Boubli et al. *Zool. Res.* 2021, 42(6): 761−771
https://doi.org/10.24272/j.issn.2095-8137.2021.143

ZR Zoological Research

Article Open Access

Ancient DNA of the pygmy marmoset type specimen *Cebuella pygmaea* (Spix, 1823) resolves a taxonomic conundrum

Jean P. Boubli[1,2,*], Mareike C. Janiak[1], Leila M. Porter[3], Stella de la Torre[4], Liliana Cortés-Ortiz[5], Maria N. F. da Silva[2], Anthony B. Rylands[6], Stephen Nash[6], Fabrício Bertuol[7], Hazel Byrne[8], Felipe E. Silva[9], Fabio Rohe[2], Dorien de Vries[1], Robin M. D. Beck[1], Irune Ruiz-Gartzia[10], Lukas F. K. Kuderna[10], Tomas Marques-Bonet[10], Tomas Hrbek[7,11], Izeni P. Farias[7], Anneke H. van Heteren[12,13,14], Christian Roos[15]

[1] *School of Science, Engineering & Environment, University of Salford, Salford M5 4WT, UK*
[2] *Instituto Nacional de Pesquisas da Amazônia, Manaus, Amazonas* 69060-001, *Brazil*
[3] *Department of Anthropology, Northern Illinois University, DeKalb, IL* 60115-2828, *USA*
[4] *College of Biological and Environmental Sciences, Universidad San Francisco de Quito, Quito* 170901, *Ecuador*
[5] *Department of Ecology and Evolutionary Biology, University of Michigan, Ann Arbor, MI* 48109, *USA*
[6] *Re:wild, Austin, TX* 78767, *USA*
[7] *Laboratório de Evolução e Genética Animal, Universidade Federal do Amazonas, Manaus, Amazonas* 69080-900, *Brazil*
[8] *Department of Anthropology, University of Utah, Salt Lake City, UT* 84112, *USA*
[9] *Research Group on Primate Biology and Conservation, Mamirauá Institute for Sustainable Development, Tefé, Amazonas* 69553-225, *Brazil*
[10] *Experimental and Health Sciences Department (DCEXS), Institut de Biologia Evolutiva, Universitat Pompeu Fabra-CSIC, Barcelona* 08002, *Spain*
[11] *Department of Biology, Trinity University, San Antonio, TX* 78212, *USA*
[12] *Bavarian State Collection of Zoology, Zoologische Staatssammlung München, Staatliche Naturkundliche Sammlungen Bayerns*, 81247 *Munich, Germany*
[13] *GeoBio-Center, Ludwig-Maximilians-Universität München*, 80333 *Munich, Germany*
[14] *Department Biologie II, Ludwig-Maximilians-Universität München*, 82152 *Munich, Germany*
[15] *Gene Bank of Primates and Primate Genetics Laboratory, German Primate Center, Leibniz Institute for Primate Research*, 37077 *Göttingen, Germany*

ABSTRACT

The pygmy marmoset, the smallest of the anthropoid primates, has a broad distribution in Western Amazonia. Recent studies using molecular and morphological data have identified two distinct species separated by the Napo and Solimões-Amazonas rivers. However, reconciling this new biological evidence with current taxonomy, i.e., two subspecies, *Cebuella pygmaea pygmaea* (Spix, 1823) and *Cebuella pygmaea niveiventris* (Lönnberg,

Received: 20 August 2021; Accepted: 11 October 2021; Online: 12 October 2021

Foundation items: This study was supported by the Conselho Nacional de Pesquisa, Brazil (563348/2010), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (3261/2013), NSF (1241066), FAPESP (12/50260-6) and NERC (NE/T000341/1)

*Corresponding author, E-mail: j.p.boubli@salford.ac.uk

1940), was problematic given the uncertainty as to whether Spix's pygmy marmoset (*Cebuella pygmaea pygmaea*) was collected north or south of the Napo and Solimões-Amazonas rivers, making it unclear to which of the two newly revealed species the name *pygmaea* would apply. Here, we present the first molecular data from Spix's type specimen of *Cebuella pygmaea*, as well as novel mitochondrial genomes from modern pygmy marmosets sampled near the type locality (Tabatinga) on both sides of the river. With these data, we can confirm the correct names of the two species identified, i.e., *C. pygmaea* for animals north of the Napo and Solimões-Amazonas rivers and *C. niveiventris* for animals south of these two rivers. Phylogenetic analyses of the novel genetic data placed into the context of cytochrome *b* gene sequences from across the range of pygmy marmosets further led us to re-evaluate the geographical distribution for the two *Cebuella* species. We dated the split of these two species to 2.54 million years ago. We discuss additional, more recent, subdivisions within each lineage, as well as potential contact zones between the two species in the headwaters of these rivers.

Keywords: Historic DNA; DNA taxonomy; Pygmy marmoset; *Cebuella pygmaea*; *C. niveiventris*; Amazon; Type specimen

INTRODUCTION

Pygmy marmosets (*Cebuella*) are the smallest of all living anthropoid primates. They have a wide geographic distribution across the upper Amazon region in northwestern Bolivia, Brazil, Peru, Ecuador, and southern Colombia. A recent molecular genetic study (Boubli et al., 2018) tested the validity of separating this taxon into two subspecies, namely, *Cebuella pygmaea pygmaea* (Spix, 1823) and *Cebuella pygmaea niveiventris* (Lönnberg, 1940), which have been recognized in various studies, including da Cruz Lima (1945), Napier (1976), van Roosmalen & van Roosmalen (1997), and Groves (2001, 2005). Using the mitochondrial cytochrome *b* (cyt *b*) gene and ddRAD nuclear genome sequences from geographically representative samples from both sides of the Solimões River (upper Amazon), Boubli et al. (2018) recovered two highly supported clades that diverged 2.25 million years ago (Ma), leading the authors to suggest the existence of two species of *Cebuella*; one comprising pygmy marmosets sampled on the northern side (left bank) of the Solimões and the other comprising samples collected on the southern side (right bank) (see also Porter et al., 2021). Reconciling the results of these molecular and morphological analyses with the current *Cebuella* taxonomy was confusing, however, due to the uncertainty of the provenance of Spix's holotype of *Iacchus pygmaeus* (Boubli et al., 2018; Rylands et al., 2009).

Johann Baptist von Spix described *pygmaea* based on a pet pygmy marmoset given to him, probably by a local Tikuna Indian, during his visit to the upper Solimões River on the Brazilian-Colombian border (von Spix, 1823; von Spix & von Martius, 1824). He assigned the type locality as near Tabatinga, then a small village in Brazil, near the Colombian border on the left bank of the Solimões. In 1940, Lönnberg described *niveiventris* as a distinct pygmy marmoset from the Lago Ipixuna, approximately midway between the Tefé and Purus rivers, a little west of Coarí on the right (south) bank of the Solimões (Lönnberg, 1940). To describe this new taxon, he compared his type series with specimens collected by the Olalla brothers near the Brazilian village of Eirunepé (then known as João Pessoa) on the right bank of the Juruá River (see Boubli et al., 2018). For him, the Juruá pygmy marmosets were typical *pygmaea,* even though Eirunepé is more than 200 km south of Tabatinga and on the opposite bank of the Solimões. In his publication, he affirmed that the Spix's type came from the right bank of the Solimões, and thus the opposite bank of Tabatinga (Lönnberg, 1940).

