than for antibody production)?
No. Proceed to section II. C.
Yes. Will tissues be collected post-mortem only?
Yes. Proceed to section II.C.
No. Complete Appendix 1.

C. Will animals be food restricted (calorically or specific constituents)?
No. Proceed to section II. D.
Yes.
What are the restriction parameters? Provide scientific justification

How will you monitor for negative effects of food restriction (include information on how you will account for animal growth)?

D. Will animals be water restricted?
No. Proceed to section II. E.
Yes.
What are the restriction parameters? Provide scientific justification

How will you monitor for negative effects of water restriction (include information on how you will account for animal growth)?

E. Will pharmacologic or toxicologic materials be used apart from surgical use, including but not limited to tranquilizers, sedatives, analgesics, and anesthetics?
No. Proceed to section II. F.
Yes. Complete the following for each material.

Agent	Dose	Route	Purpose
Ketamine	10-20 mg/kg	Intramuscular injection	sedation

Provide scientific justification: Biological samples will be collected from wild marmosets. In order to lessen the stress of sampling, animals will be immobilized with Ketamine. Ancrenaz, M., Setchell, J. M., and Curtis, D.J. (2003) Handling, anaesthesia, health evaluation, and biological sampling. *Field and Laboratory Methods: A Practical Guide,* ed. J.M. Setchell and D.J. Curtis. Cambridge University Press: Cambridge recommend this drug as an anesthetic because it is relatively safe for primates and for its ease of use.

F. Will irradiation or radioisotopes be used?
No. Proceed to section II. G.
Yes. List here and attach ASU Radioisotope Approval Form.

Agent	Dose	Route	Purpose

Provide scientific justification

G. Will toxic chemicals, carcinogens, recombinant DNA, or infectious agents be used in conjunction with animal use?

No. Proceed to section II. H.

Yes. List the agent, dose, route, and purpose in the table below ____________.

Agent	Dose	Route	Purpose

Provide the Institutional Biosafety Committee (IBC) approval #:

Provide scientific justification for the need to use these agents:

H. Will animals be exposed to trauma, injury, burning, freezing, or electric shock?

No. Proceed to section II. I.

Yes. List and justify each exposure.

Provide scientific justification:

I. Will animals be exposed to environmental stress (e.g., temperature, physical restraint, forced exercise)?

No. Proceed to section II. J.

Yes. List and scientifically justify each exposure.

J. Will animals undergo surgery?

No. Proceed to section II. K.

Yes. Attach Appendix 2.

K. Will any animals have a device (e.g., thermocouple, cannula, electrode) that extends chronically through the skin?

No. Proceed to section II. L.

Yes. Describe wound management measures to minimize chances of infection around the device where it penetrates the skin:

L. Will animals need any special husbandry considerations?

No. Proceed to section II. M.

Yes. Describe special procedures and provide scientific justification:

M. Will any animals need to be individually identified?

No. Proceed to section III.

Yes. Describe the marking technique to be used, why that technique was chosen, how it will be performed, and on what age range of animals?

Adult animals will be tattooed on the inner thigh as a permanent method of identification at the Rio de Janiero sites. Identifying marks such as scars, deformities, unique coloration or pelage patterns will be noted for identification at the Brasilia site. These marking techniques are protocols used by the graduate student's collaborators.

III. DETRIMENTAL SEQUELAE

A. Will animals possibly experience clinical signs intentionally or as a possible side effect of the study?

No. Proceed to section IV.

Yes. Complete the following.

Possible Clinical Effect	Probability of Occurrence	Treatment

IV. END POINT CRITERIA

A. What clinical signs will be used as a basis for removal of an animal from the study?

B. This is applicable for animals captured in the field: While an animal is immobilized, extremely low bodily temperature and a slow down of vital signs will cause an animal not to be sampled during captures. At the Brasilia site, if an animal experiences hypothermia, heating blankets or hot water bottles are used on the animals. Also at this site, if an animal experiences shock and reduction of vital signs, a procedure of resuscitation is followed using epinephrine, hydrocortisone, saline infusion and heating. At the Rio de Janeiro sites, an animal may be placed into a "nest" of cloth material or towel that retains heat to warm up the animal. Such animals are not further processed for sample collection. At the Bahia-Pernambuco sites, animals experiencing hypothermia are wrapped in towels and kept warm. No medications are used at the Rio de Janeiro and Bahia-Pernambuco sites.

V. EUTHANASIA

A. Chemical/Gas Methods, if any:

Agent	Dose	Route
euthanasia solution	**(100 mg/kg)**	intravenous injection

If using a chemical method for euthanasia, what secondary physical means (e.g., thoracotomy) will be used to assure euthanasia?

Thoracotomy will be used as a secondary means of euthanasia. More specifically, euthanasia (IV euthanasia solution followed by thoracotomy) will be after sedation with ketamine (using doses already described within the protocol).

