Mitogenomic Phylogeny of *Callithrix* with Special Focus on Human Transferred Taxa

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Abstract

Background
Callithrix marmosets are a relatively young non-human primate radiation, whose phylogeny is not yet full resolved. These primates are naturally para- and allopatric, but three species with highly invasive potential have been introduced into the southeastern Brazilian Atlantic Forest by the pet trade. There, these species hybridize with each other and endangered, native congeners. We aimed in this study to reconstruct a robust *Callithrix* phylogeny and divergence time estimates, as well as identify autochthonous and allochthonous *Callithrix* mitogenome lineages across Brazil. We sequenced 49 mitogenomes from four species (*C. aurita*, *C. geoffroyi*, *C. jacchus*, *C. penicillata*) and anthropogenic hybrids (*C. aurita* x *Callithrix* sp., *C. penicillata* x *C. jacchus*, *Callithrix* sp. x *Callithrix* sp., *C. penicillata* x *C. geoffroyi*) via Sanger and whole genome sequencing. We combined these data with previously published *Callithrix* mtDNA genomes to analyze five *Callithrix* species in total.

Results
We report the complete sequence and organization of the *C. aurita* mtDNA genome. Phylogenetic analyses showed that *C. aurita* was the first to diverge within *Callithrix* 3.54 million years ago (MYA), while *C. jacchus* and *C. penicillata* lineages diverged most recently 0.5 MYA as sister clades. MtDNA clades of *C. aurita, C. geoffroyi,* and *C. penicillata* show intraspecific geographic structure, but *C. penicillata* clades appear polyphyletic. Hybrids, which were identified by phenotype, possessed mainly *C. penicillata* or *C. jacchus* mtDNA haplotypes. The geographic origins of mtDNA haplotypes from hybrid and allochthonous *Callithrix* were broadly distributed across natural *Callithrix* ranges. Our phylogenetic results also evidence introgression of *C. jacchus* mtDNA into *C. aurita.*

Conclusion
Our robust *Callithrix* mitogenome phylogeny shows *C. aurita* lineages as basal and *C. jacchus* lineages among the most recent within *Callithrix*. We provide the first evidence that parental mtDNA lineages of anthropogenic hybrid and allochtonous marmosets are broadly distributed inside and outside of the Atlantic Forest. We also show evidence of cryptic hybridization between allochthonous *Callithrix* and autochthonous *C. aurita*. Our results encouragingly show that further development of genomic resources will allow to more clearly elucidate *Callithrix* evolutionary relationships and understand the dynamics of *Callithrix* anthropogenic introductions into the Brazilian Atlantic Forest.

Keywords
mtDNA, marmoset, divergence, Atlantic Forest, Brazil, Callitrichidae, primate

Background

Callithrix species represent a relatively young radiation, and divergence among lineages within the genus is estimated to be between approximately 0.7 and 2.5 million years ago (MYA) [43, 20, 32]. Two major subgroups occur within the genus, the *aurita* group (*C. aurita*/*C. flaviceps*) and the *jacchus* group (*C. geoffroyi*/*C. kuhlii*/*C. jacchus*/*C. penicillata*), but the phylogeny of these lineages is not yet fully resolved. *Callithrix* species are naturally para- and allopatric across the Brazilian Atlantic Forest, Cerrado, and Caatinga biomes (Figure 1) [52, 4], and natural hybridization occurs between some species [33]. However, *C. geoffroyi, C. jacchus,* and *C. penicillata* have high invasive potential [37,64] and have spread widely outside of their native ranges due to the legal and illegal pet trades. These species have established several allochthonous populations in the southeastern Brazilian Atlantic Forest [8, 56, 33] and hybridize with other allochthonous and autochthonous congeners [7,8,56,33], including endangered *C. aurita* and *C. flaviceps* [53,35]. Yet, determining evolutionary relationships between autochthonous, allochthonous, and hybrid *Callithrix* populations across Brazil is complicated by the unresolved *Callithrix* phylogeny.

In general, mitochondrial DNA (mtDNA) can be utilized for an initial look into evolutionary relationships among taxa [e.g., 66, 67] as well as track dispersal and gene flow patterns of allochthonous species [11]. MtDNA sequence data can also provide initial genetic insight in the direction of introgression (if sex-biased) when two species hybridize due to incongruences between phenotypes and haplotypes [e.g., 66, 67]. This genetic marker mutates relatively fast [3], with an effective population size that is one quarter of that of nuclear DNA in a diploid, bisexual population, which gives the former relatively shorter coalescent times [9]. As a result, lineage sorting and reciprocal monophyly are expected to occur faster in mtDNA than nuclear DNA, which can provide insight into shallow evolutionary relationships expected for young radiations.

One major challenge in applying genetic and genomic methods in *Callithrix* studies is an overall lack of genomic resources and sample material for most *Callithrix* species. Studies of *Callithrix* species have utilized mtDNA markers that generally resulted in polytomies and/or poorly supported branching patterns, as well as polyphyly for *C. penicillata* and *C. kuhlii* [59, 60, 38, 57, 8]. Also, the few available genetic studies of allochthonous and hybrid *Callithrix* within the Atlantic Forest, all conducted within Rio de Janeiro state, used portions of mtDNA or the Y-chromosome that could not fully resolve the evolutionary relationships of *Callithrix* lineages [e.g., 30, 8]. Nonetheless, Malukiewicz et al. [32] obtained a well-resolved phylogeny for the *jacchus* group using complete mitochondrial genomes, but they only sampled one individual/species with unknown provenances.

To build upon the above previous *Callithrix* studies, we have conducted the largest to-date geographical sampling of *Callithrix* mtDNA genomes across Brazil (Figure 1) with the following aims: (1) improve resolution of phylogenetic relationships and divergence times estimates between *Callithrix* mtDNA haplotypes; (2) determine which *Callithrix* mtDNA lineages are autochthonous across *Callithrix* ranges; and (3) identify allochthonous *Callithrix* mtDNA lineages in the southeastern Atlantic Forest and their possible origins. We provide for the first time the complete mtDNA sequence of *C. aurita,* and included 49 newly obtained mitochondrial genome sequences from four species (*C. aurita*, *C. geoffroyi*, *C. jacchus*, *C. penicillata*), and four hybrid types (*C. aurita* x *Callithrix* sp., *C. penicillata* x *C.jacchus*, *Callithrix* sp. x *Callithrix* sp., *C. penicillata* x *C. geoffroyi*) in our analysis.

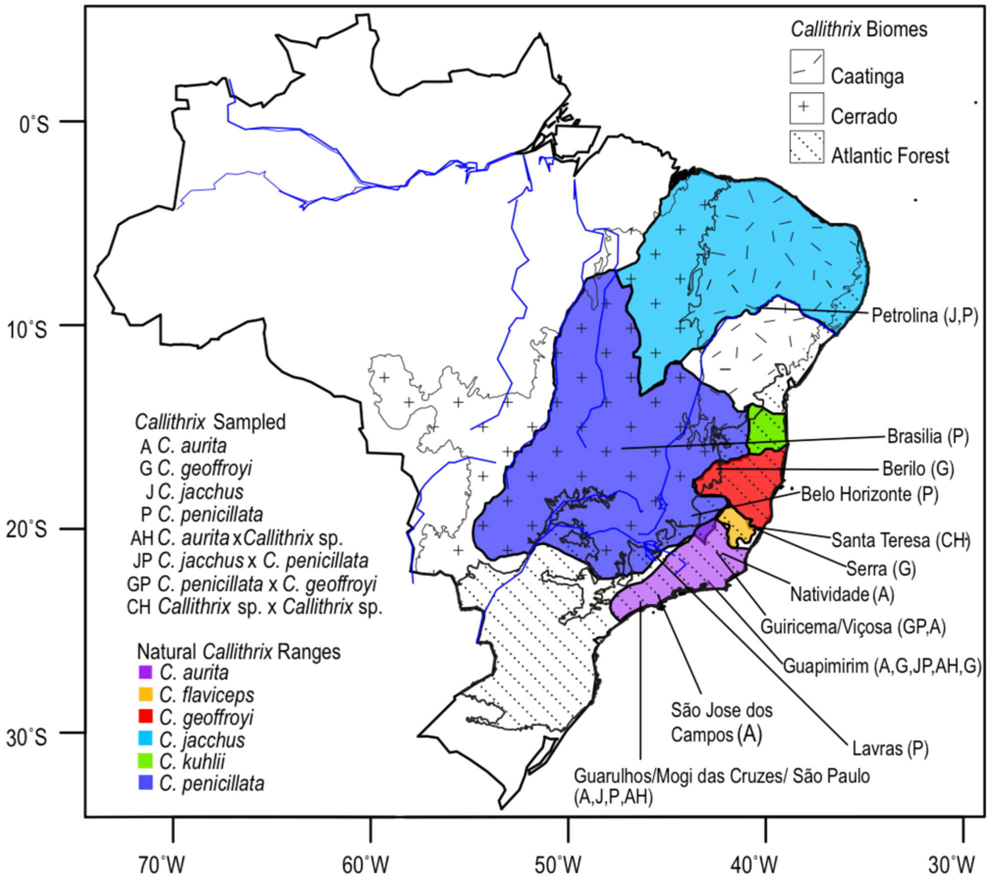
Callithrix Biomes
Caatinga
Cerrado
Atlantic Forest
0˚S
10˚S
20˚S
30˚S
70˚W
60˚W
50˚W
40˚W
30˚W
Callithrix Sampled
A C. aurita
G C. geoffroyi
J C. jacchus
P C. penicillata
AH C. aurita x Callithrix sp.
JP C. jacchus x C. penicillata
GP C. penicillata x C. geoffroyi
CH Callithrix sp. x Callithrix sp.
Natural Callithrix Ranges
C. aurita
C. flaviceps
C. geoffroyi
C. jacchus
C. kuhlii
C. penicillata
Petrolina (J,P)
Brasilia (P)
Berilo (G)
Belo Horizonte (P)
Santa Teresa (CH)
Serra (G)
Natividade (A)
Guiricema/Viçosa (GP,A)
Guapimirim (A,G,JP,AH,G)
São Jose dos Campos (A)
Lavras (P)
Guarulhos/Mogi das Cruzes/ São Paulo (A,J,P,AH)

Figure 1. Approximate distribution of *Callithrix* species in Brazil (2012 IUCN Red List Spatial Data; http://www.iucnredlist.org/technical-documents/spatial-data) and geographic origins of Brazilian samples, as indicated by capital letter symbols. Locations of three biomes where *Callithrix* occur naturally*,* the Caatinga, Cerrado, and Atlantic Forest, are also indicated.

Table 1. Number of *Callithrix* specimens newly sampled by species and hybrid phenotype. Provenance abbreviations are CRC: Callitrichid Research Center, NEPRC: New England Primate Research Center (no longer in operation), CPRJ: Centro de Primatologia do Rio de Janeiro, and CEMAFAUNA: Centro de Concervação e Manejo de Fauna da Caatinga.

Phenotype	Provenance	N
C. aurita	Guiricema, Minas Gerais, Brazil	2
C. aurita	Guarulhos Municipal Zoo, Guarulhos, São Paulo, Brazil (wild marmoset originally from Mogi das Cruzes, São Paulo)	1
C. aurita	Guarulhos Municipal Zoo, Guarulhos, São Paulo, Brazil	2
C. aurita	Guarulhos Municipal Zoo, Guarulhos, São Paulo, Brazil (wild marmoset originally from São José dos Campos, São Paulo)	1
C. aurita	CPRJ, Guapimirim, Rio de Janeiro, Brazil (wild marmosets originally from Natividade, Rio de Janeiro)	2
C. aurita	CPRJ, Guapimirim, Rio de Janeiro, Brazil	1
C. geoffroyi	Callitrichid Research Center, Omaha, Nebraska, US	1
C. geoffroyi	CPRJ, Guapimirim, Rio de Janeiro, Brazil	1
C. geoffroyi	Berilo, Minas Gerais, Brazil	1
C. geoffroyi	Santo Serra, Espírito Santo, Brazil	3
C. jacchus	Guarulhos Municipal Zoo, Guarulhos, São Paulo, Brazil	2
C. jacchus	NEPRC, Southborough, Massachusetts, US	1
C. jacchus	CEMAFAUNA, Petrolina, Pernambuco, Brazil	4
C. jacchus	CPRJ, Guapimirim, Rio de Janeiro, Brazil	1
C. penicillata	Brasília, Federal District, Brazil	2
C. penicillata	Lavras, Minas Gerais, Brazil	2
C. penicillata	Belo Horizonte, Minas Gerais, Brazil	2
C. penicillata	CEMAFAUNA, Petrolina, Pernambuco, Brazil	2
C. jacchus x *C. penicillata*	CPRJ, Guapimirim, Rio de Janeiro, Brazil	1
C. penicillata x C. geoffroyi	Viçosa, Minas Gerais, Brazil	11
C. aurita x *Callithrix sp.*	Guarulhos Municipal Zoo, Guarulhos, São Paulo, Brazil	3
C. aurita x *Callithrix sp.*	Guarulhos Municipal Zoo, Guarulhos, São Paulo, Brazil (wild marmoset originally from Maripora, São Paulo)	1
C. aurita x *Callithrix sp.*	CPRJ, Guapimirim, Rio de Janeiro, Brazil	1
C. geoffroyi x *Callithrix sp.*	Santa Teresa, Espírito Santo, Brazil	1
Total		49

Results

Using Illumina WGS and Sanger sequencing approaches, we sequenced complete mtDNA genomes from 49 *Callithrix* (Figure 1, Table 1, and Table S1). We also utilized previously published primate mitochondrial genome sequences (listed in Table S1). The *C. aurita*-only alignment consisted of 16,503 bases, while the full *Callithrix* alignment was 16,556 bases long. The organization of the *C. aurita* mtDNA genome was consistent with previously published *Callithrix* mitochondrial genomes (Malukiewicz et al., 2017), and includes 12 protein-coding genes, both rRNAs, and 14 tRNAs on the heavy strand and one protein-coding gene and eight tRNAs on the light strand, as well as the control region (Table S2). Sampled individuals that possessed the same mtDNA haplotypes are listed in Table S3.