Doubt concerning the origin of Spix's type specimen (left or right bank of the Solimões at Tabatinga) complicated the attribution of the proper names of the two clades identified by Boubli et al. (2018). In that study, the pygmy marmosets from the south (right) bank of the Solimões formed a clade to the exclusion of those from the north bank and northwards to the right bank of the Japurá River. If Spix's type came from the right bank of the Solimões —specifically the mouth of the Javarí River, as stated by Lönnberg (1940)—*niveiventris* could be a junior synonym of *pygmaea*. In this case, animals from the left bank (north) bank of the Solimões would have no available name and thus constitute a new taxon. On the other hand, if the *pygmaea* type originally came from near Tabatinga on the left bank of the Solimões, then *niveiventris* would be available for pygmy marmosets on the right bank of the Solimões and *pygmaea* would be available for pygmy marmosets on the left bank, as suggested by van Roosmalen & van Roosmalen (1997).

In the absence of novel historical evidence, obtaining genetic material from Spix's type specimen, as well as material from the supposed type locality Tabatinga, presented the best means to resolve this taxonomic deadlock. Since its first use in museum collections in the 1980s, the retrieval and amplification of ancient DNA (Higuchi et al., 1984; Pääbo, 1985) has provided new insights into the evolution, biology, and taxonomy of organisms across the tree of life (Burrell et al., 2015). Perhaps the most famous example, the recovery of the first fragments (Green et al., 2006; Noonan et al., 2006) and finally complete genomes (Prüfer et al., 2014) of Neanderthals (*Homo neanderthalensis*), provided evidence of interbreeding between this species and our own. Ancient DNA can also be a way to improve museum collections, by assigning likely origins and species designations to museum specimens of uncertain provenance (Shepherd et al., 2013) and ascertaining the accuracy of specimen data (Verry et al., 2019). Additionally, many taxonomic puzzles have been solved by analysis of DNA from museum specimens, including equids (Orlando et al., 2009), monk seals (Scheel et al., 2014), leaf monkeys (Roos et al., 2020), and most recently the reclassification of the dire wolf from *Canis dirus* to *Aenocyon dirus*, which had been largely ignored since it was first proposed in 1918 (Perri et al., 2021).

Ancient DNA from Spix's type specimen of *pygmaea* could help determine its relationship with the two clades of *Cebuella* recovered previously (Boubli et al., 2018) and establish the provenance of this specimen (i.e., right or left bank of the Solimões), allowing valid names to be attributed to the two species of pygmy marmosets.

In this study, we present the first molecular data from Spix's type specimen of *Cebuella pygmaea* (housed in the Bavarian State Collection of Zoology), as well as novel mitochondrial genomes (mitogenomes) from modern pygmy marmosets sampled in the purported type locality of Spix's specimen, near the town of Tabatinga. By placing these data in the context of available cyt *b* mitochondrial barcode gene sequences from pygmy marmosets across their distribution, we provide the first range-wide phylogenetic analysis of this taxon and confirm the correct naming of the two species of *Cebuella*, thereby resolving the present taxonomic uncertainty. We also re-evaluate the current geographical distribution hypothesis for the two *Cebuella* species (Mittermeier et al., 2013) based on our results and those of Porter et al. (2021).

MATERIALS AND METHODS

Holotype sampling and sequencing

Dried tissue skin scrapings were obtained from the stuffed type specimen of *pygmaea* from the Bavarian State Collection of Zoology, Munich, Germany. The skin scrapings were sampled from the inner side of the damaged skin together with pieces of the damaged skin. To minimize the risk of environmental (including human) and cross-sample contamination, DNA extraction and library preparation were performed in an ancient DNA laboratory, in which all standards for such laboratories were implemented (UV light decontamination, positive air pressure, separate sterile working areas, protective clothing, and negative controls during DNA extraction and library preparation for sequencing). DNA from the holotype was extracted with a column-based method that recovers small DNA fragments (Dabney et al., 2013; Rohland et al., 2004). The DNA concentration was measured with a Qubit 4.0 fluorometer (ThermoFisher Scientific, USA), and DNA quality and degradation status were checked on a Bioanalyzer 2100 (Agilent Technologies, USA). Approximately, 50 ng of genomic DNA was subjected to shotgun library preparation with a NEBNext Ultra II DNA Library Prep Kit (E7103, New England Biolabs, USA) following the standard protocols of the supplier. However, DNA fragmentation prior to library preparation was omitted as the DNA was already largely degraded (50–150 bp). A sequencing library was also prepared from the DNA extraction negative control. After end repair, adapter ligation, and ligation cleanup (without size selection) using the kit's purification beads, the libraries were indexed with multiplex oligos and then cleaned again with the purification beads. Library concentration and size distribution were measured with a Qubit fluorometer and Bioanalyzer, respectively, and molarity was quantified via quantitative polymerase chain reaction (qPCR) using a NEBNext Library Quant Kit (New England Biolabs, USA). Sequencing was conducted on an Illumina HiSeq 4000 (100 bp single-end reads) at the NGS Integrative Genomics (NIG) core unit at the University Medical Center Göttingen, Germany. Raw sequencing reads were demultiplexed with Illumina software. Subsequent bioinformatics analyses were performed with the Geneious package v11.1.2. First, we trimmed and quality-filtered the reads with BBDuk 37.64 in the BBTools package (https://jgi.doe.gov/data-and-tools/bbtools/) and removed duplicate reads with Dedupe 37.64 (BBTools package); both filtering steps were conducted with standard settings. For assembly, reads were mapped onto the mitogenome of the *C. pygmaea* reference mitogenome NC_021942.1 and a *C. niveiventris* specimen (JT95, see below) using the Geneious assembler. We applied different settings (high and custom sensitivity, 5–10 iterations), while the advanced standard settings remained unchanged. To check for potential DNA damage (C to T or G to A changes) in historical specimens (Rambaut et al., 2009), we calculated the base frequencies in our *C. pygmaea* dataset with PAUP v4.0a (Swofford, 2002) and compared the base frequencies of the holotype with those of modern specimens. The newly produced mitogenome was manually checked and then annotated with Geneious v11.1.2.