B. Physical Methods, if any:

Cervical dislocation (mice, immature rats)*
Decapitation*
Exanguination under anesthesia
For methods that are marked with an *, provide a scientific justification here for the need to use this method (provide references if possible):

C. Name(s) and qualifications of person(s) performing euthanasia:

1.Name*	**1.Qualification**

*procedure to be performed by Brazilian collaborators and their team only if necessary

APPENDIX 1: ANTEMORTEM SPECIMEN COLLECTION

I. BLOOD COLLECTION

A. Will blood be collected?
No. Proceed to section II.
Yes. Complete the following.

Site*	Volume (ml)	% BW	Max. # of collections	Min. Interval
	1-5			

*This will apply captive animals and will be collected according to the specific rules and veterinary care of the collection facility. Neither the PI nor Joanna Malukiewicz will be conducting any blood collection.

B. Will anesthetics, sedatives, or other drugs be used during blood collection?
No. Proceed to section I. C.
Yes. Complete the following.

Drug*	Dose	Route	Purpose

* This will apply captive animals and will be collected according to the specific rules and veterinary care of the collection facility. The PI nor Joanna Malukiewicz will be conducting any blood collection.

C. Describe the methods used to draw the blood including physical restraint, if any.

D. Provide scientific justification for blood collection and justification for the frequency of it.

E. Who will draw the blood?
Name:

Qualifications:

II. OTHER TISSUE/BODY FLUID COLLECTION

A. Will other tissues or body fluids be collected prior to death?
No. Appendix 1 is completed.
Yes. Complete the following. Surgical procedures should be described more fully in Appendix 2.

Tissue/Fluid	Site and Method	Amt	# of collections	Min Interval
Skin hair feces	Skin: Ear Lobe; Hair: Tail Tip; Fecal: if animal voids	3 mm biopsy skin/ 40-50 hairs/ animal; what every is available for feces	1/ animal	N/A

B. Will anesthetics, sedatives, or other drugs be used during tissue/body fluid collection?
No. Proceed to section II. C.
Yes. Complete the following.

Drug	Dose	Route	Purpose
Ketamine	10-20 mg/kg	Intramuscular injection	Immobilization

C. Describe the methods used to collect the samples, including physical restraint, if any.

40-50 hairs with root will be plucked with flame-sterilized forceps from the animal's tail; Using a sterilize skin biopsy punch, a 3mm diameter biopsy will be taken from the ear of the immobilized animal. Fecal materials will be collected manually from area where animal voids. During collection in the Bahia/Pernambuco hybrid zone, animals will be placed into a restraint board.

D. Provide scientific justification for the sample collection(s) and justification for the frequency of it

Collected biological samples will be used for DNA extraction. Skin and hair were found to tissues with low levels of chimerism in *Callithrix kuhli (*Ross et al., 2007). Lower levels of chimerism allows for easier genotyping of individuals at genetic markers that will be used in this study. Preliminary genetic data generated by the graduate student also found that genotyping of DNA from skin is easier than genotyping DNA from blood from the same individual. Blood tends to be more chimeric than epithelial tissue based on the findings of Ross et al. (2007), and the graduate student's findings generally supported this notion. Skin and hair tissues will be collected following the methods of Faulkes et al. (2003) and Di Fiore and Gagneux (2003).

References:

Di Fiore, A., & Gagneux, P. (2007). Molecular primatology. In C. J. Campbell, A. Fuentes, K. C. MacKinnon, M. Panger & S. K. Bearder (Eds.), *Primates in perspective* (pp. 369-393). New York: Oxford University Press.

Faulkes, C. G., Arruda, M. F., & Da Cruz, A. O. M. (2003). Matrilineal genetic structure within and among populations of the cooperatively breeding common marmoset, *Callithrix jacchus. Molecular Ecology, 12*(4), 1101-1108.

Ross, C. N., French, J. A., & Orti, G. (2007). Germ-line chimerism and paternal care in marmosets (Callithrix kuhlii). *Proceedings of the National Academy of Sciences of the United States of America, 104*(15), 6278-6282.

E. Provide name and qualifications of individual who will collect tissues or body fluids.
Name: Joanna Malukiewicz

Qualifications: BS, Evolutionary Anthropology; MA, Biological Anthropology; PhD Candidate, Biology
1-month experience conducting all day follows and recording of behavioral and nutritional data on *Saguinus midas.*
1 day experience collecting hair and 2 mm samples from one time collection of skin samples from cadavers of *Leontopithecus rosalia, Callimico goeldii,* and *Saguinus midas*
Additional training to be provided by the Brazilian veterinarians involved with the Marmoset Hybridization study.