Phylogenetic Trees and Divergence Times among Taxa

ML and Bayesian inference produced well-supported phylogenetic trees that show mostly congruent phylogenetic relationships between the *aurita* and *jacchus* groups (Figure 2, Figures S1-S3). A number of nodes in the ML tree possessed 100% bootstrap support but most had bootstrap scores of >70% (Figure S1). Most nodes in the Bayesian trees had posterior probabilities of 1 (Figure 2, Figures S2-S3). Major node names and divergence times within and outside the *Callithrix* clade are shown in Figure 2, Figure S3, Table 2, and Table S3. *Callithrix* diverged from *Cebuella* approximately 6.83 million years ago (MYA) (node E) and the initial split within *Callithrix*, separating *C. aurita* and the *jacchus* group, occurred approximately 3.54 MYA (node D) (Table 2). Thus, *C. aurita* formed the *Callithrix*

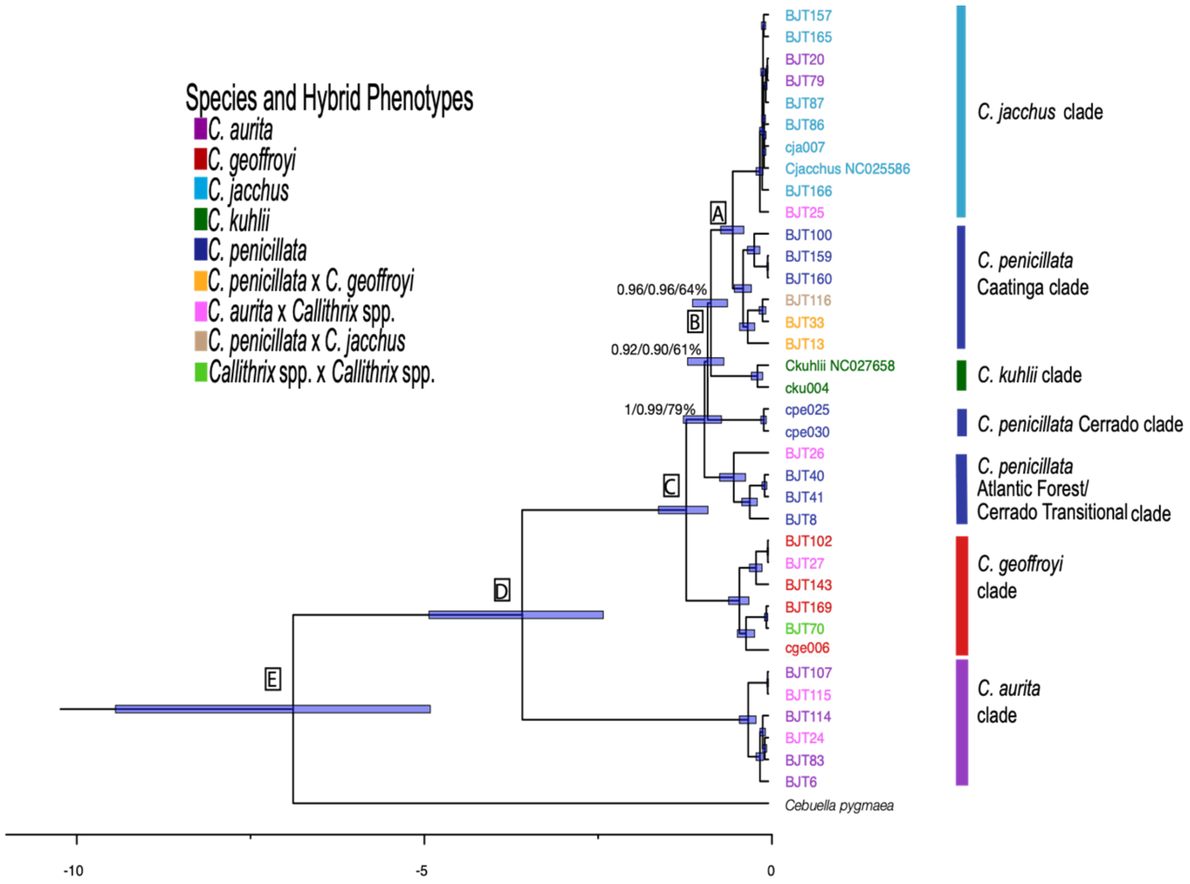
Species and Hybrid Phenotypes
C. aurita
C. geoffroyi
C. jacchus
C. kuhlii
C. penicillata
C. penicillata x C. geoffroyi
C. aurita x Callithrix spp.
C. penicillata x C. jacchus
Callithrix spp. x Callithrix spp.
BJT157
BJT165
BJT20
BJT79
BJT87
BJT86
cja007
Cjacchus NC025586
BJT166
BJT25
BJT100
BJT159
BJT160
BJT116
BJT33
BJT13
Ckuhlii NC027658
cku004
cpe025
cpe030
BJT26
BJT40
BJT41
BJT8
BJT102
BJT27
BJT143
BJT169
BJT70
cge006
BJT107
BJT115
BJT114
BJT24
BJT83
BJT6
Cebuella pygmaea
A
B
C
D
E
0.96/0.96/64%
0.92/0.90/61%
1/0.99/79%
C. jacchus clade
C. penicillata Caatinga clade
C. kuhlii clade
C. penicillata Cerrado clade
C. penicillata Atlantic Forest/ Cerrado Transitional clade
C. geoffroyi clade
C. aurita clade
-10
-5
0

Figure 2. Phylogenetic relationships and divergence ages in million years (Ma) among *Callithrix* haplotypes as calculated from complete mtDNA genomes (complete tree with outgroups is presented in Figure S3). Major nodes are identified by capital letters, and blue bars at nodes indicate 95% highest posterior densities (HPD) of divergence times. Node support is shown for major nodes where either posterior probability was < 1 in the BEAST tree, posterior probability was < 1 in the MRBAYES tree, or bootstrap support < 70% in the ML tree. Haplotype colors at tips correspond to the ‘Species and Hybrid Phenotypes’ legend, and indicate phenotypes associated with each given haplotype.

basal clade, and *C. geoffroyi* formed the most basal clade within the *jacchus* group by arising 1.18 MYA (node C). The next *Callithrix* clade to diverge within the *jacchus* group was the *C. penicillata* Atlantic Forest-Cerrado transition clade at 0.92 MYA. Afterward, the *C. penicillata* Cerrado clade appeared at 0.87 MYA, followed by the *C. kuhlii* clade at 0.82 MYA (node B). The *C. penicillata* Caatinga clade and the *C. jacchus* clades represent the two youngest clades within the phylogeny, splitting about 0.51 MYA (node A).

Within the *C. aurita* clade, haplotypes fall into two main groups with one including *C. aurita*-phenotype individuals sampled in the state of Rio de Janeiro and one haplotype from a *C. aurita* x *Callithrix* sp. hybrid. The other *C. aurita* subgroup contained a mix of haplotypes from *C. aurita*-phenotype individuals with known origins within Minas Gerais and São Paulo states, and it also included a haplotype from a *C. aurita* x *Callithrix* sp. hybrid. The two subgroups found within the *C. geoffroyi* clade may represent geographic clusters, with one cluster containing haplotypes of *C. geoffroyi*-phenotype individuals from the state of Espírito Santo, and the other cluster containing haplotypes from *C. geoffroyi*-phenotype individuals from the state of Minas Gerais. Two haplotypes from hybrids, that of a *C. aurita* x *Callithrix* sp. hybrid and a *Callithrix* sp. x *Callithrix* sp. hybrid from Santa Teresa, also grouped within the *C. geoffroyi* clade.

The most complex clustering was found for *C. penicillata,* as haplotypes grouped into three polyphyletic clades that clustered into groups corresponding to three different biome regions, an Atlantic Forest-Cerrado transition area, Cerrado, and Caatinga. The first of these clades, the *C. penicillata* Atlantic Forest-Cerrado transition clade, contained several haplotypes from *C. penicillata*-phenotype individuals that originate from the Atlantic Forest-Cerrado transition zone of Minas Gerais as well as a hybrid sampled in São Paulo state. The *C. penicillata* Cerrado clade includes haplotypes from *C. penicillata*-phenotype individuals sampled in Brasília. Then two *C. kuhlii* haplotypes grouped together, and this clade diverged after the *C. penicillata* Cerrado transition clade. The most recent clade for *C. penicillata,* the Caatinga clade, includes haplotypes from *C. jacchus*-phenotype animals sampled in Petrolina. The two haplotypes, representing eleven *C. penicillata* x *C. geoffroyi* hybrids sampled in Viçosa as well as a *C. jacchus* x *C. penicillata* hybrid, clustered within the *C. penicillata* Caatinga group.

The *C. jacchus* clade showed some of the shallowest branch tips among *Callithrix* haplotypes, and there was not enough phylogenetic resolution within the *C. jacchus* clade to discern any clear haplotype clustering. However, a ParsimonySplits network of the *C. jacchus* clade (Figure 2) showed one cluster of haplotypes from marmosets with *C. jacchus* phenotypes sampled at CEMAFAUNA and the Guarulhos Zoo, as well as three *C. aurita* phenotype individuals sampled within São Paulo. Additionally, a haplotype from a *C. aurita* x *Callithrix* sp. hybrid sampled in São Paulo state as well as a haplotype from a CEMAFAUNA *C. jacchus*-phenotype individual also grouped themselves close to this larger cluster.

Discussion

Callithrix *Mitochondrial Phylogenetic Relationships and Divergence Times*

Our ML and Bayesian phylogenies were generally well supported and corroborated *Callithrix* divergence patterns from previous nuclear and mtDNA studies [43, 58, 32,17]. These previous phylogenies were generally based on one sample per *Callithrix* species, while we utilized multiple haplotypes per species. In ours and these previous phylogenies, the *C. aurita* clade was the most basal within the genus, the *C. geoffroyi* clade was most basal within the *jacchus* group, and *C. penicillata* and *C. jacchus* were the most recently diverged sister

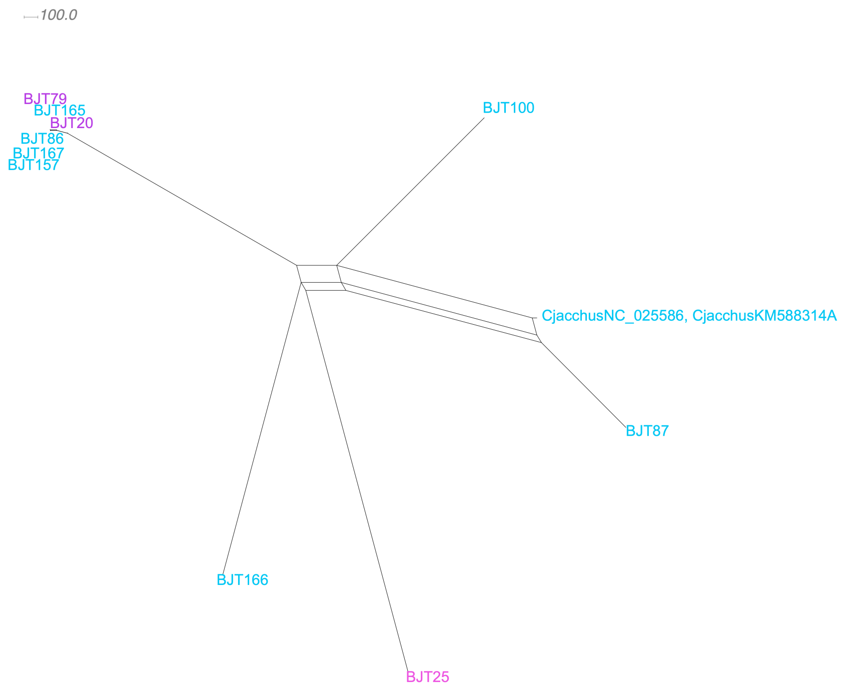
100.0
BJT79
BJT165
BJT20
BJT86
BJT167
BJT157
BJT100
CjacchusNC_025586, CjacchusKM588314A
BJT87
BJT166
BJT25

Figure 2. ParimonySplits network of haplotypes in from phylogenetic *C. jacchus* clade. Haplotype colors at tips follow Figure 1 ‘Species and Hybrid Phenotypes’ legend, and indicate phenotypes associated with each given haplotype.

Table 2. Divergence times in million years (Ma) for *Callithrix* species and select nodes (MRCA = Most recent common ancestor; values in brackets = 95% highest posterior density). Node names follow major node destinations show in Figure 2 in capital letters.

Node	Taxa Diverging at Node	Age (Ma)
A	*C. jacchus* - *C. penicillata* (Caatinga clade)	0.51 [0.35-0.69]
B	*C. kuhlii* - *C. jacchus* + *C. penicillata* (Caatinga clade)	0.82 [0.59-1.09]
C	*C. geoffroyi* - *C. kuhlii* + *C. jacchus* + *C. penicillata*	1.18 [0.87-1.58]
D	*C. aurita* - *C. geoffroyi* + *C. kuhlii* + *C. jacchus* + *C. penicillata*	3.54 [2.37-4.88]
E	*Callithrix* - *Cebuella*/*Mico*	6.83 [4.86-9.39]

clade. Further, our mtDNA clades reflected biogeographic patterns of recognized *Callithrix* species [e.g., 4]. Finally, our mtDNA analysis also showed that *C. penicillata* is polyphyletic, similar to the study by Tagliaro et al. [60] and Malukiewicz et al. [30]. The latter two studies also showed *C. kuhlii* mtDNA haplotypes as polyphyletic. Given the recent divergence times of *Callithrix* species and due to geographical clustering of mtDNA haplotypes, *Callithrix* polyphyly may be explained by incomplete lineage sorting when ancestral polymorphisms at a given locus are not fixed before population divergence [47].

The *Callithrix* divergence time estimates from our study are within the range of previously published estimates [58,33,17], and these time estimates place the divergence of *Callithrix* species into the Pleistocene. In this epoch, climatic oscillations that may have promoted para- and allopatric speciation in South America through repeated contractions and expansions of forested refuge [28,63]. Biogeographic and phylogenetic analyses support a

Callithrix origin in the southeastern Brazilian Atlantic Forest and then a northward expansion [43, 58, 4, 17, 32].

Geographical Distributions of Autochthonous Callithrix *Mitogenomes Show Multiple Origins of Allochthonous and Anthropogenic Hybrid Marmosets*

When considering sampled mitogenome lineages of known provenance, grouping of our reconstructed phylogenetic clades was strongly influenced by the geographic origin of our samples across natural *Callithrix* ranges. For example, within the *C. geoffroyi* clade, the Minas Gerais state lineage formed a separate clade from the Espírito Santo state lineages. *Callithrix penicillata* possesses the largest natural geographic distribution of all *Callithrix* species [52] and the paraphyletic clades were defined by where samples were collected within the Cerrado, Atlantic Forest, and Caatinga biomes. Although most sampled *C. jacchus* clade haplotypes did not possess known provenance and showed shallow tips, all haplotypes from *C. jacchus* phenotype samples collected at CEMAFAUNA grouped together, possibly forming a Caatinga subclade. Further evidence that *Callithrix jacchus* mtDNA lineages tend to group geographically under denser sampling was shown by Malukiewicz et al. [30] with a geographically broader sampling of *C. jacchus* mtDNA D-loop sequences.

We can harness such data as above to infer the origins of parental populations of anthropogenic hybrid *Callithrix* and allochthonous *Callithrix* species found in the southeastern Brazilian Atlantic Forest. Overall, grouping patterns of our reconstructed mitogenome clusters show that the parental populations of anthropogenic hybrid *Callithrix* and allochthonous *Callithrix* species likely possess multiple geographic origins from within and outside this region. For example, mtDNA haplotypes of three *C. aurita* x *Callithrix* sp. hybrids we sampled in São Paulo state clustered, respectively, with the *C. geoffroyi* haplotype originating form Minas Gerais state, a *C. penicillata* Atlantic Forest-Cerrado Transitional clade haplotype, and a *C. jacchus* clade haplotype recovered from the Caatinga. On the other hand, a haplotype from a *C. aurita* x *Callithrix* sp. sample was the sister lineage to a haplotype from a *C. aurita* phenotype sample, and both samples have a provenance of Guapimirim, Rio de Janeiro state. The three haplotypes from *C. penicillata* x *C. geoffroyi* hybrids we sampled in Minas Gerais state as well as a *C. jacchus* x *C. penicillata* hybrid sampled from Rio de Janeiro state clustered closely together within the *C. penicillata* Caatinga clade. Overall, these patterns suggest that denser sampling and cataloguing of autochthonous mitogenomes and nuclear data within *Callithrix* native ranges will allow for identification source populations of allochthonous *Callithrix* species and anthropogenic hybrids at a much greater resolution.

Implications of Biological Invasions for Callithrix *Genetic Integrity, Hybridization, and Conservation*

Several species of non-native fauna and flora have been introduced to the Brazilian Atlantic Forest [21] - one of the most anthropogenically disturbed, yet highly biodiverse biomes on Earth [45,46]. Deliberate and accidental relocations of species beyond their natural geographic ranges by humans may lead to the establishment of non-autochthonous populations and biological invasions within new geographic localities. Such introductions alter the ecological relationships among taxa, and in cases of closely related species, gene flow may occur due to hybridization [13,42,12].

Allochthonous *Callithrix* species began appearing in portions of the southeastern Brazilian Atlantic Forest within approximately the last 20-30 years [51, 22, pers. obs. C. Igayara]. Contemporary anthropogenic hybrid *Callithrix* and allochthonous *Callithrix* species are normally found in urban or peri-urban areas of southeastern Brazil, due to releases of exotic pet marmosets into such locales [51, 8, 56, 33], where they may encounter autochthonous *Callithrix* species. Indeed, cases exist of native *C. aurita* and *C. flaviceps* meeting up and interbreeding with hybrid and allochthonous *Callithrix* at urban fringes [7,8,14,15,56,33]. Such interactions likely facilitate gene flow from invasive *C. jacchus* and *C. penicillata* into marmoset populations in southeastern Brazil, with consequences that may include outbreeding depression, admixture, hybrid swamping, or introgressive replacement [48, 65,1,34].

Indeed, our analyses show, for the first time, evidence of introgression of genetic material from allochthonous *Callithrix* species into the genetic background of an endangered, autochthonous *Callithrix* species. Two mtDNA haplotypes that grouped within the *C. jacchus* clade were associated with three individuals with pure *C. aurita* phenotypes sampled within São Paulo state. Two of these individuals were sampled two different regions of São Paulo state in municipalities (Mogi Das Cruzes and São Jose dos Campos) that lie 60 km apart. These data not only show the first genetic evidence for cryptic hybridization within the *aurita* group marmosets, but also suggest two independent occurrences of a *C. jacchus* female mating with a *C. aurita* male that led to genetic introgression. Under scenarios of biological invasions, theoretical and empirical data show that hybridization between allochontone species and endangered, native species creates extinction risk for the latter [62]. Our initial mtDNA data strongly prompt for the development of diagnostic genetic markers to detect the actual extent of allochthonous *Callithrix* genetic introgression in *C. aurita* populations, particularly within São Paulo state.