Modern specimen sampling and sequencing

For mitogenome analysis, we used samples of pygmy marmosets from the town of Tabatinga, Brazil (Tabatinga 1 henceforth, TAB1), and from the upper Japurá River (JAP720, JAP723) and Jutaí River (JT56, JT57, JT79, JT95). These specimens were deposited in the Zoological Collection of the Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus. We extracted total genomic DNA from muscle tissues preserved in alcohol using standard protocols (Sambrook et al., 1989). Complete mitogenomes for these specimens were generated via shotgun high-throughput sequencing using a NEBNext Ultra II FS DNA Library Prep Kit (New England Biolabs, USA) (TAB1, JAP720, JT95) or the Qiagen MagAttract HMW DNA Kit according to the manufacturer's specifications (JAP723, JT56, JT57, JT79). Before library preparation, 100 ng of DNA was fragmented (200–450 bp) during the kit's fragmentation step. All follow-up steps adhered to the kit protocols. Sequencing at NIG was conducted on an Illumina HiSeq 4000 (50 bp single-end reads) (TAB1, JAP720, JT95) or an Illumina NovaSeq6000 machine (150 bp paired-end reads (JAP723, JT56, JT57, JT79). Read mapping and mitogenome assembly were conducted as described for Spix's holotype.

The mitochondrial cyt *b* gene was amplified for a second individual from Tabatinga (Tabatinga 02) and another individual from the Jutaí River (JT32) by PCR with the primers MonkeyGluF1 (5'-CCATGACTAATGATATGAAAARCC-3') and MonkeyProR1 (5'-AGAATSTCAGCTTTGGGTGTTG-3') (see Boubli et al., 2018). The PCR products were purified using ExoSap (Werle et al., 1994) and subjected to fluorescent dye-terminator (ddNTP) sequencing following the manufacturer's recommended protocols for BigDye sequencing chemistry (Applied Biosystems, USA) and using the primers MonkeyCytbF2 (5'-GGATCAARYAAYCCRTCAGG-3'), MonkeyCytbR1 (5'-GCBCCTCAGAADGATATTTG-3'), and MonkeyCytbR2 (5'-CGTAGRATTGCRTATGCRAA-3') (Boubli et al., 2018). After the cycle sequencing reaction, the products were precipitated with 100% ethanol/125 mmol/L EDTA solution, re-suspended in Hi-Di formamide, and resolved on an ABI 3130xl automatic sequencer (Applied Biosystems, USA). Sequences were assembled, edited, and trimmed using Geneious v8.1.8.

Additional sequences

We supplemented the newly sequenced mitochondrial data with cyt *b* sequencing data from previous publications (Boubli et al., 2018; Porter et al., 2021), as well as mitogenomes available in GenBank. Our final dataset consisted of cyt *b* sequences from 65 individuals and full mitogenomes from 15 individuals. Of the 65 individuals included in the cyt *b* alignment, 52 belonged to the genus *Cebuella*, including both contemporary samples collected from wild primates and museum samples, covering most of the geographical distribution of pygmy marmosets (Table 1, Figures 1, 2). Importantly, this included a pygmy marmoset from the town of Benjamin Constant, Brazil, which is located on the right (south) bank of the Solimões, directly across the river from Tabatinga (Figure 2). Additionally, we included 13 cyt *b* sequences from other platyrrhine species (*Aotus*, *Saguinus*, *Callimico*, *Callithrix*, *Mico*, and *Callibella*) to provide a broader phylogenetic context. Alignment of the complete mitogenomes included the newly sequenced pygmy marmosets from Tabatinga (TAB1), Japurá (JAP720, JAP723), Jutaí (JT56, JT57, JT79, JT95), and Spix's *pygmaea* holotype (aDNA578), as well as publicly available genomes from *Cebuella pygmaea*, *Callithrix jacchus*, *Callimico goeldii*, *Saguinus oedipus*, *Aotus azarai*, *A. lemurinus*, and *A. nancymaae*. Details and accession numbers for all sequences included in this study are presented in Supplementary Table S1.

Phylogenetic analyses

All sequencing alignments were performed using the MAFFT v7.309 (Katoh & Standley, 2013) alignment plugin in Geneious v9.1.8 and checked by eye. Using the alignment of cyt *b* sequences from 65 individuals, including 52 *Cebuella* samples and 13 other platyrrhines, we jointly estimated phylogeny and diversification times under a strict clock model implemented in BEAST v2.6.3 (Bouckaert et al., 2019). A strict clock model was chosen as it is most appropriate for single locus mitochondrial data, where rates are not expected to vary across branches. To date the tree, we used a fossil calibration dating the split between Aotinae and Callitrichidae based on the presence of the stem Callitrichidae *Lagonimico* (de Vries & Beck, in prep.). The Aotinae and Callitrichidae split was given a hard minimum bound of 13.4 Ma following the age published for *Lagonimico* (following the age listed by Kay, 2015), and a generous soft maximum bound of 35.0 Ma based on the age of the oldest stem catarrhine *Catopithecus browni* (using the maximum age of the L-41 locality in the Fayum Depression proposed by Seiffert, 2006). The calibration was given a log-normal distribution with an S parameter of 0.8 and M parameter of 1.755 to set the 95% quantile of the maximum age at 35.0 Ma. We partitioned the cyt *b* sequence alignment into three partitions based on codon position and used bModelTest (Bouckaert & Drummond, 2017) to assign appropriate substitution models to each partition. BEAST2 was allowed to run for 50 million generations, with sampling at every 5 000 trees and the first 10% discarded as burn in. Additionally, we compared our topology to a smaller subset of samples using full mitogenomes under a maximum-likelihood approach in RAxML v8 (Stamatakis, 2014). Robustness of the RAxML analyses was assessed via 1 000 bootstrap replicates. Alignment of the full mitochondrial sequences, the xml files used as input for BEAST2, and the command used to run RAxML are provided in the Supplementary Materials.

RESULTS

Holotype sampling and sequencing

We successfully retrieved mitochondrial DNA from Spix's *Cebuella pygmaea* type specimen and sequenced a total of 14 726 unambiguous base pairs, representing 89.1% of the complete mitogenomes, with an average sequencing depth of 3.8x. From the cyt *b* gene, we retrieved unambiguous base pairs for 59.6% of the sequence length. Base frequencies in the mitogenome of the *C. pygmaea* holotype (A=33.21%, C=26.31%, G=13.27%, and T=27.21%) were similar to those of modern specimens (mean: A=33.17%, C=26.51%, G=13.09%, and T=27.23%), indicating that DNA damage in the *C. pygmaea* holotype was minimal to zero.

Modern sampling and sequencing

We sequenced the mitogenomes for one of the newly collected specimens of pygmy marmosets from Tabatinga (TAB1) and six samples from other locations (JAP720, JAP723 JT56, JT57, JT79, and JT95) and retrieved full mitogenomes (100% coverage) from all modern samples (TAB1: 1717.7x; JAP720: 61.4x; JAP723: 4778.3x; JT56: 606.7x, JT57: 1007.1x, JT79: 3555.2; JT95: 90.0x). The newly sequenced mitogenomes of the holotype and modern samples were deposited in GenBank and are available under accession numbers MW733803–MW733806 and MZ747451–MZ747454 (Supplementary Table S1).