APPENDIX 2: SURGICAL PROCEDURES

I. GENERAL INFORMATION

A. Species

B. Surgical Procedure(s)

C. Room/location of surgery

D. Name(s) and qualifications of person(s) performing surgery

II. SURGICAL PROCEDURE:

Survival Nonsurvival

A. Describe and justify scientifically each surgical procedure (e.g., approach, tissue manipulation, closure):

B. Will pre-anesthetic drugs be used?
No. Proceed to section II. C.
Yes. Complete the following.

Drug & concentration (e.g., mg/ml)	Dose (e.g., mg/kg)	Route	Purpose

C. Anesthetic regimen:

Drug and concentration	Dose	Route

Describe measures used to indicate surgical plane of anesthesia to keep animals from getting both too light and/or too deep:

D. Additional pharmacological agents used during surgery (include analgesics, supportive medications, and research drugs):

Drug and concentration	Dose	Route	Frequency	Purpose

E. Who will administer the drugs?

E. Describe the steps taken to maintain an aseptic surgery:

F. What is the maximum duration of surgery?

G. Will any animals recover from surgery?
No. This involves terminal, or non-survival, procedures; Appendix 2 is complete.
Yes. Complete Section III.

III. POST-SURGICAL CARE

A. Is post-operative pain or distress anticipated?
No. Proceed to section C.
Yes.

B. Will analgesics be used?
(For analgesic use in rodents see The Guidelines for Rodent Analgesia:
http://researchadmin.asu.edu/compliance/iacuc/sop/rodentanalgesia.htm)
No. Provide a scientific justification:

Yes. Complete the following.

Drug	Dose	Frequency	Route

Who will administer these drugs?

C. Post-operative routine care:

What drugs will be administered, if any (e.g., antibiotics, fluids)?

Drug and concentration	Dose	Route	Frequency	Purpose

What other post-operative support and monitoring will be provided, how often, for how long, and by whom?

D. Is post-operative intensive care required?
No. Proceed to section E.
Yes.
What special care is required?

Who will provide special care and what are their qualifications?

For how long will special care be needed?

E. Will animals undergo multiple survival surgical procedures?
No. Appendix 2 is complete.
Yes. Describe which surgeries, the sequence (timeline), and frequency. Provide scientific justification:

Institutional Animal Care and Use Committee (IACUC)
Office of Research Integrity and Assurance
Arizona State University
660 South Mill Avenue, Suite 315
Tempe, Arizona 85287-6111
Phone: (480) 965-4387 *FAX:* (480) 965-7772

Animal Protocol Review

ASU Protocol Number: **11-1150R**
Protocol Title: **Phylogenetics and Population History of the Callitrichidae**
Principal Investigator: **Anne Stone**
Date of Action: **03/11/2011**

The animal protocol review was considered by the Committee and the following decisions were made:

☐ The original protocol was APPROVED as presented.
☐ The revised protocol was APPROVED as presented.
☐ The protocol was APPROVED with RESTRICTIONS or CHANGES as noted below. The project can only be pursued, subject to your acceptance of these restriction or changes. If you are not agreeable, contact the IACUC Chairperson immediately.
☐ The Committee requests CLARIFICATIONS or CHANGES in the protocol as described in the attached memorandum. The protocol will be considered when these issues are clarified and the revised protocol is submitted.
☐ The protocol was approved, subject to the approval of a WAIVER of provisions of NIH policy as noted below. Waivers require written approval from the granting agencies.
☐ The protocol was DISAPPROVED for reasons outlined in the attached memorandum.
☐ The Committee requests you to contact ____________________ to discuss this proposal.
☐ A copy of this correspondence has been sent to the Vice President for Research.
☒ Amendment was approved by Designated Review to add an additional procedure to the protocol.

RESTRICTIONS, CHANGES OR WAIVER REQUIREMENTS:
Total # of Animals: 325 **Pain Level: D** **Species:** **Marmosets**
Approval Period: 11/22/2010 – 11/21/2013

Signature: ______________________________ Date: 3/11/11
IACUC Chair or Designee

Original: Principal Investigator
Cc: IACUC Office
IACUC Chair

Date: 3-7-11

ARIZONA STATE UNIVERSITY

IACUC REQUEST FOR AMENDMENT

Protocol No. 11-1150R
Title: Phylogenetics and Population History of the Callitrichidae
Principal Investigator: Anne C. Stone

Requested Change (check all that apply):

New procedures to be performed – complete Part A and sign assurance.
New species and or an increase in the number of animals to be used – complete Part A and sign assurance.
X New location of housing or procedures – complete Part A and sign assurance.
New personnel – complete Part B and sign assurance.
Other – complete Part A and sign assurance.

A. Description of Requested Changes

Describe the changes you are requesting. If you are adding a procedure that could create pain or distress, you need to include a literature search for alternatives. If you are requesting an increase in animal numbers, provide justification with supportive statistics):

Collection of Cheek Swab Samples from Wild Caught Common and Black Tufted Marmosets and their Hybrids

Pain Category – same as in the original protocol.