Invasive *C. jacchus* and *C. penicillata* represent a potential risk for genetic extinction of the other two *jacchus* group species, *C. geoffroyi* and *C. kuhlii,* and Silva et al. [56] recently showed that *C. penicillata* is encroaching on the range of *C. geofforyi*. We sampled one *Callithrix* sp. x *Callithrix* sp. hybrid in Santa Teresa, Espírito Santo state, a city that straddles the native ranges of *C. flaviceps* and *C. geoffroyi.* Although this hybrid possessed a *C. geoffroyi* clade mitogenome lineage, the individual had a novel phenotype (see Supplementary Information) that strongly suggested *C. penicillata* or *C. jacchus* ancestry in hybrids- a white "star" on the forehead [22]. Anthropogenic hybridization of *jacchus* group species generally results in the formation of hybrid swarms, admixed populations that lost parental phenotypes and genotypes [22,31,33]. Should large numbers of exotic *C. jacchus* or *C. penicillata* ever invade native ranges of *C. kuhlii* or *C. geoffroyi,* the latter two species may be threatened with genetic swamping by the former two species, a process through which parental lineages are replaced by hybrids that have admixed genetic ancestry [62]. As *C. kuhlii* is considered a vulnerable [40], biological invasions by other marmosets present potential conservation risks for this species.

Conclusions

We provide a robust *Callithrix* phylogeny based on the largest to-date geographical sampling of *Callithrix* mtDNA genomes across Brazil, showing that the *aurita* group is basal to the *jacchus* group. Our divergence time estimates show these two groups diverged approximately 3.54 MYA, and within the *jacchus* group, *C. jacchus* diverged most recently from the *C. penicillata* Caatinga clade approximately 0.51 MYA. With future sampling of *C. flaviceps,* full mtDNA genomes can likely be utilized to fully resolve the *Callithrix* phylogeny. Nonetheless, we used our current well-supported phylogenies to elucidate, for the first time, evolutionary relationships among autochthonous, allochthonous, and anthropogenic hybrid marmosets across Brazil. We show that parental populations of allochthonous and anthropogenic hybrid marmosets within the southeastern Brazilian Atlantic Forest incorporate local populations and populations broadly distributed outside of the regions. We also show, for the first time, evidence of allochthonous *Callithrix* species genetic introgression into the genetic background of endangered, autochthonous *C. aurita*. At this time, further determination is needed of the ancestry of *Callithrix* anthropogenic hybrids in southeastern Brazil as well as the fitness and viability of these hybrids. Such data will help determine to what extent anthropogenic hybrids and allochthonous *Callithrix* species threaten the genetic integrity, or ability of a population to preserve its genotypes over generations [61], of autochthonous Atlantic Forest *Callithrix* species.

Methods

Samples

In 2011, skin samples were collected from two *C. penicillata* individuals that were captured in in Brasília, Federal District. Between 2010 and 2016, skin tissue was collected from: (1) wild marmosets in Minas Gerais and Espírito Santo states as well as the Brazilian Federal District; (2) captive-born, wild-caught, and confiscated marmosets housed at the Guarulhos Municipal Zoo, Guarulhos, São Paulo, CEMAFAUNA (Centro de Manejo de Fauna da Caatinga), Petrolina, Pernambuco, and Centro do Primatologia do Rio de Janeiro (CPRJ), Guapimirim, Rio de Janeiro; (3) a wild group from Natividade, Rio de Janeiro that was caught and housed at CPRJ; (4) a captive-born *C. geoffroyi* sample donated by the Callitrichid Research Center (CRC), Omaha, Nebraska; (5) a captive born *C. jacchus* donated by the New England Primate Research Center (NEPRC, now closed), Southborough, Massachusetts, US. Sampling information and locations are given in Table 1, Table S1, and Figure 1. Table S1 also lists information on utilized sequences that were published elsewhere. Marmoset capture and sampling methodology has been described elsewhere [30]. Specimens were classified phenotypically as pure *C. aurita*, *C. geoffroyi*, *C. jacchus* and *C. penicillata* or hybrid (*C. aurita* x *Callithrix* sp., *C. jacchus* x *C. penicillata*, and *C. penicillata* x *C. geoffroyi*) based on published descriptions [24,22,30,8,33]. A summary of key phenotypic characteristics of the above species and their hybrids is included in the Supplementary Material.

Laboratory Protocols

DNA from skin samples was extracted using a standard proteinase K/phenol/chloroform protocol [55]. Buffers used for extraction, precipitation and elution of DNA from blood and skin tissue are listed elsewhere [30]. DNA from the Callitrichid Research Center samples was extracted at Arizona State University (ASU). DNA from Brasília individuals was extracted at Northern State Fluminense University, Rio de Janeiro State, Brazil, and then exported to ASU (CITES permit #11BR007015/DF). DNA from all other individuals was extracted at the Federal University of Viçosa (UFV), Viçosa, Minas Gerais, Brazil.

Mitochondrial genomes were obtained for a subset of the samples (Table S1) following the long-range PCR (LR-PCR) methodology of Malukiewicz et al. (2017), and sequenced on an ABI 3730 sequencer with the BigDye Cycle Sequencing Kit (Applied Biosystems) by the ASU School of Life Science DNA Core Laboratory. The remainder of mitochondrial genomes was obtained from whole genome sequencing (WGS). Individual WGS sequencing libraries were prepared at UFV and ASU with Illumina Nextera DNA Flex Library Prep Kits (catalog #20018704) following manufacturer's instructions. Individual libraries were barcoded with Illumina Nextera DNA CD Indexes (catalog # 20018707), and pooled in equimolar amounts and sequenced on an Illumina NextSeq using v2 chemistry for 2 x 150 cycles at the ASU Genomic Core Facilities.

Mitochondrial Genome Alignment and Data Analysis

Genetic samples collected since 2015 have been registered in the Brazilian CGen SISGEN database (Supplementary Table S4) and newly sequenced mitochondrial genomes have been deposited in Genbank (Table S1). Trace files of resulting forward and reverse reads from LR-PCR products for each individual sequence were inspected by eye and merged into a single contig for each sampled individual using SEQMAN PRO software from the DNAStar Lasergene Core 10 suite (DNASTAR, Madison, WI). Mitochondrial genomes from WGS data were assembled with NOVOPlasty 2.6.4 [16] (scripts available at https://github.com/Callithrix-omics/Callithrix_mtDNA.git). We downloaded mitochondrial genome sequences of several primate species from Genbank (Supporting Information Table S1). All mitochondrial genomes were aligned in MAAFT (https://www.ebi.ac.uk/Tools/msa/mafft/) with default settings. The alignment was manually adjusted in Mesquite 3.5 [29], and the newly generated marmoset mitochondrial genomes were manually annotated based on the Genbank record of *C. kuhlii* (Accession number KR817257). To check for the presence of nuclear copies of mitochondrial genes (numts) in mitochondrial sequence data, we followed the strategy described in Malukiewicz et al. [32].

We kept mitochondrial genomes in their entirety, but trimmed part of tRNA-Phe, 12s rRNA and the control region to accommodate the length of all utilized sequences. Mitochondrial genome haplotypes were determined with DnaSP 6.12.03 [50], and haplotypes were used for phylogenetic reconstruction. Individuals that possess identical mtDNA haplotypes are listed in Table S3, and these groups are represented in phylogenetic reconstructions by a single haplotype. We added data from several other New World monkeys (Table S2) and reconstructed phylogenetic trees with maximum-likelihood (ML) and

Bayesian algorithms using IQ-TREE 1.5.2 [41] and MrBayes 3.2.6 [25,49], respectively. For the ML phylogeny, we used the optimal substitution model (TIM2+I+G) as calculated with ModelFinder [10,27] in IQ-TREE under the Bayesian Information Criterion (BIC). We performed the ML analysis in IQ-TREE with 10,000 ultrafast bootstrap (BS) replications [36]. The Bayesian tree was reconstructed via a Markov Chain Monte Carlo (MCMC) run with 1 million generations and tree and parameter sampling occurring every 100 generations. The first 25% of generations were discarded as burn-in. To check convergence of all parameters and the adequacy of the burn-in, we assessed the uncorrected potential scale reduction factor (PSRF) [23] as calculated by MrBayes. We calculated posterior probabilities (PP) and a phylogram with mean branch lengths from the posterior density of trees using MrBayes. Phylogenetic trees were visualized and edited with FigTree 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

The divergence time calculation was performed with the BEAST 2.4.8 package [2] using a relaxed lognormal clock model of lineage variation [18] and by applying a Yule tree prior and the best-fit model of sequence evolution as obtained by ModelFinder. To calibrate the molecular clock, we applied fossil data to constrain the splits between Cebinae and Saimirinae and between Callicebinae and Pitheciinae with hard minimum and soft maximum bounds using a log normal prior following settings and fossils described in detail in Byrne et al. [5]. Briefly, for the Cebinae - Saimirinae split, we used an offset of 12.6, mean of 1.287 and standard deviation of 0.8, which translates into a median divergence of 16.2 million years ago (Ma) (95% highest posterior density [HPD]: 13.4-30.0 Ma). For the Callicebinae - Pitheciinae split, we used an offset of 15.7, mean of 1.016 and standard deviation of 0.8, resulting in a median divergence of 18.5 Ma (95% HPD: 16.3-28.9 Ma). We performed two independent runs each with 50 million generations and tree and parameter sampling setting in every 5000 generations. To assess the adequacy of a 10% burn-in and convergence of all parameters, we inspected the trace of the parameters across generations using Tracer 1.6 [44]. We combined sampling distributions of both replicates with LogCombiner 2.4.8 and summarized trees with a 10% burn-in using TreeAnnotator 2.4.8 (both programs are part of the BEAST package). A ParimonySplits network of a subset of mtDNA haplotypes was made with default settings in SplitsTree4 [26].

Declarations

Ethics approval and consent to participate

Tissues were collected under the approval of the Arizona State University Institutional Animal Care and Use Committee Animals (ASU IACUC, protocols #11-1150R, 15-144R), Brazilian Environmental Ministry (SISBIO protocols #47964-2 and #28075-2), and a CPRJ internal review.

Consent for publication

Not applicable.

Availability of data and materials

Newly sequenced and annotated *Callithrix* mitochondrial genomes have been deposited in NCBI Genbank. Accession numbers for each sequence are given in Table S1.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

JM formulated the idea for the study, collected samples, obtained funding, conducted wet and dry laboratory work, and wrote the original manuscript. RAC provided study guidance, and logistical support. NHAC provided field assistance and logistical support. JAD provided study guidance, and logistical support. CM assisted in phenotypic identification of hybrids and generation of Appendix A. CSI gave access and provided logistical support to collect samples from animals kept at Guarulhos Zoo. SBM provided logistical support and veterinary assistance to collect samples from animals kept at the Rio de Janeiro Primatology Center. AN gave assistance with data processing. PAN gave access and provided logistical support to collect samples from animals kept at CEMAFAUNA. MP provided field assistance and logistical support. LCMP gave access and provided logistical support to collect samples from animals kept at CEMAFAUNA. AP gave access and provided logistical support to collect samples from animals kept at CPRJ. CRRM and DZ were major contributor in writing the manuscript. DLS performed DNA extractions, methodological optimization, and carried out PCR. ACS was major contributor in writing the manuscript, provided study guidance, and logistical support. CR gave assistance in data processing, carried out divergence analysis, gave significant guidance and input in the development of this study. All authors read and approved the final manuscript.

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Supplement Material

Table S1. Metadata for newly collected samples as well as primate mitochondrial genome sequences obtained from Genbank. The ‘Sample’ column gives ID of each new sampled individual or the species for sequences obtained from previous studies. The ‘Accession’ column gives Genbank accession numbers for each sequence, the ‘Assembly Method This Study’ column states the manner in which new *Callithrix* mitochondrial genomes were sequenced and assembled (S = Sanger; N = NOVOPlasty2.6.4). The ‘Phenotype’ column indicates whether the sampled individual possessed a pure species or hybrid phenotype, and capital letters in parentheses next to *Callithrix penicillata* x *Callithrix geoffroyi* category are specific phenotype classifications following Figure 5 in Fuzessy et al. (2014). The ‘mtDNA Genome Lineage’ column indicates phylogenetic classification of the mitochondrial genome of the sampled individual. The ‘Sampling Location’ column indicates where each individual was sampled. Nearest cities are located for individuals sampled from the wild, and facilities are indicated for individuals sampled in captivity. The Guarulhos Municipal Zoo is located in Guarulhos, São Paulo, Brazil; CRC (Callitrichid Research Center) is located in Omaha, Nebraska, US; NEPRC (New England Primate Research Center, no longer in operation) was located in Southborough, Massachusetts, US; CPRJ (Centro de Primatologia do Rio de Janeiro) is located in Guapimirim, Rio de Janeiro, Brazil; CEMAFAUNA (Centro de Concervação e Manejo de Fauna da Caatinga) is located in Petrolina, Pernambuco. Abbreviations for Brazilian states in the ‘Sampling Location’ column are as follows: Espírito Santo (ES), Minas Gerais (MG), Rio de Janeiro (RJ), São Paulo (SP). DF is the Brazilian Federal District. (NA = No data Available)

Sample	Accession Number	Assembly method	Phenotype	mtDNA lineage	Sampling location	Latitude/ Longitude
BJT6	MN787074	N	*C. aurita*	*C. aurita*	Guiricema, MG	-21.010, -42.720
BJT20	MN787075	S	*C. aurita*	*C. jacchus*	Mogi das Cruzes, SP (Removed from wild and housed at Guarulhos Municipal Zoo)	-23.523, -46.179
BJT65	MT041703	N	*C. aurita*	*C. aurita*	Guiricema, MG	-21.010, -42.720
BJT79	MN787077	S	*C. aurita*	*C. jacchus*	Guarulhos Municipal Zoo	NA
BJT83	MN787078	N	*C. aurita*	*C. aurita*	Sao Jose dos Campos, SP (Removed from wild and housed at Guarulhos Municipal Zoo)	-23.070, -45.933

BJT84	MN787079	S	*C. aurita*	*C. jacchus*	Guarulhos Municipal Zoo	NA
BJT107	MN787080	N	*C. aurita*	*C. aurita*	Natividade, RJ (Removed from wild and housed at CPRJ)	-21.043, -41.978
BJT109	MN787081	N	*C. aurita*	*C. aurita*	Natividade, RJ (Removed from wild and housed at CPRJ)	-21.043, -41.978
BJT114	MN787082	N	*C. aurita*	*C. aurita*	Guapirim, RJ (Removed from wild and housed at CPRJ)	-22.488, -42.912
Cgeoffroyi_NC_021	NC_021941	NA	*C. geoffroyi*	*C. geoffroyi*	NA	NA
cgeo006	LR745201	S	*C. geoffroyi*	*C. geoffroyi*	CRC	NA
BJT102	MN787083	N	*C. geoffroyi*	*C. geoffroyi*	CPRJ	NA
BJT143	MN787084	N	*C. geoffroyi*	*C. geoffroyi*	Berilo, MG	-16.953, -42.466
BJT169	MN787086	N	*C. geoffroyi*	*C. geoffroyi*	Serra, ES	-20.213, -40.267
BJT170	MN787085	N	*C. geoffroyi*	*C. geoffroyi*	Serra, ES	-20.213, -40.267
BJT171	MN787087	N	*C. geoffroyi*	*C. geoffroyi*	Serra, ES	-20.213, -40.267
cja007	LR745200	S	*C. jacchus*	*C. jacchus*	NEPRC	NA
Cjacchus KM588314A	KM588314	NA	*C. jacchus*	*C. jacchus*	NA	NA
Cjacchus NC_025586	NC_025586	NA	*C. jacchus*	*C. jacchus*	NA	NA
BJT86	MN787088	N	*C. jacchus*	*C. jacchus*	Guarulhos Municipal Zoo	NA
BJT87	MN787089	N	*C. jacchus*	*C. jacchus*	Guarulhos Municipal Zoo	NA
BJT100	MN787090	N	*C. jacchus*	*C. jacchus*	CPRJ	NA
BJT157	MN787091	N	*C. jacchus*	*C. jacchus*	CEMAFAUNA	NA
BJT165	MN787092	N	*C. jacchus*	*C. jacchus*	CEMAFAUNA	NA
BJT166	MN787093	N	*C. jacchus*	*C. jacchus*	CEMAFAUNA	NA
BJT167	MN787094	N	*C. jacchus*	*C. jacchus*	CEMAFAUNA	NA
cpe025	MN787101	N	*C. penicillata*	*C. penicillata*	SWPW Quadra 15, Conjunto 5, Brasília, DF	-15.911, -47.953
cpe030	MN787102	N	*C. penicillata*	*C. penicillata*	Jardim Botânico, Brasília, DF	-15.861, -47.829
BJT8	MN787095	N	*C. penicillata*	*C. penicillata*	Lavras, MG	-21.227, -44.980
BJT10	MN787096	N	*C. penicillata*	*C. penicillata*	Lavras, MG	-21.227, -44.980
BJT40	MN787097	N	*C. penicillata*	*C. penicillata*	Belo Horizonte, MG	-19.921, -43.990
BJT41	MN787098	N	*C. penicillata*	*C. penicillata*	Belo Horizonte, MG	-19.921, -43.990
BJT159	MN787099	N	*C. penicillata*	*C. penicillata*	CEMAFAUNA	NA
BJT160	MN787100	N	*C. penicillata*	*C. penicillata*	CEMAFAUNA	NA
cku004	KR817257	N	*C. kuhlii*	*C. kuhlii*	CRC	NA