Phylogenetic analyses

BEAST2 analysis of the cyt *b* alignment supported a clear separation of two *Cebuella* clades and dated the split between clades at 2.54 Ma (95% highest posterior density (HPD) interval: 1.51–3.82 Ma) (Figure 3). The same topology was recovered by RAxML analysis of the full mitogenome alignment of the sample subset, with 100% bootstrap support (Figure 4). In both analyses, the sequence from Spix's holotype grouped with contemporary animals sampled in Tabatinga on the north bank of the Solimões River, and other localities north of the Amazon/Solimões, including the Japurá River, while the pygmy marmoset from the south bank of the Solimões, near Benjamin Constant, grouped with the second clade. Of note, disregarding the missing data, the cyt *b* sequence of the holotype was identical to that of the two Tabatinga specimens.

Our results indicated a geographic distribution for *C. pygmaea* that is limited by the Napo and Solimões rivers in the south, the Andes in the west, and the Japurá-Caquetá in the north (however, there are some discrepancies that are discussed below). The distribution of *C. niveiventris* is concordantly limited by the Napo and Solimões rivers in the

Table 1 List of voucher specimens and tissue samples used in this study and their localities

Sample ID	Genus	Species	Collection site	Latitude	Longitude
EC_H1	*Cebuella*	*pygmaea*	Flor del Pantano (Group 1), Orellana, Ecuador	–0.4517989	–76.864899
EC_H2	*Cebuella*	*pygmaea*	Flor del Pantano (Group 3), Orellana, Ecuador	–0.4517989	–76.864899
EC_H3	*Cebuella*	*pygmaea*	Flor del Pantano (Group 4), Orellana, Ecuador	–0.4517989	–76.864899
EC_H4	*Cebuella*	*pygmaea*	San Pablo, Sucumbíos, Ecuador	–0.2735964	–76.421896
EC_H5	*Cebuella*	*pygmaea*	San Pablo, Sucumbíos, Ecuador	–0.2735964	–76.421896
EC_H6	*Cebuella*	*niveiventris*	Tiputini Biological Station, Orellana, Ecuador	–0.6381041	–76.149596
AMNH_72033	*Cebuella*	*pygmaea*	Curaray, Maynas, Loreto, Peru	–2.3667	–74.0833
AMNH_72035	*Cebuella*	*pygmaea*	Curaray, Maynas, Loreto, Peru	–2.3667	–74.0833
AMNH_72035	*Cebuella*	*pygmaea*	Curaray, Maynas, Loreto, Peru	–2.3667	–74.0833
AMNH_72037	*Cebuella*	*pygmaea*	Curaray, Maynas, Loreto, Peru	–2.3667	–74.0833
AMNH_72038	*Cebuella*	*pygmaea*	Curaray, Maynas, Loreto, Peru	–2.3667	–74.0833
AMNH_73751	*Cebuella*	*niveiventris*	Orosa, Mariscal Ramon Castilla, Loreto, Peru	–3.5333	–72.1833
AMNH_74054	*Cebuella*	*niveiventris*	Orosa, Mariscal Ramon Castilla, Loreto, Peru	–3.5333	–72.1833
AMNH_74055	*Cebuella*	*niveiventris*	Orosa, Mariscal Ramon Castilla, Loreto, Peru	–3.5333	–72.1833
AMNH_74056	*Cebuella*	*niveiventris*	Orosa, Mariscal Ramon Castilla, Loreto, Peru	–3.5333	–72.1833
AMNH_74366	*Cebuella*	*pygmaea*	Apayacu, Maynas, Loreto, Peru	–3.4833	–72.1833
AMNH_74367	*Cebuella*	*pygmaea*	Apayacu, Maynas, Loreto, Peru	–3.4833	–72.1833
AMNH_74368	*Cebuella*	*pygmaea*	Apayacu, Maynas, Loreto, Peru	–3.4833	–72.1833
AMNH_74369	*Cebuella*	*pygmaea*	Apayacu, Maynas, Loreto, Peru	–3.4833	–72.1833
AMNH_75280	*Cebuella*	*niveiventris*	Sarayacu, Ucayali, Loreto, Peru	–6.7833	–75.1167
AMNH_76327	*Cebuella*	*pygmaea*	Sarayacu, Ucayali, Loreto, Peru	–6.7833	–75.1167
AMNH_76328	*Cebuella*	*pygmaea*	Sarayacu, Ucayali, Loreto, Peru	–6.7833	–75.1167
AMNH_98312	*Cebuella*	*pygmaea*	Iquitos, Maynas, Loreto, Peru	–3.7667	–73.25
FMNH_54290	*Cebuella*	*pygmaea*	Río Copataza, Pastaza, Ecuador	–2.11667	–77.449997
FMNH_71003	*Cebuella*	*pygmaea*	Leticia, Amazonas, Colombia	–4.15	–69.950003
FMNH_87136	*Cebuella*	*niveiventris*	Río Maniti, Santa Cecilia, Maynas, Peru	–3.4333354	–72.766674
FMNH_87137	*Cebuella*	*niveiventris*	Río Maniti, Santa Cecilia, Maynas, Peru	–3.4333354	–72.766674
FMNH_88997	*Cebuella*	*niveiventris*	Alto Yavari Mirim, boca Yaque, Mariscal Ramon, Peru	–4.4499988	–71.783336
FMNH_88998	*Cebuella*	*niveiventris*	Alto Yavari Mirim, boca Yaque, Mariscal Ramo, Peru	–4.4499988	–71.783336
FMNH_122750	*Cebuella*	*niveiventris*	Quistococha, Maynas, Loreto, Peru	–3.8333284	–73.266669
FMNH_122752	*Cebuella*	*niveiventris*	Quistococha, Maynas, Loreto, Peru	–3.8333284	–73.266669
UMMZ_82856	*Cebuella*	*pygmaea*	Río Napo, Intillama, Napo, Ecuador	–0.9829959	–77.817001
UMMZ_82857	*Cebuella*	*pygmaea*	Río Napo, Intillama, Napo, Ecuador	–0.9829959	–77.817001
JAP720	*Cebuella*	*pygmaea*	Río Japurá, Amazonas, Brazil	–1.8424722	–69.022833
JAP723	*Cebuella*	*pygmaea*	Río Japurá, Amazonas, Brazil	–1.8424722	–69.022833
JAP724	*Cebuella*	*pygmaea*	Río Japurá, Amazonas, Brazil	–1.8424722	–69.022833
Tabatinga_01	*Cebuella*	*pygmaea*	Tabatinga, Amazonas, Brasil	–4.241472	–69.944472
Tabatinga_02	*Cebuella*	*pygmaea*	Tabatinga, Amazonas, Brasil	–4.238944	–69.944667
CTGA-M170	*Cebuella*	*niveiventris*	Igarapé do Jacinto, Tapauá, Amazonas, Brazil	–5.7	–63.2
FR20	*Cebuella*	*niveiventris*	Lago Xadá, Amazonas, Brazil	–5.2620278	–60.722944
CCM19	*Cebuella*	*niveiventris*	Benjamin Constant, Amazonas, Brazil	–4.382494	–70.008512
MNFS1019	*Cebuella*	*niveiventris*	Ocidente, Acre, Brazil	–8.5722222	–72.8
MNFS1020	*Cebuella*	*niveiventris*	Ocidente, Acre, Brazil	–8.5722222	–72.8
MNFS1361	*Cebuella*	*niveiventris*	Ocidente, Acre, Brazil	–8.5722222	–72.8
CCM23	*Cebuella*	*niveiventris*	Codajas, Amazonas, Brazil	–3.894248	–62.071256
CCM251	*Cebuella*	*niveiventris*	Lago Matupiri, Río Madeira	–5.5986111	–61.006944
JT79	*Cebuella*	*niveiventris*	Río Jutaí, Brazil	–3.31174	–67.532681
JT95	*Cebuella*	*niveiventris*	Río Jutaí, Brazil	–3.735624	–67.469317
JT57	*Cebuella*	*niveiventris*	Río Jutaí, Brazil	–3.218021	–67.334289
JT56	*Cebuella*	*niveiventris*	Río Jutaí, Brazil	–3.218021	–67.334289
JT32	*Cebuella*	*niveiventris*	Río Jutaí, Brazil	–3.21801	–67.334296
Holotype	*Cebuella*	*pygmaea*	Adjacent to the town of Tabatinga, Brazil		