We are requesting the addition of this method to our current protocol to increase the amount of DNA we will be able to sample from animals captured in the wild. The procedure will be implemented while animals are immobilized with ketamine (as described in the original protocol). A sterile Whatman cheek swab will be carefully and gently scraped on the inside of the cheek of each animal for 60 seconds. Afterward, the cheek swab will be placed in a test tube containing lysis buffer. The lysis buffer is made up of 50 mM Tris, pH 8.0, 50 mM EDTA, 50 mM sucrose, 100 mM NaCl, and 1% SDS. The lysis buffer will preserve DNA samples at room temperature until the samples are brought into the genetics laboratory of Dr. Adriana Grativol at Universidade Estadual Fluminense do Norte Darcy Ribeiro at Campos dos Goytacazes, Brazil. In the laboratory, DNA will be extracted from collected cheek swab samples using a phenol:chloroform procedure.

B. Addition of Personnel

Name	Title	Role in Protocol (What procedures will each person be doing?)	Species with which individual will have direct contact ("all" or list species)*	IACUC USE ONLY Training (mm/yy)

For each new individual, describe the individual's training and years experience with all listed species and procedures:

_____

Assurance

As Principal Investigator of this protocol, I assure that all procedures will be conducted as described in this amendment and that personnel will receive appropriate additional training prior to conducting any new procedures that are not listed above.

SIGNED: Anne C. Stone

Principal Investigator

3/7/11

Date

Chantelle Miller

From: Dale DeNardo
Sent: Wednesday, March 09, 2011 9:09 AM
To: Debra Murphy; Chantelle Miller
Subject: FW: Amendment request
Attachments: Stone-1150R Amendment-JT-030911V.doc

Please put this thru the DR process with me as the DR. Assuming the process gets approved, I apoprove the amendment request as written.

Dale

****
Dale DeNardo, DVM, PhD
Associate Professor
School of Life Sciences
Chair, Institutional Animal Care and Use Committee
Arizona State University
Tempe, AZ 85287-4501
tel: 480-965-3325
fax: 480-965-0698
denardo@asu.edu
****

From: Joanne Tetens
Sent: Wednesday, March 09, 2011 8:55 AM
To: Debra Murphy; Chantelle Miller
Cc: Dale DeNardo; Anne Stone; Joanna Malukiewicz; Juan Jordan
Subject: Amendment request

Dear Deb and Chantelle

Please find attached Dr. Stone's amendment request which has completed veterinary pre-review.

Sincerely
Joanne

Debra Murphy

From: Debra Murphy
Sent: Wednesday, March 09, 2011 9:41 AM
To: Dale DeNardo; Christopher Buneo; Carol Johnston; Karen Kibler; Stephen Massia; Federico Sanabria; Joanne Tetens; Brent Vernon; Yung Chang; Dianna Hubbard
Cc: Chantelle Miller
Subject: Action Required - Designated Reveiw for Stone 11-1150R
Attachments: Amendment Stone-1150R Amendment-JT-030911V.doc

Tracking:

Recipient	**Response**
Dale DeNardo ✓	Y-email
Christopher Buneo	Y-email
Carol Johnston ✓	Yes: 3/9/2011 10:37 AM
Karen Kibler ✓	Y-ema
Stephen Massia ✓	Y email
Federico Sanabria ✓	Y-email
Joanne Tetens ✓	Yes: 3/9/2011 9:46 AM
Brent Vernon	
Yung Chang	
Dianna Hubbard	Yes: 3/9/2011 12:06 PM
Chantelle Miller	
Tol Cheshko	3/11 by phone

PI: Anne Stone
Protocol: 11-1150R
Title: Phylogenetics and Population History of the Callitrichidae
Designated Reviewer: Dale DeNardo

A request for an amendment to the referenced protocols has been submitted. The request for amendment is attached and the various related protocols are available at the SharePoint site. The Designated Review process allows IACUC members, by voting, to permit the Designated Reviewer to approve or disapprove an amendment request.

Select "YES" if you approve the use of the designated review process for this amendment.

Select "NO" if you disapprove the use of designated review process regarding this amendment request. The amendment will then be reviewed at the next monthly IACUC meeting.

Please indicate your approval or disapproval of the request for designated review by using the YES or NO button in the toolbar at the top of this message. The use of "YES or NO" buttons allows you to submit comments along with your choice.

You may also send your comments to me directly or to the primary reviewer without using the selection buttons. Please copy me on all correspondence and email related to this request.

Thank you,

Please think about the environment before you print this email.

Debra Murphy, CRA
Director, Office of Research Integrity and Assurance
480-965-2179
480-965-7772 (fax)
http://Researchintegrity.asu.edu