Ckuhlili KR869628	KR869628	NA	*C. kuhlii*	*C. kuhlii*	NA	NA
Ckuhlii NC_027658	NC_027658	NA	*C. kuhlii*	*C. kuhlii*	NA	NA
BJT24	MN787103	N	*C. aurita* x sp.	*C. aurita*	Guarulhos Municipal Zoo (apprehended animal)	NA
BJT25	MN787104	N	*C. aurita* x sp.	*C. jacchus*	Guarulhos Municipal Zoo (apprehended animal)	NA
BJT26	MN787105	N	*C. aurita* x sp.	*C. penicillata*	Guarulhos Municipal Zoo (apprehended animal)	NA
BJT27	MN787106	N	*C. aurita* x sp.	*C. geoffroyi*	Guarulhos Municipal Zoo (apprehended animal)	NA
BJT115	MN787107	N	*C. aurita* x sp.	*C. aurita*	Guapirim, RJ	-22.488, -42.912
BJT116	MN787108	N	*C. penicillata* x *C. jacchus*	*C. penicillata*	Guapirim, RJ	-22.488, -42.912
BJT70	MN787109	N	*Callithrix* sp. x *Callithrix* sp.	*C. geoffroyi*	Santa Teresa, ES	-19.935, -40.596
BJT13	MN787110	N	*C. penicillata* x *C. geoffroyi* (D)	*C. penicillata*	Viçosa, MG	-20.755, -42.872
BJT14	MN787120	N	*C. penicillata* x *C. geoffroyi* (B)	*C. penicillata*	Viçosa, MG	-20.755, -42.872
BJT15	MN787117	N	*C. penicillata* x *C. geoffroyi* (D)	*C. penicillata*	Viçosa, MG	-20.755, -42.872
BJT16	MN787114	N	*C. penicillata* x *C. geoffroyi*	*C. penicillata*	Viçosa, MG	-20.755, -42.872
BJT31	MN787111	N	*C. penicillata* x *C. geoffroyi* (C)	*C. penicillata*	Viçosa, MG	-20.758, -42.860
BJT33	MN787112	N	*C. penicillata* x *C. geoffroyi* (B)	*C. penicillata*	Viçosa, MG	-20.778, -42.863
BJT38	MN787113	N	*C. penicillata* x *C. geoffroyi* (A)	*C. penicillata*	Viçosa, MG	-20.755, -42.857
BJT39	MN787116	N	*C. penicillata* x *C. geoffroyi* (D)	*C. penicillata*	Viçosa, MG	-20.755, -42.857
BJT75	MN787115	N	*C. penicillata* x *C. geoffroyi*	*C. penicillata*	Viçosa, MG	-20.759, -42.866
BJT150	MN787118	N	*C. penicillata* x *C. geoffroyi* (A)	*C. penicillata*	Viçosa, MG	-20.759, -42.866
BJT151	MN787119	N	*C. penicillata* x *C. geoffroyi* (A)	*C. penicillata*	Viçosa, MG	-20.759, -42.866
Ateles belzebuth	KC757386	NA	NA	NA	NA	NA
Alouatta caraya	KC757384	NA	NA	NA	NA	NA
Aotus azarai	KC757385	NA	NA	NA	NA	NA
Aotus lemurinus	FJ785421	NA	NA	NA	NA	NA
Aotus nancymaae	NC_018116	NA	NA	NA	NA	NA

Brachyteles arachnoides	JX262672	NA	NA	NA	NA	NA
Cacajao calvus	KC959985	NA	NA	NA	NA	NA
Cebuella pygmaea	KC757389	NA	NA	NA	NA	NA
Cebus albifrons	NC_002763	NA	NA	NA	NA	NA
Chiropotes albinasus	KC757393	NA	NA	NA	NA	NA
Lagothrix lagotricha	KC757398	NA	NA	NA	NA	NA
Leontopithecus rosalia	KC757399	NA	NA	NA	NA	NA
Plecturocebus cupreus	KC959986	NA	NA	NA	NA	NA
Plecturocebus donacophilus	FJ785423	NA	NA	NA	NA	NA
Saguinus oedipus	KC757409	NA	NA	NA	NA	NA
Saimiri boliviensis boliviensis	NC_018096	NA	NA	NA	NA	NA
Saimiri oerstedii citrinellus	HQ644336	NA	NA	NA	NA	NA
Saimiri sciureus macrodon	HQ644338	NA	NA	NA	NA	NA
Saimiri oerstedii oerstedii	HQ644337	NA	NA	NA	NA	NA
Saimiri boliviensis peruviensis	HQ644340	NA	NA	NA	NA	NA
Saimiri sciureus sciureus	HQ644334	NA	NA	NA	NA	NA
Sapajus apella	NC_016666	NA	NA	NA	NA	NA
Sapajus xanthosternos	KC757410	NA	NA	NA	NA	NA

Table S2. Organization of the *C. aurita* mitochondrial genome based on 16,471 sequenced bases of individual BJT065 (Accession number MT041703).

Region	Position		Length	Strand	Codon	
	From	To			Start	Stop
tRNA-Phe	1	69	69	H		
12s-rRNA	70	1019	950	H		
tRNA-Val	1024	1091	68	H		
16s-rRNA	1092	2644	1553	H		
tRNA-Leu (UUR)	2646	2720	75	H		
ND1	2723	3679	957	H	GTG	TAA
tRNA-Ile	3679	3747	69	H		
tRNA-Gln	3745	3816	72	L		
tRNA-Met	3820	3887	68	H		
ND2	3890	4928	1039	H	ATC	TAG
tRNA-Trp	4929	4995	67	H		
tRNA-Ala	5004	5072	69	L		
tRNA-Asn	5074	5146	73	L		
tRNA-Cys	5179	5245	67	L		
tRNA-Tyr	5245	5311	67	L		
COI	5319	6875	1557	H	ATG	AGG
tRNA-Ser(UCN)	6864	6931	68	L		
tRNA-Asp	6936	7003	68	H		
COII	7004	7691	688	H	ATG	TA
tRNA-Lys	7692	7758	67	H		
ATP8	7759	7965	207	H	GTG	TAG
ATP6	7920	8600	681	H	ATG	TAA
COIII	8600	9383	784	H	ATG	T
tRNA-Gly	9384	9450	67	H		
ND3	9451	9796	346	H	ATA	T
tRNA-Arg	9797	9862	66	H		
ND4L	9866	10162	297	H	ATG	TAA
ND4	10156	11530	1375	H	ATG	T
tRNA-His	11531	11599	69	H		
tRNA-Ser(AGY)	11600	11658	59	H		
tRNA-Leu(CUN)	11659	11729	71	H		

ND5	11733	13538	1806	H	ATA	TAA/TAG
ND6	13535	14068	534	L	ATG	TAA
tRNA-Glu	14069	14137	69	L		
CYT B	14142	15281	1140	H	ATG	TAG
tRNA-Thr	15284	15352	69	H		
tRNA-Pro	15354	15422	69	L		
Control region	15427	16471	1045	H		

Table S3. Each cell lists individuals that possess the same mtDNA haplotypes

BJT6, BJ65
Cge006, Cgoeffroyi NC21941
BJT107, BJT109
BJT169, BJT170, BJT171
BJT13, BJT14, BJT15, BJT16, BJT31, BJT38, BJT39, BJT75, BJT150, BJT151
Cjacchus NC_025586, Cjacchus KM588314A
BJT166, BJT167
BJT8, BJT10
Ckuhlii NC_027658, Ckuhlii KR869628

Table S3. Divergence times for *Callithrix* species and select nodes (MRCA = Most recent common ancestor; values in brackets = 95% highest posterior density). Node names follow major node designations shown in Figure S3 as capital letters.

Node	**Taxa Diverging at Node**	**Divergence Time**
A	*C. jacchus* - *C. penicillata* (Caatinga clade)	0.51 [0.35-0.69]
B	*C. kuhlii* - *C. jacchus* + *C. penicillata* (Caatinga clade)	0.82 [0.59-1.09]
C	*C. geoffroyi* - *C. kuhlii* + *C. jacchus* + *C. penicillata* (all clades)	1.18 [0.87-1.58]
D	*C. aurita* - *C. geoffroyi* + *C. kuhlii* + *C. jacchus* + *C. penicillata* (all clades)	3.54 [2.37-4.88]
E	*Callithrix* - *Cebuella/Mico*	6.83 [4.86-9.39]
F	*Saguinus- Cebuella+ Callithrix*	13.42 [9.66,18.82]
G	*Leontopithecus -Saguinus + Cebuella+ Callithrix*	15.69 [11.73-22.06]
H	Callitrichidae -*Aotus+Sapajus+Saimiri*	18.63 [15.28-22.80]
I	Atelidae - Cebidae + Callitrichidae	19.76 [16.43-23.48]
J	Pithecidae - Atelidae + Cebidae + Callitrichidae	21.68 [18.56-27.23]

Table S4. Summary of record numbers for collected samples that have been entered into the Brazilian CGEN SISGEN sample database (ES = Espírito Santo, MG = Minas Gerais, PE = Pernambuco, RJ = Rio de Janeiro, SP = São Paulo).

City	State	Wild Animal	Translocated/ Captive	Facility	SISGEN Record Number
Santa Teresa	ES	x			ABD1A11
Serra	ES	x			AE61F27
Guiricema	MG	x			A94942B
Berilo	MG	x			A3CB657
Lavras	MG	x			A679090
Belo Horizonte	MG	x			A679090
Vicosa	MG	x			A998C84
Petrolina	PE		x	CEMAFAUNA	A3F0F46, A679090
Guapirim	RJ		x	Rio de Janeiro Primate Center	A2AEE0E, AD432CF, A6F3E89, A3F0F46, A6F3E89
Campos	RJ		x	SERCAS	AD2F796
Silva Jardim	RJ	x			A4A29D7
Guarulhos	SP		x	Guarulhos Zoo	A18C1CE, A3F0F46, A679090, A20E733

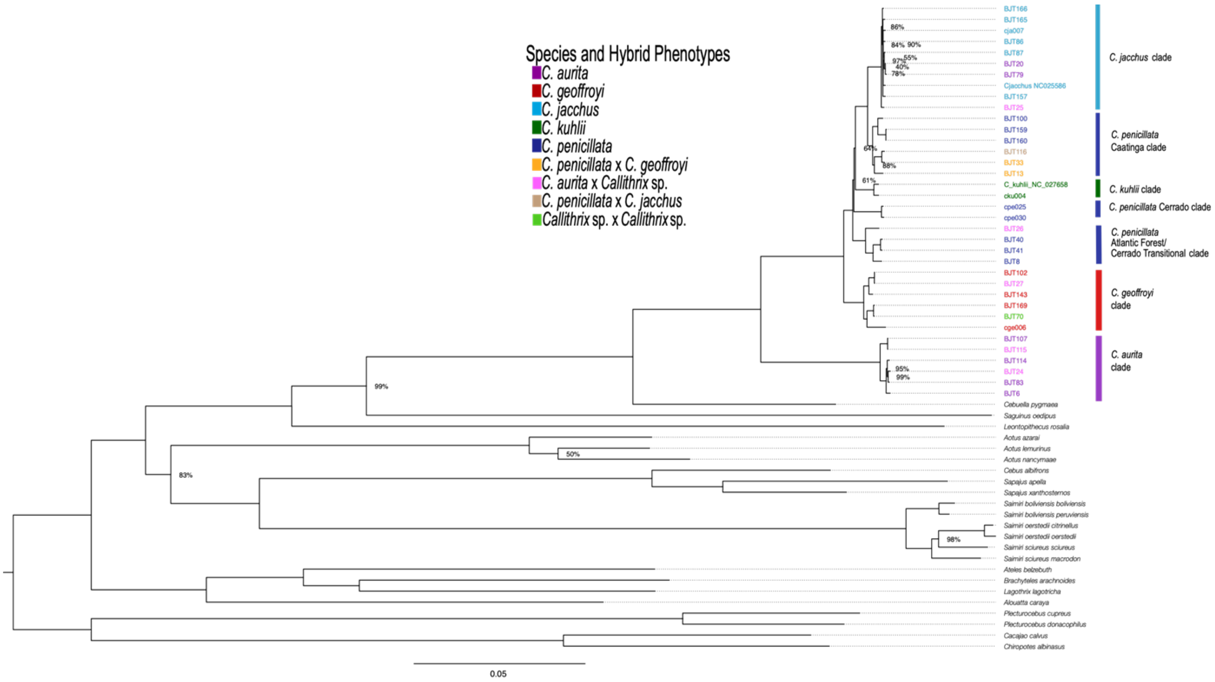
Species and Hybrid Phenotypes
C. aurita
C. geoffroyi
C. jacchus
C. kuhlii
C. penicillata
C. penicillata x C. geoffroyi
C. aurita x Callithrix sp.
C. penicillata x C. jacchus
Callithrix sp. x Callithrix sp.
BJT166
BJT165
cja007
BJT86
BJT87
BJT20
BJT79
Cjacchus NC025586
BJT157
BJT25
BJT100
BJT159
BJT160
BJT116
BJT33
BJT13
C_kuhlii_NC_027658
cku004
cpe025
cpe030
BJT26
BJT40
BJT41
BJT8
BJT102
BJT27
BJT143
BJT169
BJT70
cge006
BJT107
BJT115
BJT114
BJT24
BJT83
BJT6
Cebuella pygmaea
Saguinus oedipus
Leontopithecus rosalia
Aotus azarai
Aotus lemurinus
Aotus nancymaae
Cebus albifrons
Sapajus apella
Sapajus xanthosternos
Saimiri boliviensis boliviensis
Saimiri boliviensis peruviensis
Saimiri oerstedii citrinellus
Saimiri oerstedii oerstedii
Saimiri sciureus sciureus
Saimiri sciureus macrodon
Ateles belzebuth
Brachyteles arachnoides
Lagothrix lagotricha
Alouatta caraya
Plecturocebus cupreus
Plecturocebus donacophilus
Cacajao calvus
Chiropotes albinasus
86%
84%
90%
97%
55%
40%
78%
64%
88%
61%
95%
99%
99%
50%
83%
98%
C. jacchus clade
C. penicillata Caatinga clade
C. kuhlii clade
C. penicillata Cerrado clade
C. penicillata Atlantic Forest/ Cerrado Transitional clade
C. geoffroyi clade
C. aurita clade
0.05

Figure S1. Maximum-likelihood (ML) tree showing phylogenetic relationships among *Callithrix* haplotypes as calculated from mtDNA genome sequences. Numbers at nodes indicate bootstrap support for a given node, otherwise node bootstrap support was 100%. Haplotype colors at tips correspond to the 'Species and Hybrid Phenotypes' legend, and indicate phenotypes associated with each given haplotype.