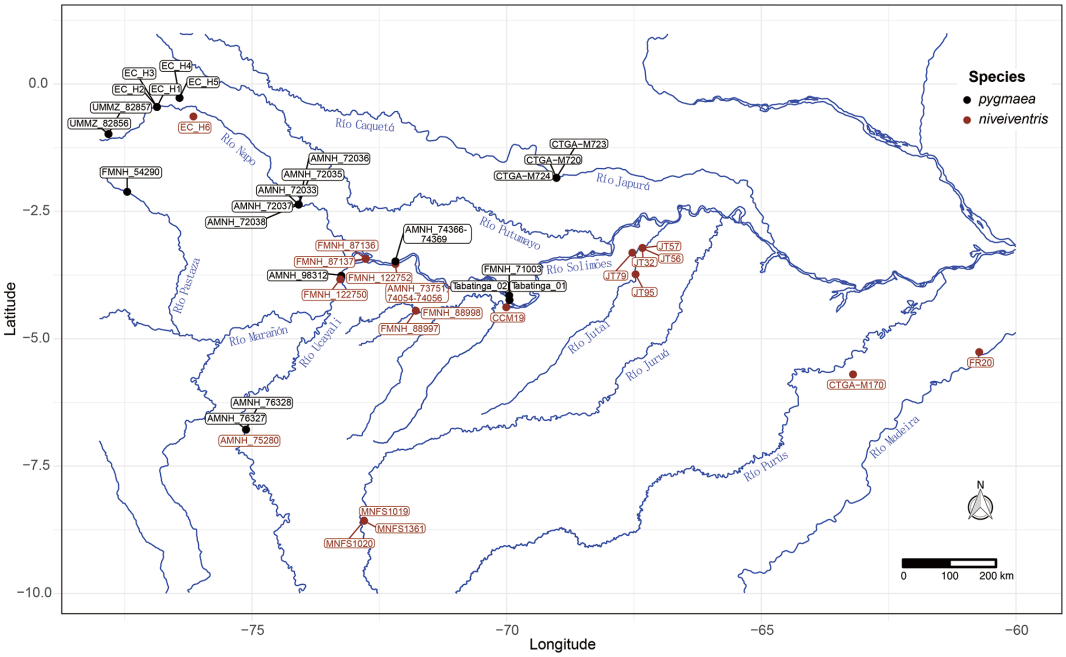

Figure 1 Geographical location of all pygmy marmoset samples used in this study

Black labels represent specimens with *C. pygmaea* mitochondrial haplotype and red labels represent specimens with *C. niveiventris* mitochondrial haplotype.

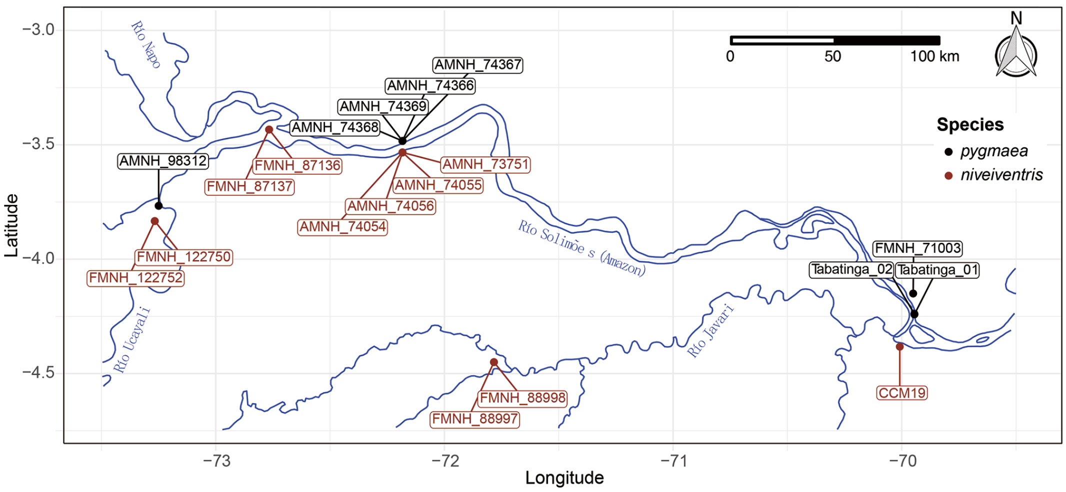

Figure 2 Close up map of western Amazonia showing precise locations of samples used in this study collected between the mouth of the Javari and Ucayali rivers, which are both south bank tributaries of the Solimões (Amazonas)

north and the Madeira River in the east. At this stage, our data do not offer unambiguous evidence to propose a southern limit for *C. niveiventris* (Figure 5).

Our BEAST2 tree indicated further sub-structuring within both *C. pygmaea* and *C. niveiventris*. Within the *C. pygmaea* clade, animals from the region between the Putumayo and Japurá rivers clustered separately from the animals collected south of the Putumayo. In the *C. niveiventris* clade, animals from the eastern extent of the species range, in the area between the Purus and Madeira rivers (CTGA-M170, FR20, CCM251), formed a cluster mostly separate from animals west of the Purus. While *C. niveiventris* west of the Ucayali and south of the Napo formed a subcluster in the phylogeny (Figure 3).