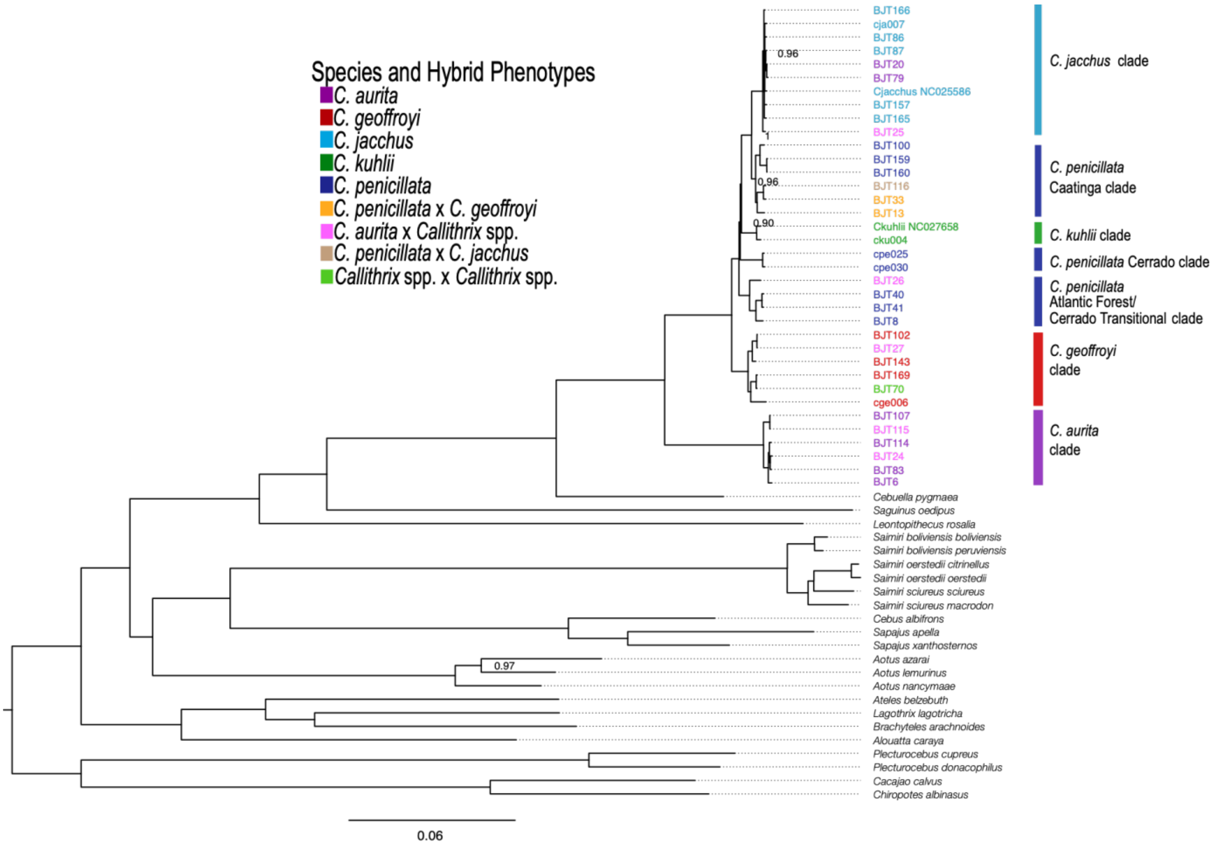
Species and Hybrid Phenotypes
C. aurita
C. geoffroyi
C. jacchus
C. kuhlii
C. penicillata
C. penicillata x C. geoffroyi
C. aurita x Callithrix spp.
C. penicillata x C. jacchus
Callithrix spp. x Callithrix spp.
BJT166
cja007
BJT86
BJT87
BJT20
BJT79
Cjacchus NC025586
BJT157
BJT165
BJT25
BJT100
BJT159
BJT160
BJT116
BJT33
BJT13
Ckuhlii NC027658
cku004
cpe025
cpe030
BJT26
BJT40
BJT41
BJT8
BJT102
BJT27
BJT143
BJT169
BJT70
cge006
BJT107
BJT115
BJT114
BJT24
BJT83
BJT6
Cebuella pygmaea
Saguinus oedipus
Leontopithecus rosalia
Saimiri boliviensis boliviensis
Saimiri boliviensis peruviensis
Saimiri oerstedii citrinellus
Saimiri oerstedii oerstedii
Saimiri sciureus sciureus
Saimiri sciureus macrodon
Cebus albifrons
Sapajus apella
Sapajus xanthosternos
Aotus azarai
Aotus lemurinus
Aotus nancymaae
Ateles belzebuth
Lagothrix lagotricha
Brachyteles arachnoides
Alouatta caraya
Plecturocebus cupreus
Plecturocebus donacophilus
Cacajao calvus
Chiropotes albinasus
0.96
1
0.96
0.90
0.97
C. jacchus clade
C. penicillata Caatinga clade
C. kuhlii clade
C. penicillata Cerrado clade
C. penicillata Atlantic Forest/ Cerrado Transitional clade
C. geoffroyi clade
C. aurita clade
0.06

Figure S2. Bayesian tree showing phylogenetic relationships among *Callithrix* species and hybrid haplotypes from mtDNA genome sequences. Numbers at nodes indicate posterior probability for a given node, otherwise node posterior probability was 1. Haplotype colors at tips correspond to the ‘Species and Hybrid Phenotypes’ legend, and indicate phenotypes associated with each given haplotype.

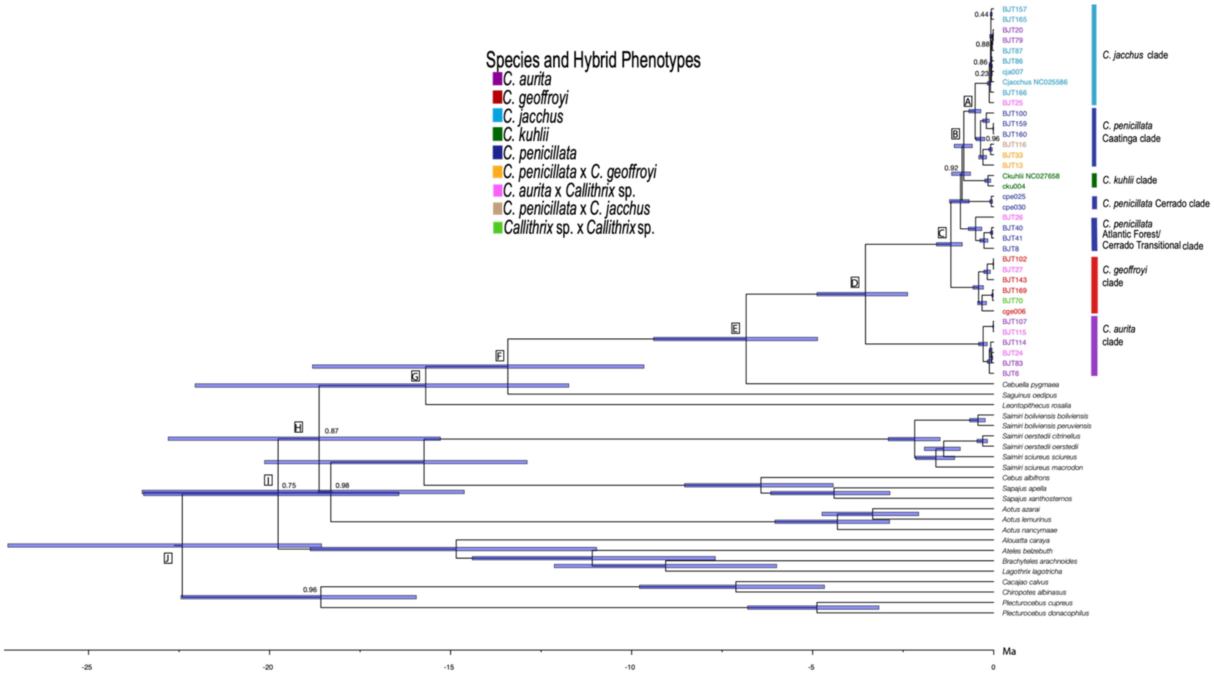
Species and Hybrid Phenotypes
C. aurita
C. geoffroyi
C. jacchus
C. kuhlii
C. penicillata
C. penicillata x C. geoffroyi
C. aurita x Callithrix sp.
C. penicillata x C. jacchus
Callithrix sp. x Callithrix sp.
0.44
0.88
0.86
0.23
A
B
0.96
0.92
C
D
E
F
G
H
0.87
I
0.75
0.98
J
0.96
BJT157
BJT165
BJT20
BJT79
BJT87
BJT86
cja007
Cjacchus NC025586
BJT166
BJT25
BJT100
BJT159
BJT160
BJT116
BJT33
BJT13
Ckuhlii NC027658
cku004
cpe025
cpe030
BJT26
BJT40
BJT41
BJT8
BJT102
BJT27
BJT143
BJT169
BJT70
cge006
BJT107
BJT115
BJT114
BJT24
BJT83
BJT6
Cebuella pygmaea
Saguinus oedipus
Leontopithecus rosalia
Saimiri boliviensis boliviensis
Saimiri boliviensis peruviensis
Saimiri oerstedii citrinellus
Saimiri oerstedii oerstedii
Saimiri sciureus sciureus
Saimiri sciureus macrodon
Cebus albifrons
Sapajus apella
Sapajus xanthosternos
Aotus azarai
Aotus lemurinus
Aotus nancymaae
Alouatta caraya
Ateles belzebuth
Brachyteles arachnoides
Lagothrix lagotricha
Cacajao calvus
Chiropotes albinasus
Plecturocebus cupreus
Plecturocebus donacophilus
C. jacchus clade
C. penicillata Caatinga clade
C. kuhlii clade
C. penicillata Cerrado clade
C. penicillata Atlantic Forest/ Cerrado Transitional clade
C. geoffroyi clade
C. aurita clade
-25
-20
-15
-10
-5
0
Ma

Figure S3. BEAST tree showing phylogenetic relationships and divergence ages in million years (Ma) among *Callithrix* haplotypes and other New World primates as calculated from mtDNA genome sequences. Major nodes are identified by capital letters, and blue bars at all nodes indicate 95% highest posterior densities (HPD) of divergence times. Haplotype colors at tips correspond to the 'Species and Hybrid Phenotypes' legend, and indicate phenotypes associated with each given haplotype.

Supplementary Material- An Overview of Species and Hybrid Phenotypes in Callithrix

This appendix is a guide to phenotypes of species and hybrids studied by Malukiewicz et al. (2020). Facial marks are important for differentiating between speices and hybrids, and these marks are pointed out in the first section below following Fuzessey et al. (2014). Phenotypic identification of species is based on Hershkovitz (1977), *C. jacchus* x *C. penicillata* hybrids on Hershkovitz (1977) and Malukiewicz et al. (2014), *C. penicillata* x *C. geoffroyi* hybrids on Hershkovitz (1977) and Fuzessy et al (2014), and *C. aurita* x *Callithrix* sp. hybrids on Carvalho (2015) and Malukiewicz (pers obs.).

Key facial landmarks

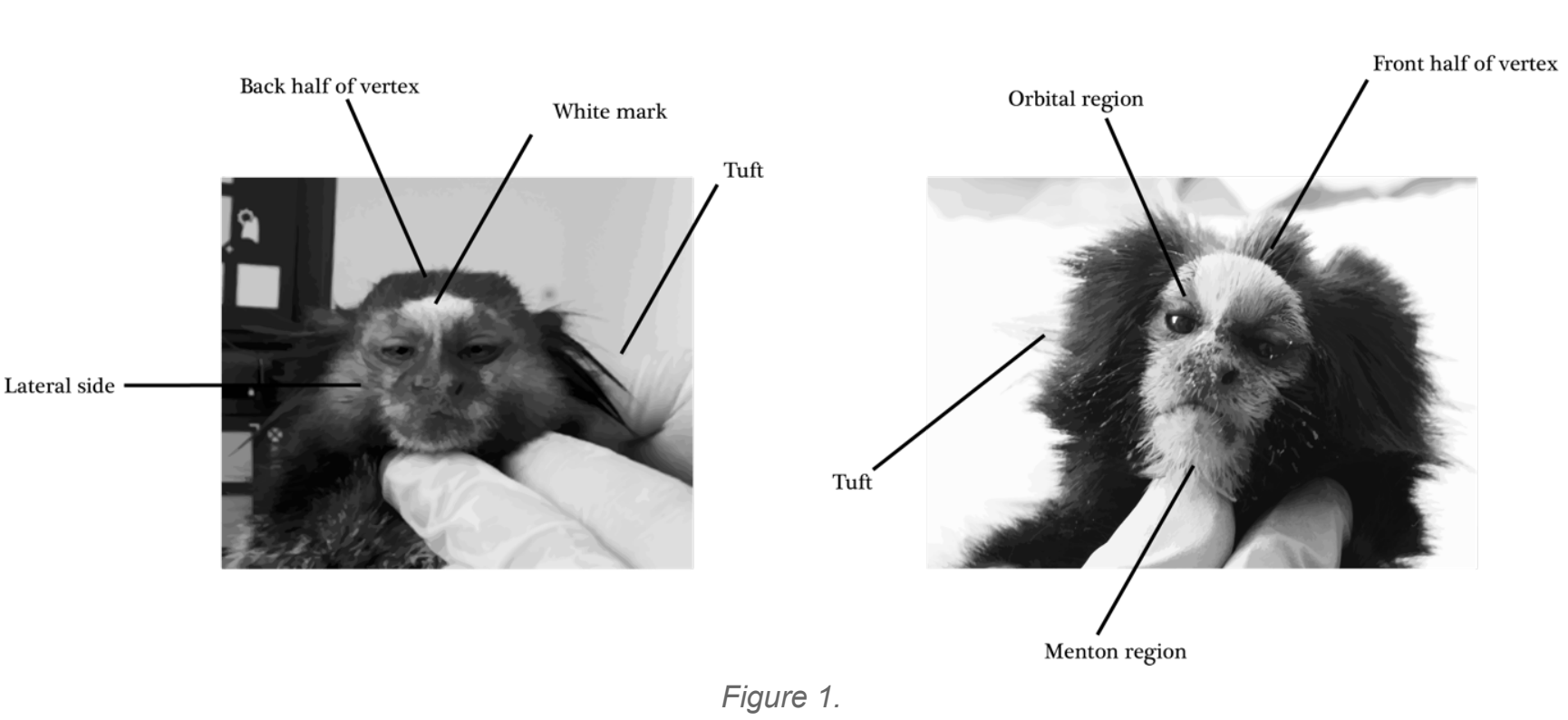

Figure 1.

Callithrix aurita

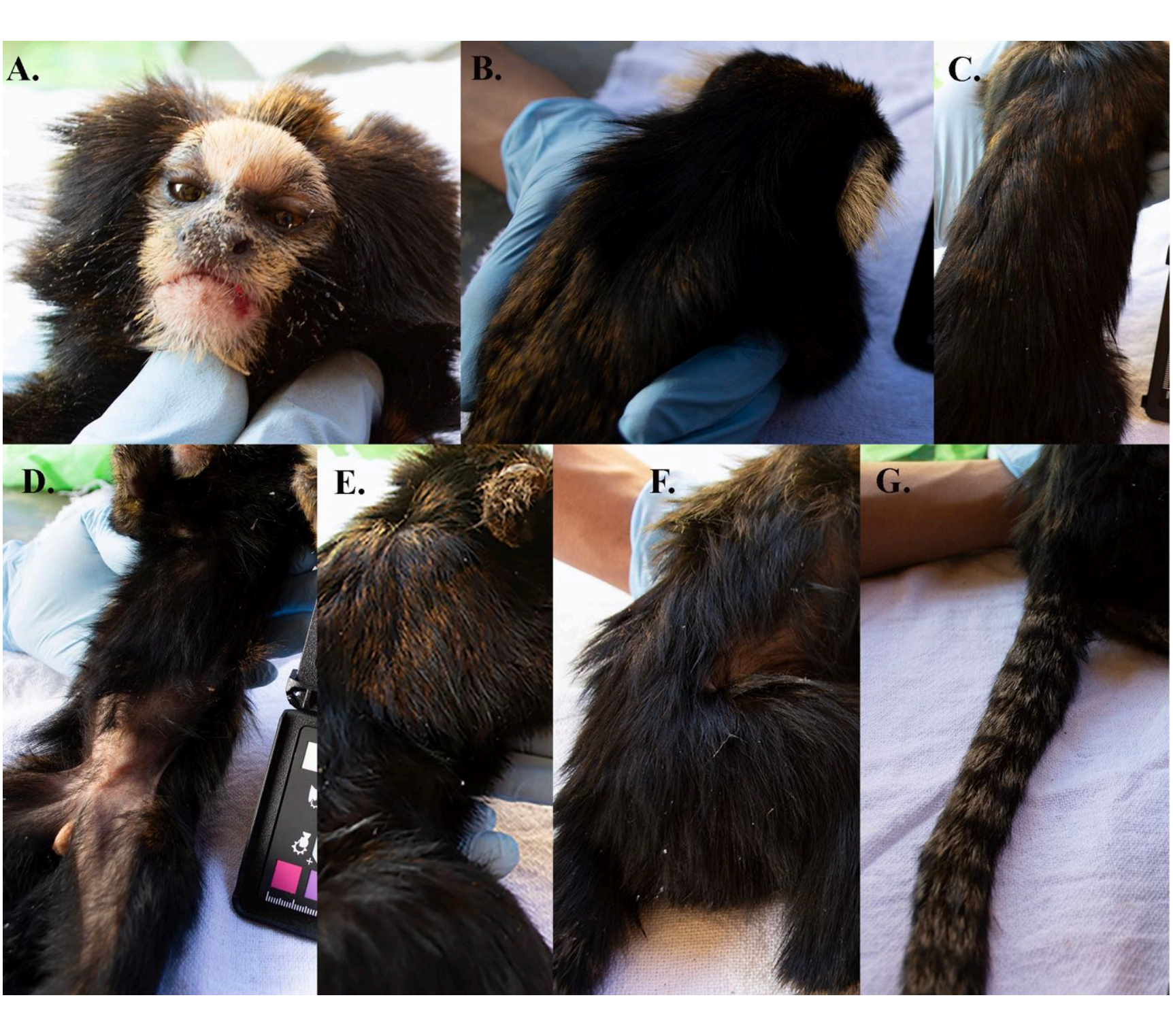

Figure 2. ***Callithrix aurita*** *phenotype*

Front of vertex (Figure 2A): Pelage is beige to orangish.
Back of vertex (Figure 2A): Pelage coloration follows the pattern found on the back and back of neck.
Menton Region (Figure 2A): This region has yellowish to orangish pelage.
Orbital Region (Figure 2A): Contains a mix of yellowish and peachy pelage
Tufts (Figure 2A): The pelage is not as full or dense as that seen in *C. jacchus*, and less dense in volume. Tufts can be black, yellow, or orange, or a mix of black with yellow tips at tuft ends.
Lateral sides of face (Figure 2A): Black pelage in this region.
White star on forehead (Figure 2A): There is no white star on the forehead as the face (forehead, orbital, nasal, infraorbital and mentonian region) has beige to light orange hairs.
Neck (Figure 2B): Region towards cranium has black hair, and caudal region has pelage that follows the pattern described for the back.
Back region (Figure 2C): Does not form a pattern of striae, but there is a mixture of orange patches (here the color is more intense than C. jacchus and C. penicillata) among black pelage. The hair in its proximal region is black in color with orange tips. The orange coloration is less intense towards the base of the tail, becoming predominating the black.
Stomach (Figure 2D): Black pelage some with slightly orange tips.
Region proximal to arms and legs (Figure 2E and 2F): Legs have black pelage with some with orange tips, on the base arm of black hair with orange tips (more evident orange than on the legs).
Tail (Figure 2G): Pelage has a black, grey, and orange striated pattern.

Callithrix geoffroyi

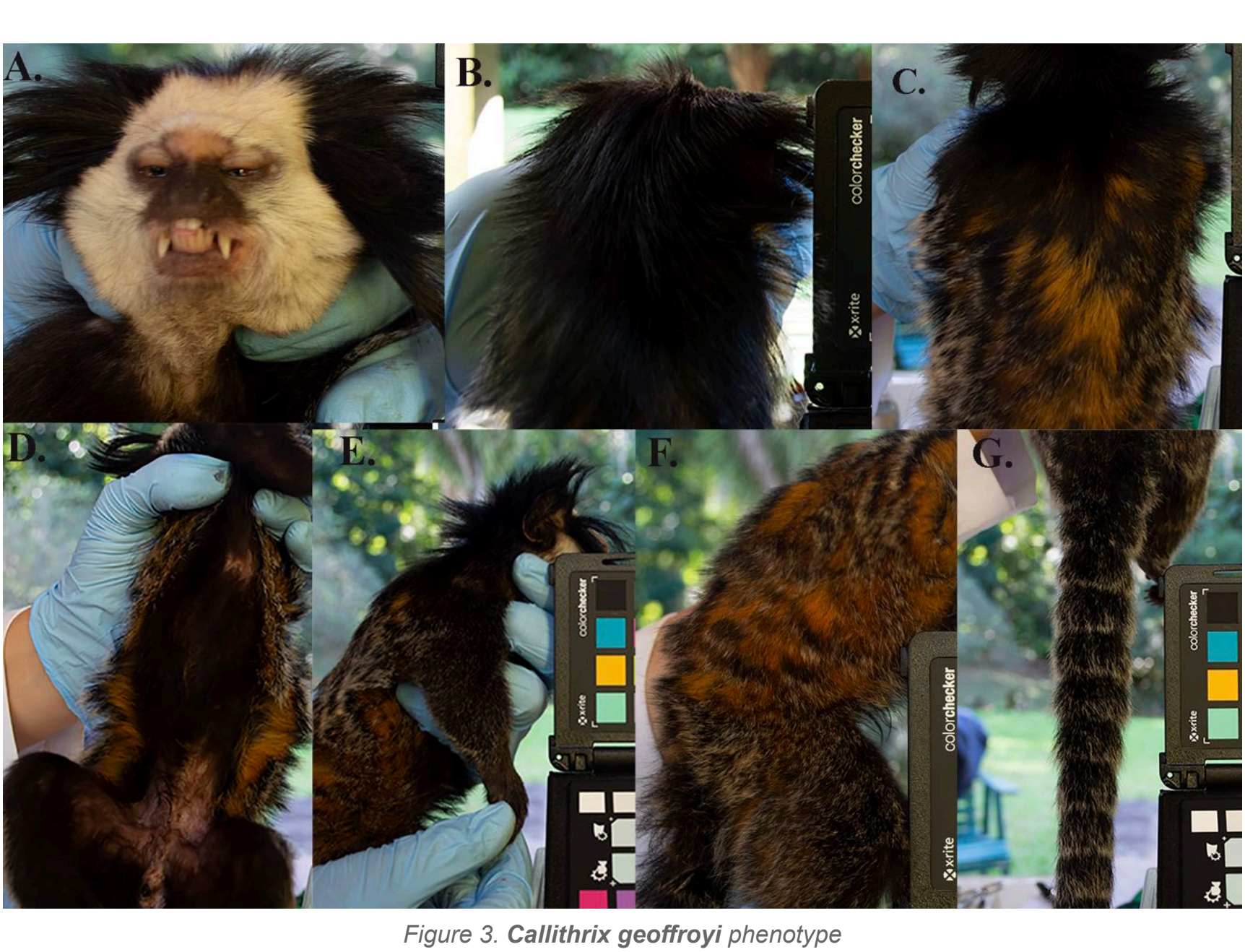

Figure 3. ***Callithrix geoffroyi*** *phenotype*

Front of vertex (Figure 3A): Pelage is white.
Back of vertex (Figure 3A): Pelage is black.
Menton Region (Figure 3A): Pelage is beige or white.
Orbital Region (Figure 3A): Pelage is peachy.
Tufts (Figure 3A): The pelage is very dense as in *C. jacchus*, and similar in volume. Tufts are black, and hairs closer to top of head are shorter and hairs closer to neck are longer.
White star on forehead (Figure 3A): The forehead and most of the fac around the orbital, nasal, infraorbital and mentonian regions is also white.
Neck (Figure 3B): Pelage in this region is black.
Back region (Figure 3C): Striations can be either black and orange or black and grey. The orange coloration is very obvious and prominent.
Region proximal to arms and legs (Figure 3E and 3F): Legs and arms are black and can be speckled with a whitish-grey.
Tail (Figure 3G): Pelage has a black, grey and orange striated pattern.

Callithrix jacchus

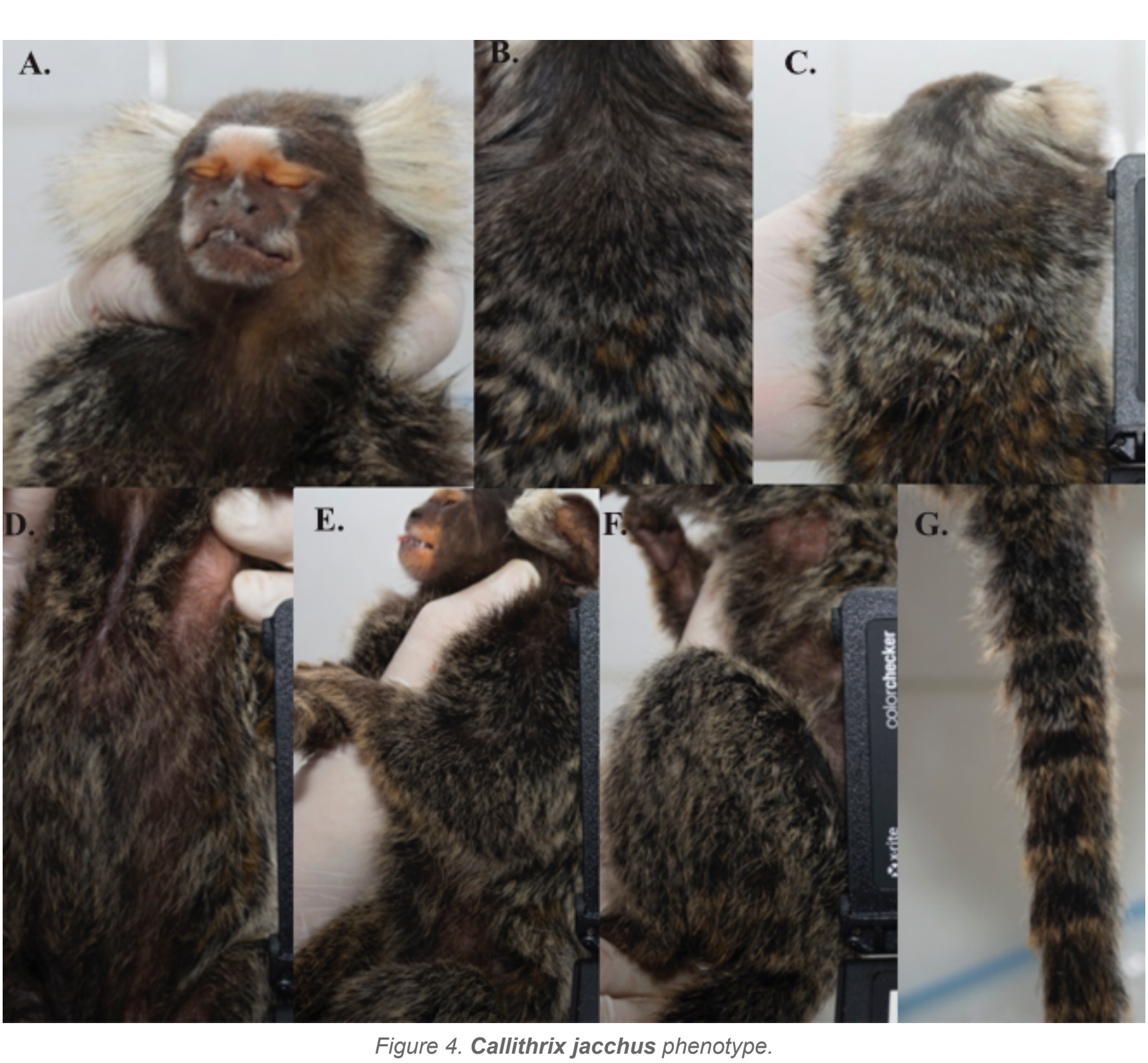

Figure 4. ***Callithrix jacchus*** *phenotype.*

Front half of vertex (Figure 4A): Pelage is brown to orange with tips of grey hair.
Back of vertex (Figure 4A): Pelage is brown with tips of grey hair.
Menton Region (Figure 4A): Pelage is grey.
Orbital Region (Figure 4A): Area is more peachy and buff colored than in *C. penicillata*.
Tufts (Figure 4A): Tufts are white, very lush, and full. At the very end of tips of tufts, pelage may be black.
Lateral sides of face (Figure 4A): Pelage ranges from dark brown to a little orange with some hairs that may have greyish tips.
White star on forehead (Figure 4A): The white mark is present and prominent.
Neck (Figure 4B): Upper neck region has dark brown coloration and lower neck region at the base
Back region (Figure 4C): Striations present with black and whitish-grey top coat and orangish undercoat.
Region proximal to arms and legs (Figure 4E and 4F): Arms have black to dark brown pelage and tips of pelage hairs are grey to orangish. The legs follow striation pattern of back region.
Tail (Figure 4G): Pelage has black, grey and orange striated pattern.

Callithrix penicillata

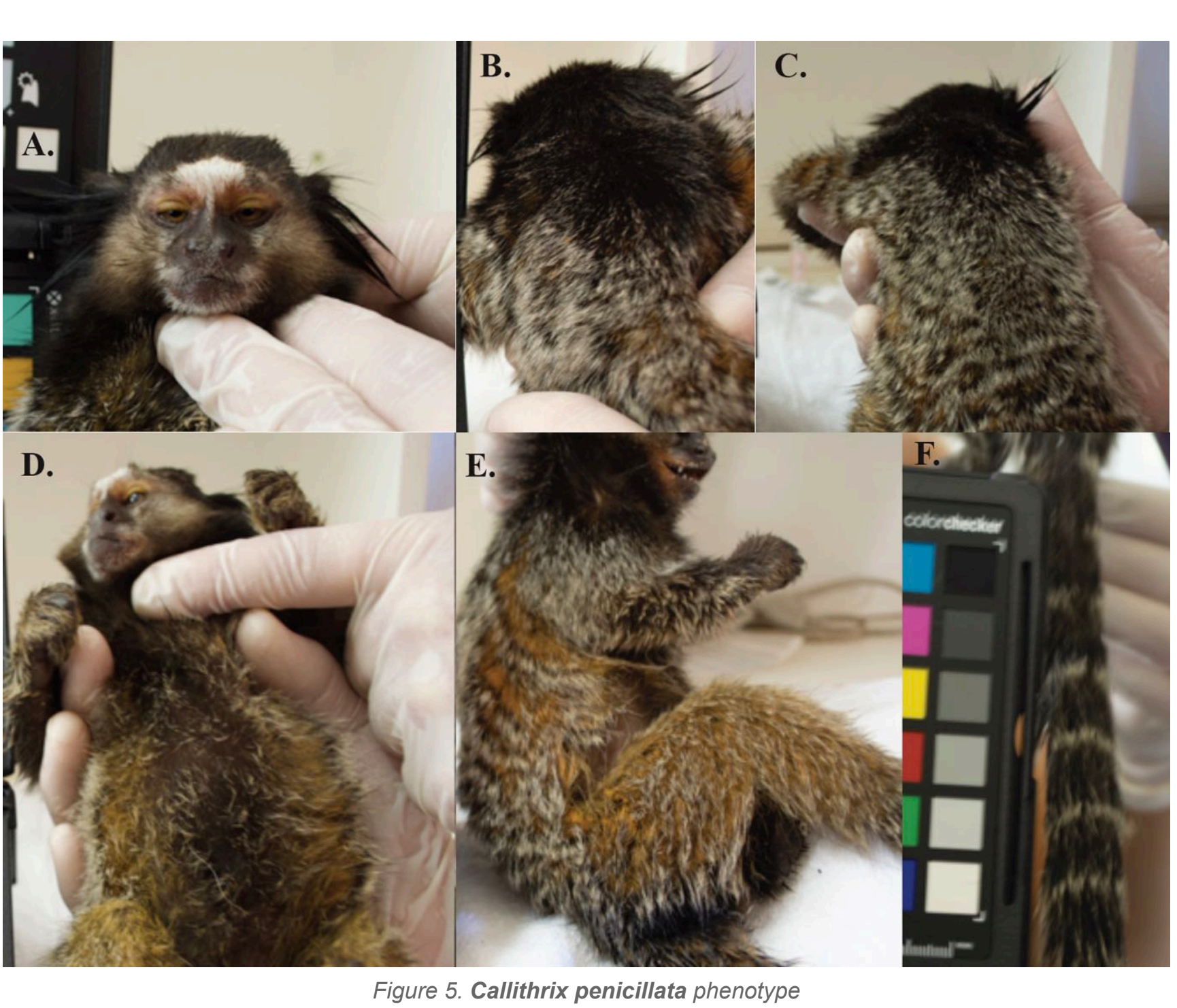

Figure 5. ***Callithrix penicillata*** *phenotype*

Front half of vertex (Figure 5A): Pelage is dark brown to black.
Back of vertex (Figure 5A): Pelage is dark brown to black.
Menton Region(Figure 5A): Pelage is whitish-grey.
Orbital Region (Figure 5A): Pelage is creme-buffy colored.
Tufts (Figure 5A): This region has thin, downward facing pelage that is black.
White star on forehead (Figure 5A): The white mark is clearly present and visible.
Lateral sides of face (Figure 5A): Pelage is whitish-grey to dark brown.
Neck (Figure 5B): Pelage has dark brown and black coloration, with possible presence of specks of whitish-grey
Back Region (Figure 5C): Striations present for which coloration combines an orangish undercoat and black and whitish-grey topcoat.
Stomach (Figure 5D): Orangish and black pelage is present in central stomach region.
Region proximal to arms and legs (Figure 5E): Arms are predominantly whitish-grey, and legs follow striation pattern of back region.
Tail (Figure 5F): Pelage has black and whitish-grey striations.