Four of the museum specimens labeled as having been collected south of the Napo and Solimões rivers fell within the *C. pygmaea* clade (Figure 3, marked with asterisks, Figure 1): FMNH (Field Museum of Natural History) 54290, AMNH (American Museum of Natural History) 76327, AMNH 76328, and AMNH 98312.

DISCUSSION

Based on their phylogenetic analysis using newly generated genomic data for a relatively large sample of individuals, Boubli et al. (2018) proposed the division of pygmy marmosets into two species of *Cebuella* (see Boubli et al., 2018; Garbino

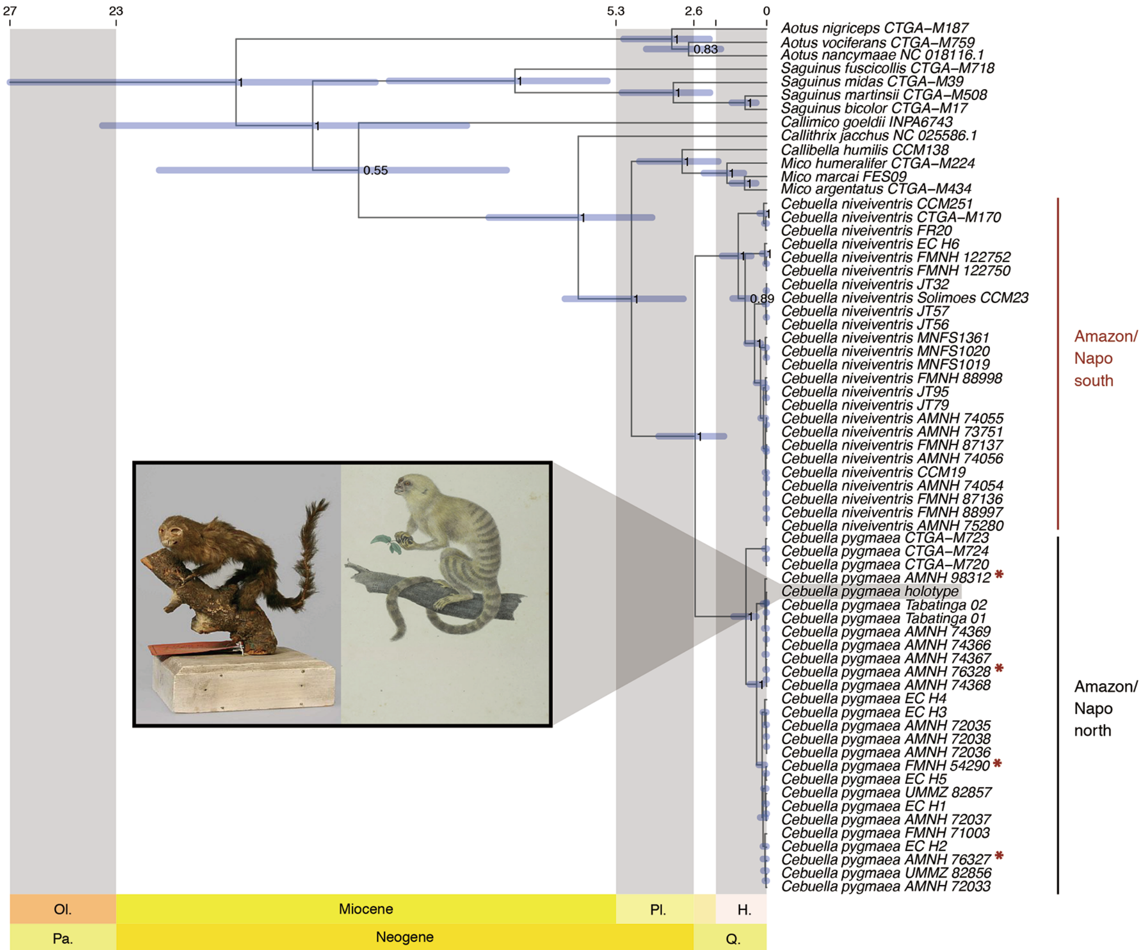

Figure 3 BEAST2 cytochrome *b* time-tree for 65 primate samples, including 52 pygmy marmosets and 13 other taxa as outgroups

Numbers in nodes correspond to posterior support and error bars represent 95% HPD intervals. Inset is picture of *Cebuella pygmaea* type and original drawing by von Spix (1823). See Figures 1 and 2 for map showing localities for all specimens used in this phylogenetic analysis. Asterisks mark museum samples that were originally labeled as collected in south of the Amazon and Napo rivers but grouped with *C. pygmaea*.

et al., 2019; see also Porter et al., 2021) separated by the Solimões-Amazonas River. However, as pointed out by these authors, confirming the names to be attributed to these two newly identified clades was hindered by the uncertainty of the type locality of Spix's *Cebuella pygmaea* (Lönnberg, 1940; see Boubli et al., 2018; Garbino et al., 2019). Garbino et al. (2019) provided an interpretation of the historic literature and concluded that the type specimen was, in fact, obtained north of the Solimões River, contra Lönnberg (1940), who stated that its origin was the mouth of the Javari River, south of the Solimões. Our results resolve this issue definitively, as we show that Spix's type specimen is more closely related to contemporary pygmy marmosets from Tabatinga, on the north bank of the Solimões River, than to animals from the south bank. We also reaffirm that museum collections are a valuable source for genetic and taxonomic investigations of primates, particularly of name-bearing types, and highly damaged DNA, as is typically extracted from such old material, can be analyzed with modern high-throughput sequencing technologies.

The holotype and modern Tabatinga pygmy marmosets formed a clade with samples from the right bank of the Japurá River and other locations north of the Solimões River that split from the southern clade, south of the Solimões approximately 2.54 Ma, confirming the separation of the genus *Cebuella* into two species: i.e., *Cebuella pygmaea* (Spix, 1823), north of the Solimões River, and *Cebuella niveiventris* Lönnberg, 1940, south of the Solimões in that region (in agreement with Boubli et al., 2018).

The morphological analyses of Garbino et al. (2019) and Porter et al. (2021) identified the Napo River as the southern range limit of *C. pygmaea* in Peru and Ecuador. Previously, it had been thought that the divide was marked by the Amazonas-Marañón rivers, extending west to the left bank of the Pastaza River (Mittermeier et al., 2013; van Roosmalen & van Roosmalen, 1997). Pygmy marmosets sampled south of the Solimões-Amazonas and Napo rivers largely grouped with the *C. niveiventris* clade. The sample from Tiputini, Ecuador (with haplotype EC_H6, Porter et al., 2021) collected on the right bank of the upper Napo was clearly nested in the *C. niveiventris* clade.