Callithrix jacchus x *Callithrix penicillata*

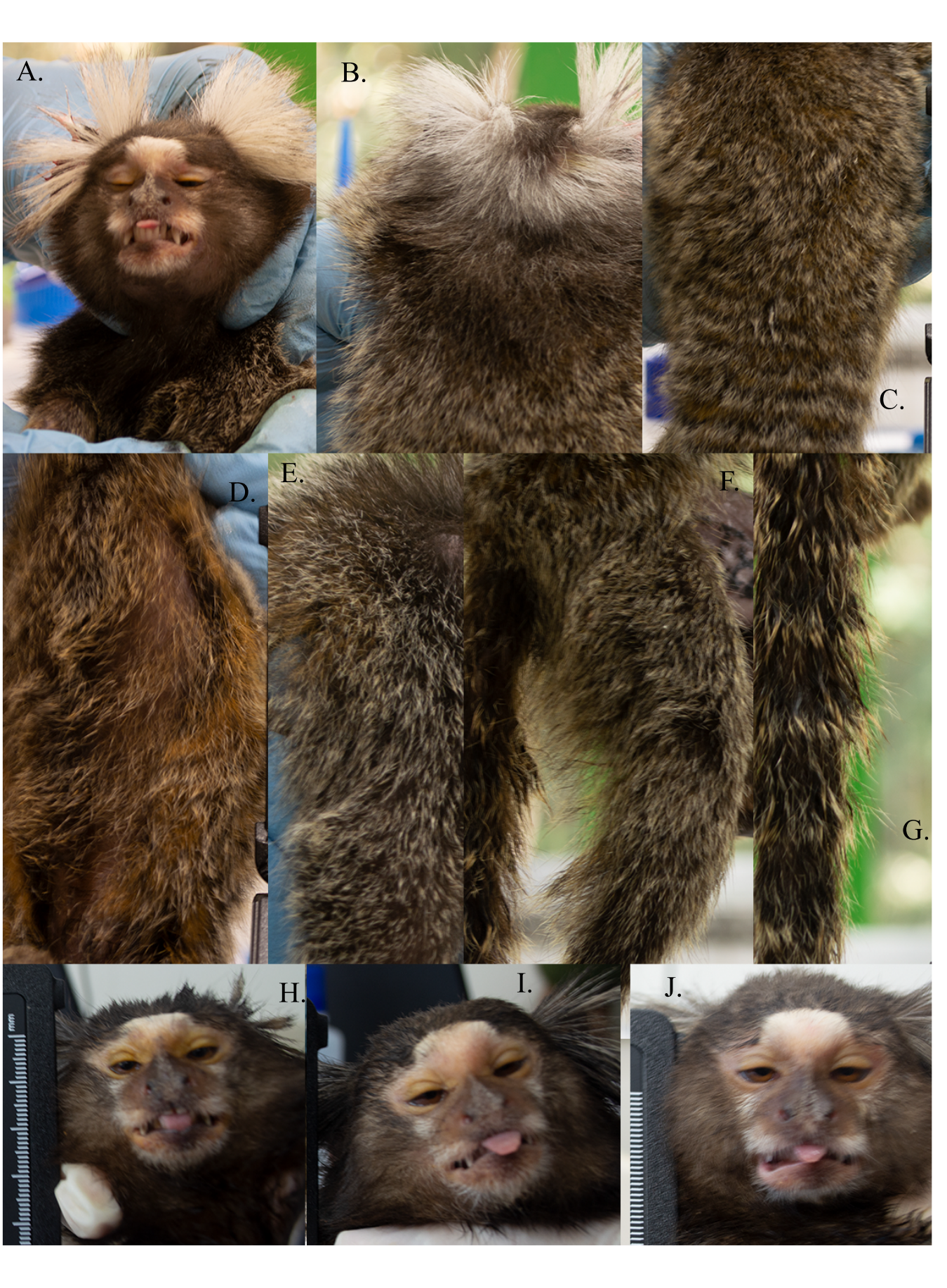

Figure 6. ***Callithrix jacchus*** *x* ***Callithrix penicillata*** *phenotype. Figures 6A-G is BJT116, and Figures 6H-J are unpublished images of other hybrids.*

Front half of vertex (Figure 6A,H-J): Pelage is composed of grey and black hair of varying intensities.
Back half of vertex (Figure 6A): Pelage coloration follows neck pattern.
Menton region (Figure 6A): Pelage is grey.
Orbital region (Figure 6A): Pelage is variable shades of orange, and may even be pink.
Tufts (Figure 6A): Tufts are usually less voluminous than in *C. jacchus*, and coloration ranges from black with grey tips to grey with some black hair.
White start on forehead (Figure 6A): This mark is present, but varies in size.
Lateral sides of the face (Figure 6A): Pelage has greyish coloration with some black and orange hairs.
Neck (Figure 6B): The coloration of pelage may range from black to greyish and/or orange.
Back region (Figure 6C): Pelage has streaks interspersed with orange, black and grey coloration. Striation patterns may not be as uniform as in parental species. Orange in some individuals is more or less intense. The hair in its proximal region is black, followed by orange, black and grey in the distal portion. The size of the proximal black and orange band may vary by individual.
Stomach (Figure 6D): Pelage varies in intensity from black to orange. These two colors are striated.
Proximal region of legs and arms (Figure 6E and 6F): Legs follow pattern of the back region, and the base of the arms has black to dark brown fur with grey tips.
Tail (Figure 6G): Pelage has black, grey and orange striated pattern, varying in color intensity.

Malukiewicz et al. (2014) contains further examples of facial phenotypes of *Callithrix jacchus* x *Callithrix penicillata* hybrids.

Callithrix penicillata x *Callithrix geofforyi*

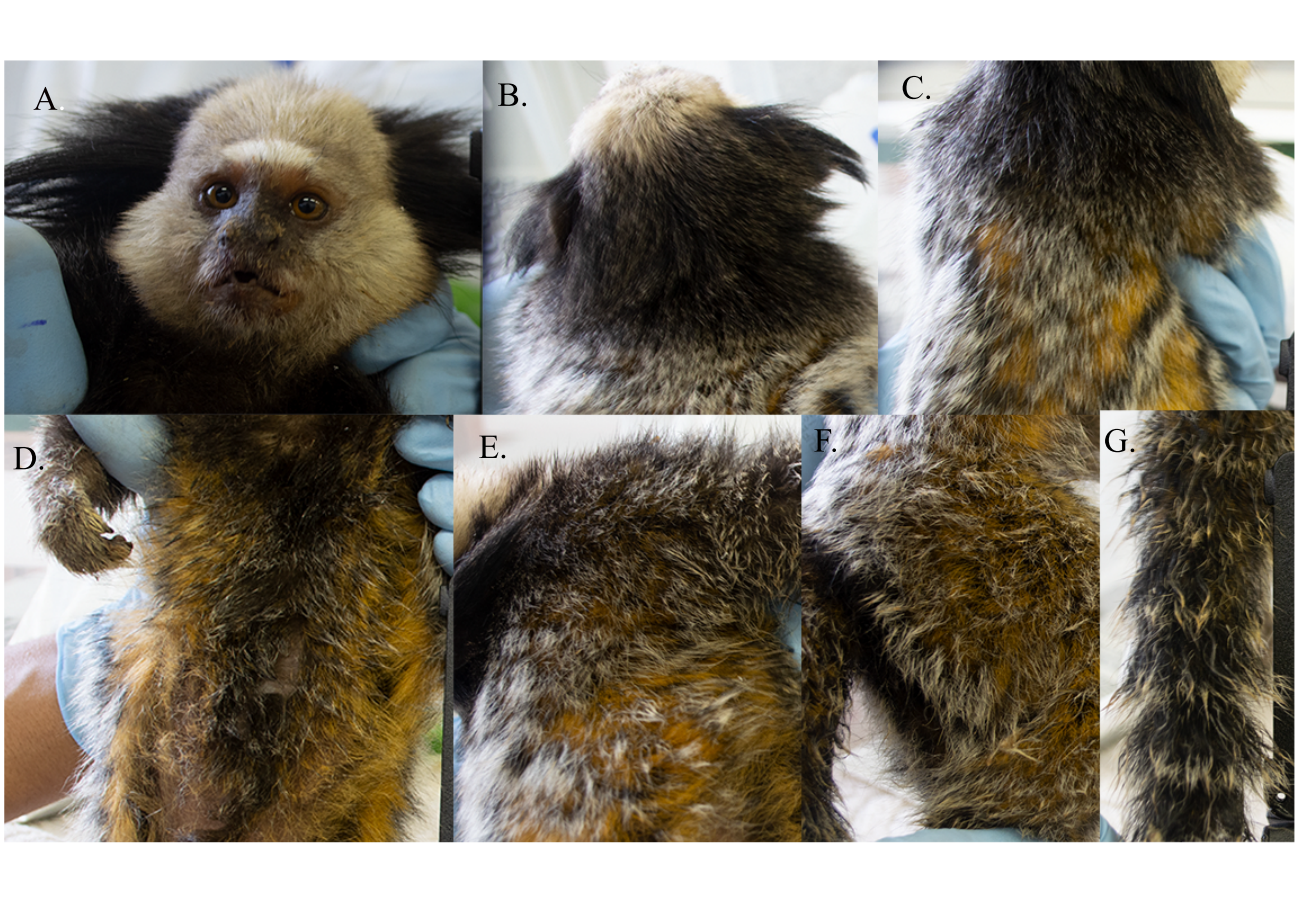

Figure 7. ***Callithrix penicillata*** *x* ***Callithrix geofforyi*** *phenotype*

Front half of vertex: Pelage follows pattern of the lateral sides of the face.
Back half of vertex: Pelage follows neck pattern.
Menton region: Pelage follows pattern of lateral sides of face.
Orbital region: Pelage is slightly orange.
Tufts: The pelage color is black but volume of tufts varies between that of the parental species.
White mark on forehead: The mark is present but varies in intensity between individual hybrids.
Lateral sides of the face: Pelage varies in intensity from white to grey.
Back region: Pattern of black, grey and orange streaks is similar to that of *C. jacchus* and *C. penicillata*. The orange coloration tends to be very evident.
Stomach: Black hairs are found in the central part of the stomach, but the hairs are intense orange in the outer parts.
Proximal region of legs and arms: Legs follow the pattern of the back, the arm hairs are black with some grey tips.
Neck: In the upper part, the hair will vary from white to dark grey. In the lower part, the hair will be black and may have grey tips.
Tail: Pelage shows a black, grey and orange striated pattern.

Fuzessy et al. (2014) contains further examples of facial phenotypes of *Callithrix penicillata* x *Callithrix geofforyi* hybrids.

Callithrix aurita x *Callithrix* sp.