Interestingly, as stated by Porter et al. (2021), four of the museum specimens are noteworthy exceptions and fall within the *C. pygmaea* clade, despite sampling locations south of the

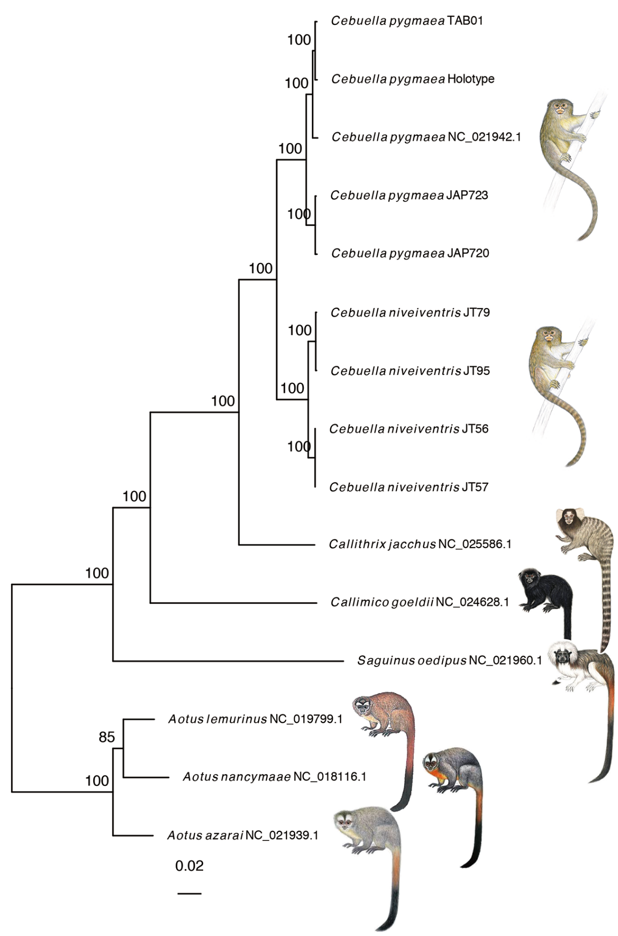

Figure 4 RAxML phylogenetic tree for mitogenome dataset of 15 samples, including Spix's *pygmaea* type

Numbers at nodes correspond to bootstrap support. See Figures 1 and 2 for map showing all localities for specimens used in this phylogenetic analysis. Drawings by Stephen Nash.

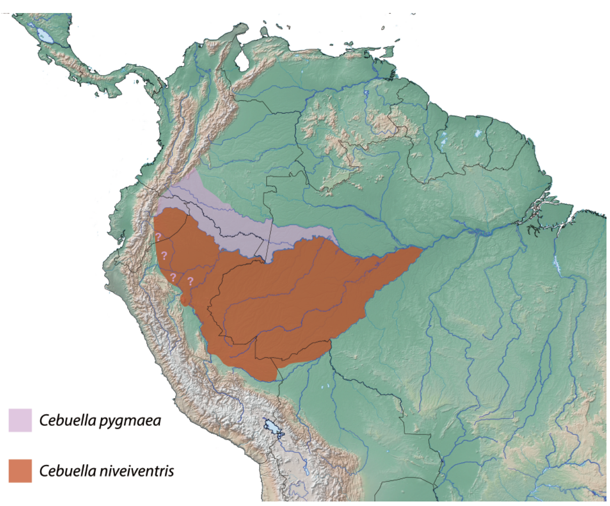

Figure 5 Distribution of two species of pygmy marmosets (*Cebuella*)

Purple question marks indicate range extension of *C. pygmaea* as proposed by Porter et al. (2021).

Napo River. We have highlighted these specimens with asterisks in Figure 3. Sample FMNH 54290 was collected in Ecuador, near the headwaters of the Copataza River on 8 April 1939 by R. Olalla, according to Chicago's FMNH records. The collecting locality may be inaccurate, or the individual may have been moved by people who use them as pets (something that continues today, de la Torre pers. obs.), or changes in rivers due to meandering caused individuals from one bank to be isolated on islands that later connected to the other bank, allowing admixture between populations (see Boubli et al., 2018; Porter et al., 2021). FMNH 54290 was classified by Garbino et al. (2019) as *C. niveiventris* (Type "1") based on its pelage pattern (Figure 6). Considering that it carries a *C. pygmaea* mitochondrial haplotype, this could be a case of admixture causing mitochondrial introgression. Such admixture events are not uncommon in the headwaters of Amazonian rivers (Naka et al., 2012; Weir et al., 2015), but further sampling in this area is needed to test this hypothesis.

The case for samples AMNH 76327, AMNH 76328, and AMNH 98312 is harder to reconcile as they were collected at the southern edge of the distribution of *C. niveiventris*, yet all three grouped with the *C. pygmaea* clade in our analysis. Samples AMNH 76327 and AMNH 76328 were collected for the AMNH by "Olalla & Hijos" on 16 March and 4 April 1927, respectively. The collection sites for these specimens are close to that of a third specimen, AMNH 75280, which was collected by the same group later that year (Haven Wiley, 2010). Sample AMNH 98312 was collected on 28 January 1929 and is labeled as coming from near Iquitos, a known wildlife trading hub.

It is possible that the location information available for these specimens is inaccurate. In fact, the provenance of other specimens collected by the Olalla brothers has previously been called into question (Haven Wiley, 2010; Marsh, 2014). The most problematic specimens appear to be those first purchased by Harvey Bassler (e.g., AMNH 98312, included here) before coming to the AMNH, as the Olalla brothers did not reliably record which riverbank was sampled until late 1926 (Haven Wiley, 2010). Several of the samples included here were collected during an expedition when the Olalla brothers appear to have realized the importance of river boundaries and began purposefully collecting on opposite sides of a river, detailing the bank clearly in their sample notes (Haven Wiley, 2010). Samples AMNH 74366–74369 and AMNH 73751, 74054–74056 were collected during this

Figure 6 Underpart view of Chicago's Field Museum of Natural History voucher specimen FMNH 54290, *Cebuella niveiventris* Type "1" (*sensu* Garbino et al., 2019, photo by J.E.S. Villavicencio)

expedition and clearly separated into *pygmaea* and *niveiventris* based on our analysis, in accordance with provenance (Figure 3). For samples collected prior to this, it is often unclear whether they were collected from the left or right bank, as in the case of AMNH 72033–38 collected in late 1925. Our results, however, provide strong evidence that these were collected from the left (northern) bank of the Napo River (see Porter et al., 2021).