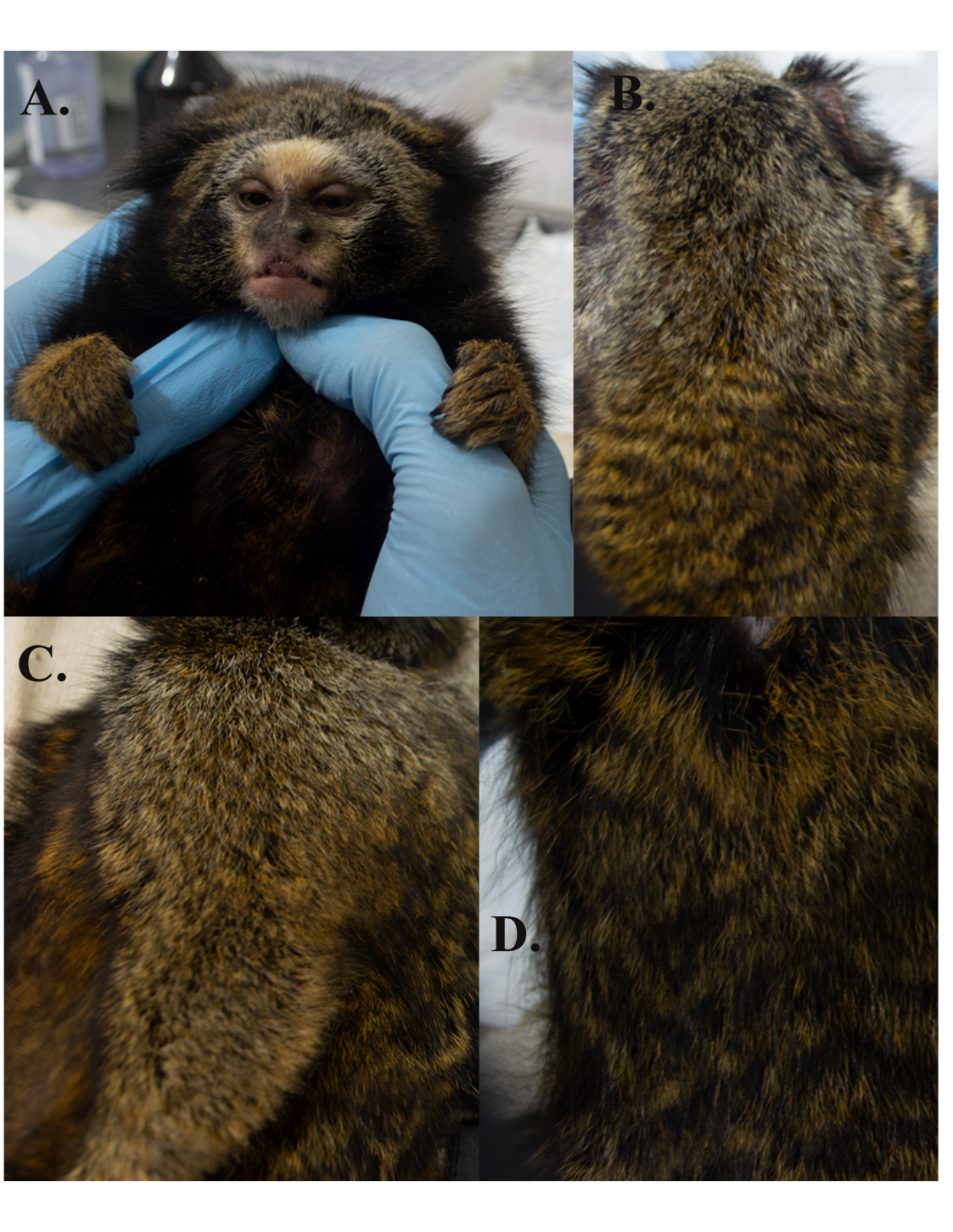

Figure 8. BJT024

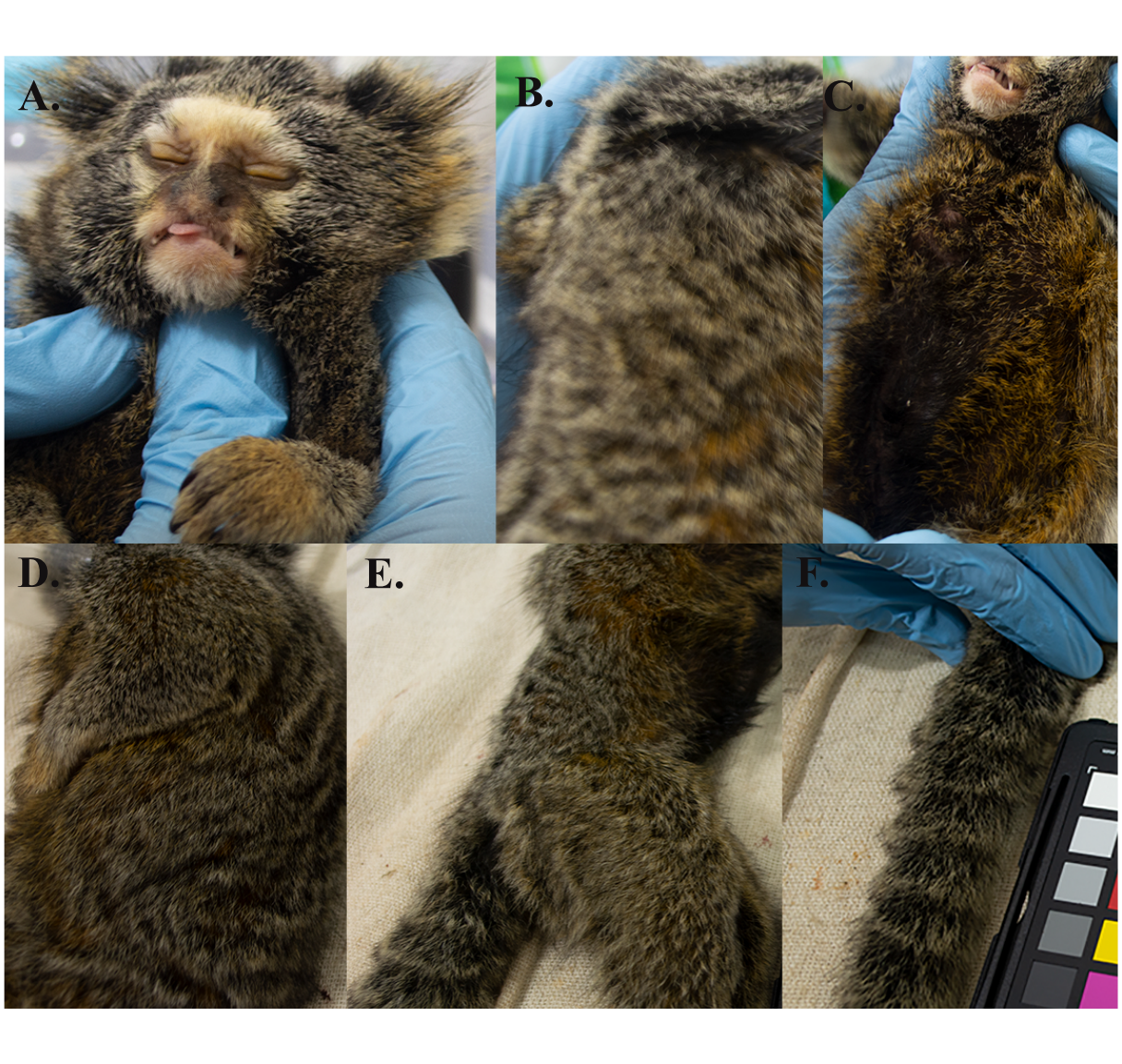

Figure 9. BJT025

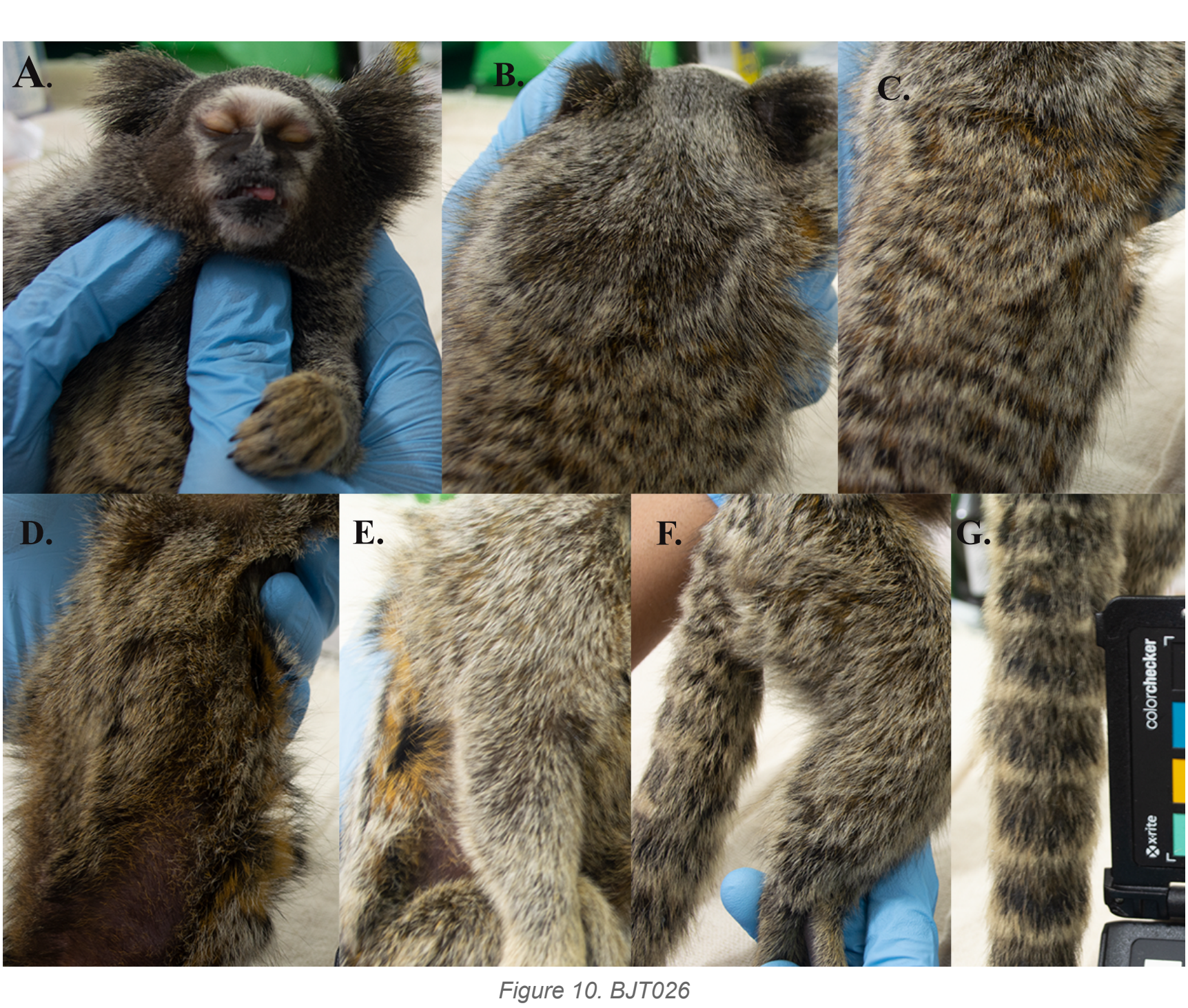

Figure 10. BJT026

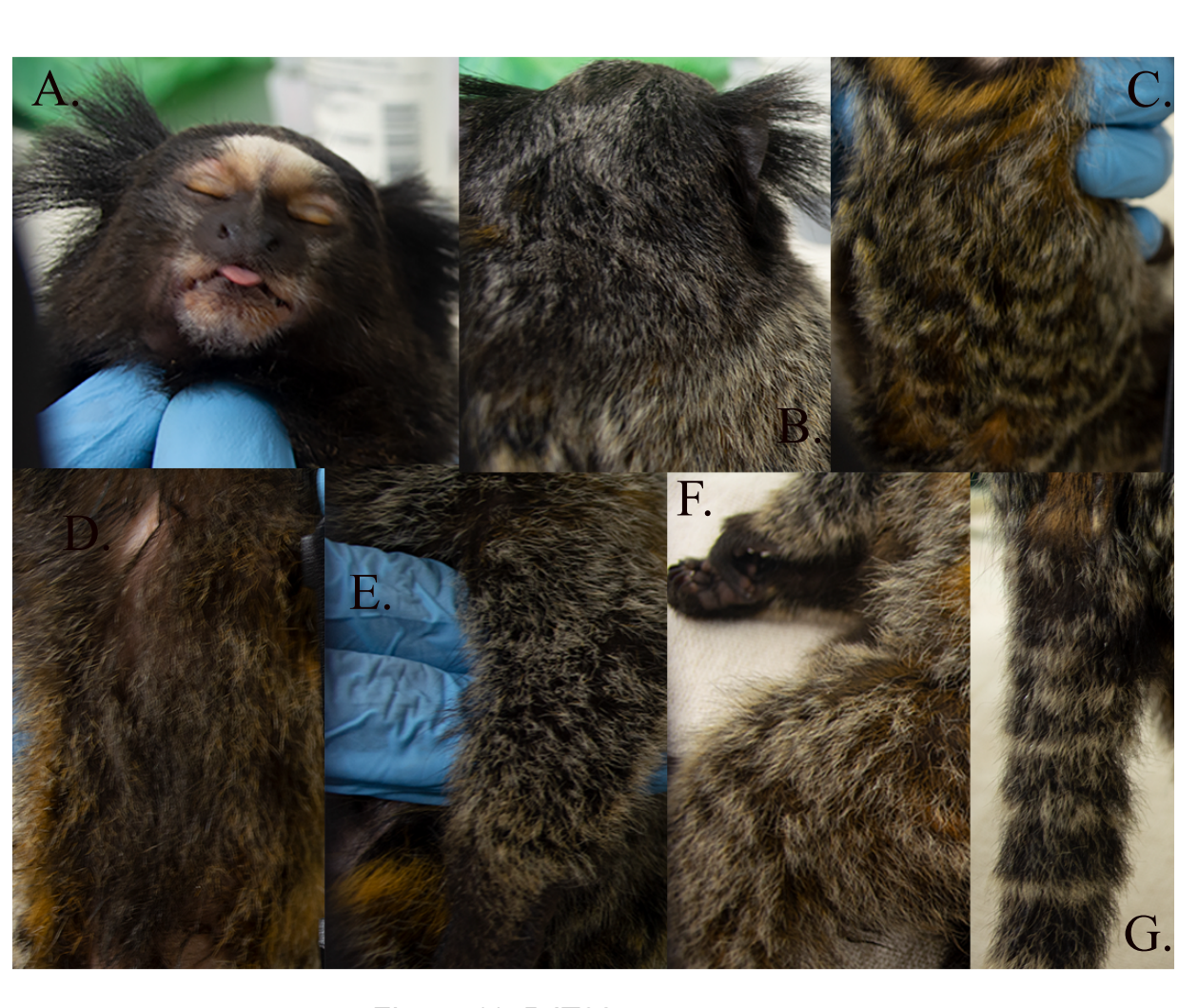

Figure 11. BJT027

Front half of vertex: Pelage has black and grey hairs, with orange tips.
Back half of vertex: Pelage coloration follows neck pattern.
Menton region: Pelage is whitish-grey.
Orbital region: Pelage may be peachy as in *C. jacchus* and *C. penicillata*, or yellowish like *C. aurita*.
Tufts: Pelage ay be light like *C.aurita* or very dense like *C. jacchus*, varying in the amount of black, grey, and orange hair at the tips.
White mark on forehead: Some hybrids have the mark of the forehead. Others will have an *aurita*-like pattern where the face (forehead, orbital, nasal, infraorbital, and mentonian) has beige to light orange hairs.
Lateral sides of the face: Black to dark brown hair that may or may not have grey tips.
Back region: Unlike *C. aurita*, hybrids show striation patterns that are similar to that of *C. penicillata* and *C. jacchus*. The striations may contain a mixture of black, grey and orange patterns or black and whitish-grey streaks. In these hybrids, the orange color tends to be more intense than in *C. aurita*, and greys are more yellowish or orangish instead of whitish than in *C. penicillata* and *C. jacchus*.
Stomach: ***Coloration is highly variable between hybrids.***
Proximal region of legs and arms: ***Legs follow the pattern of the back, but the base arm of the black fur with grey to orange tips.***
Neck: ***Pelage contains black hair with grey tips, there may be variation in the intensity of the grey. Some hybrid individuals will have patches of whitish grey and grey mixed in with the darker black pelage hairs.***
*Tail**: Pelage has black and grey striated pattern and there may be orange tips.

Note: The hands of these hybrids tend to have an orange or yellow tone, similar to *C. aurita*.

Callithrix geoffroyi x *Callithrix sp.*

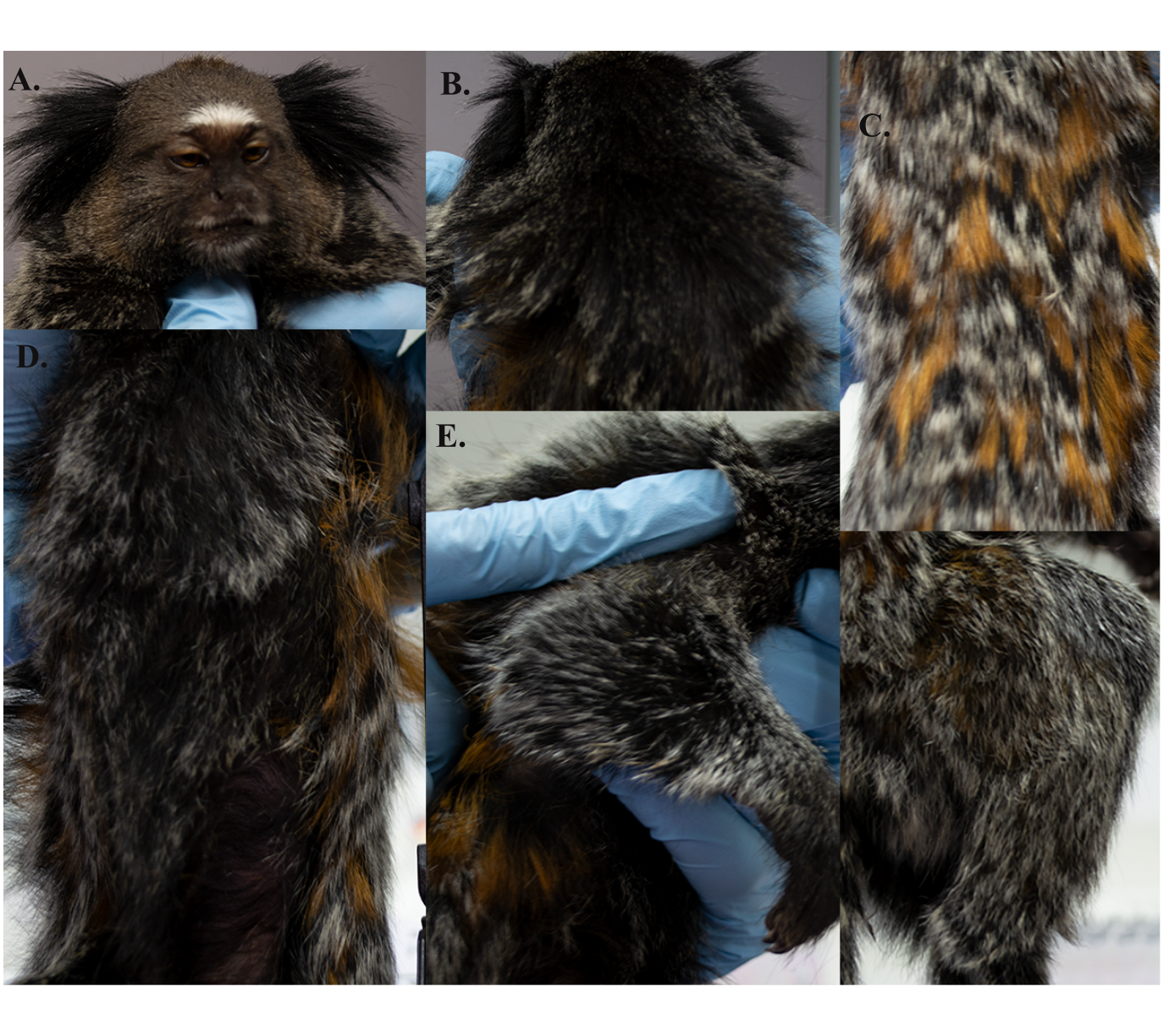

Figure 12. BJT070

Front of vertex (A): Pelage is yellowish with mix of grey and black speckles.
Back of vertex (A): Pelage is black with greyish speckles.
Menton Region (A): Pelage is dark.
Orbital Region (A): Pelage is black close to eyes and peachy below white forehead star.
Tufts (A): The pelage is very dense as in *C. geffroyi*, and similar in volume. Tufts are black, and hairs closer to top of head are shorter and hairs closer to neck are longer.
White star on forehead (A): The forehead possesses a small white star.
Neck (B): Frontal region with black hair, in the dorsal region there are greyish tips.
Back region (C): Pelage striation is black and orange and black and grey. The orange coloration is very obvious and prominent in the hairs of the pelage.
Stomach:Pelage contains straitions of black and grey.
Region proximal to arms and legs (E and F): Legs and arms are black and whitish-grey hairs.

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