As for samples AMNH 76327–28 and 75280 (discussed above), it is possible that they were collected on opposite banks of the Ucayali River, but not explicitly marked as such, despite being collected after 1926. In this case, AMNH 75280 more likely originated from the right (eastern) bank, as it groups closely with other samples collected on this side (e.g., FMNH 88997, AMNH 74054). In fact, a detailed analysis of the precise collection sites in that Olalla expedition by Haven Wiley (2010) shows that AMNH 76327 and AMNH 76328 were collected from a different basecamp site than AMNH 75280. The AMNH 76327–28 specimens were collected when the Olalla brothers' camp was located at site #2 on the left bank of the Ucayali, and AMNH 75280 was collected at site #3 on the right bank of the Ucayali (see Haven Wiley, 2010, Figure 5, pp. 21). While this would explain the lack of relatedness between AMNH 75280 and AMNH 76327 and 76328, the identity of the latter two as *C. pygmaea* remains puzzling. Porter et al. (2021) stated that this may be due to a continuous distribution of *C. pygmaea* around the headwaters of the Napo and Pastaza rivers, along the eastern foothills of the Andes and up to the western bank of the Ucayali in a horseshoe-shaped distribution. In this case, the distribution of *C. niveiventris* in western Amazonia would be nested within the broad distribution of *C. pygmaea* (see Figure 4 of Porter et al. 2021 and question marks in Figure 5 here). We agree with the intriguing hypothesis of Porter et al. (2021) regarding the geographical distribution of *C. pygmaea*, which would accommodate our suggestion that FMNH 54290 is potentially an admixed individual as there are no natural barriers separating the two species where their ranges meet around the foothills of the Andes and upper reaches of the rivers in this region.

Hershkovitz (1977) and Garbino et al. (2019) reported great variability in *Cebuella* pelage coloration to the extent that Hershkovitz (1977) concluded that there was only one pygmy marmoset species, and all variation was intraspecific. On the other hand, Garbino et al. (2019) restricted this variability to *C. niveiventris*. After examining 44 museum voucher specimens of *Cebuella*, they proposed classification of the *C. niveiventris* vouchers into three somewhat discrete morphotypes. Predominant morphotype "3" accounted for 71% of the examined vouchers and consisted of the typical, white-bellied *C. niveiventris sensu* Lönnberg (1940). Morphotypes "1" and "2" consisted of animals with much darker underparts with varying degrees of white fur present and accounted for the minority of the samples. This is the case for FMNH 54290, which was classified by Garbino et al. (2019) as morphotype "1" (see above).

Animals collected by the Olalla brothers in Eirunepé on the upper Juruá in Brazil have dark underparts and were classified by Garbino et al. (2019) as *C. niveiventris* under character states "1" and "2". This is what led Lönnberg (1940) to classify them as typical *C. pygmaea* (see Boubli et al., 2018). Likewise, Boubli et al. (2018) proposed a new morph of pygmy marmosets from the upper Juruá in Acre, Brazil, and identified them as *C.* cf. *pygmaea*. These specimens (MNFS_1019-20, MNFS_1361) are morphologically similar to those collected by the Olalla brothers in Eirunepé, in that their underparts are darker than the typical *C. niveiventris* (see Boubli et al., 2018). Such variability in underpart coloration in *C. niveiventris* led Porter et al. (2021) to disregard such variation as meaningful for taxonomic classification. In fact, Soini (1988) and de la Torre (pers. obs.) report seeing great within-population pelage color variation in Ecuador. If we consider the distribution hypothesis for *C. pygmaea* proposed by Porter et al. (2021) (see above), then we should expect possible contact between the two species in the southern and western edges of the distribution of *C. niveiventris* and potential gene flow between the two species in the upper reaches of the Napo, Pastaza, Ucayali, and possibly Juruá, which could account for the color pattern variation observed in pygmy marmosets in these regions. Previous studies have reported on gene flow and hybridization among populations of different primate species in the New World, e.g., between *Saguinus midas* and *S. bicolor* (Farias et al., 2015), *Plecturocebus cinerascens*, *P. parecis*, and *P. bernhardi* (Byrne et al., 2021), and *P. moloch* and *P. vieirai* (Boubli et al., 2019), as well as for other organisms on other continents (e.g., frogs in Southeast Asia – Chan et al., 2020; Darwin's finches in the Galapagos – Lamichhaney et al., 2020; Gazelles in Africa – Garcia-Erill et al., 2021). As such, this hypothesis deserves further investigation using nuclear DNA data.

Our study revealed greater lineage diversity in pygmy marmosets than ever before. In addition to the clear separation of pygmy marmosets in two distinct species, Porter et al. (2021) and our analysis also identified further structuring in both species, thus revealing four reciprocally monophyletic lineages in *Cebuella*: i.e., two *C. pygmaea* lineages separated by the Putumayo River and two *C. niveiventris* lineages separated by the Purus River. However, such structuring should be considered with caution as only one locus was used in some cases (cyt *b*). Thus, more robust nuclear data are needed to better understand the phylogenetic diversity of pygmy marmosets.

CONCLUSIONS

In this study, we resolved a long-standing taxonomical conundrum surrounding the origin of Spix's *pygmaea* type by sequencing its mitogenomes from historical DNA. Unambiguously, our results showed that the type is closely related to the pygmy marmosets that currently live around the town of Tabatinga in Brazil. Thus, our data support the classification of pygmy marmosets as *C. pygmaea* and *C. niveiventris*. This is the first study to successfully use historic DNA from a type specimen to address an important taxonomical question in New World primates, thus paving the way for future studies addressing similar issues in other platyrrhines.

Our results and those of Porter et al. (2021) considerably expand the range of *C. niveiventris* to the west and raise the possibility that the headwaters of these western Amazon tributaries may not, in their uppermost reaches, be barriers, and thus the distribution of *C. pygmaea* may be much larger than previously thought.

Once considered a single and widespread species, we show that *Cebuella* is a diverse taxon, with two full species and further cryptic diversification within them. As such, we now have four or even five (see Porter et al., 2021) evident lineages of significance as units for conservation management (sensu Moritz, 1994).

SCIENTIFIC FIELD SURVEY PERMISSION INFORMATION

Permission to conduct fieldwork and to collect tissue samples was granted by Instituto Brasileiro do Meio Ambiente (License No. 005/2005–CGFAU/LIC) and Insituto Chico Mendes da Biodiversidade in Brazil, and by the Ecuadorian Ministry of the Environment (Contrato Marco de Acceso a los Recursos Genéticos Nro. MAE-DNBCM-2015-0019 to Stella de la Torre —Universidad San Francisco de Quito and Liliana Cortés-Ortiz—University of Michigan) in Ecuador.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

J.P.B. conceived and designed the study, acquired funding and organized sample acquisition, interpreted the findings, and led writing of the manuscript. M.C.J. led genomic analyses, interpreted findings, participated in writing of the manuscript, and created figures with input from J.P.B. and the other authors. L.M.P., S.dlT., and L.C.-O. collected, extracted, and sequenced the samples, acquired funding, and edited the manuscript. M.N.F.dS. conceived of the study, interpreted findings, and collected samples. A.B.R. conceived of the study, interpreted findings, and participated in writing of the manuscript. S.N. participated in interpretation of the findings and prepared the illustrations. F.B. and F.R. collected, extracted, and sequenced samples. H.B. participated in interpreting the findings and editing of the manuscript. F.E.S. collected samples and edited the manuscript. D.dV. and R.M.D.B. collected data and participated in writing and editing of the manuscript. T.H. and I.P.F. acquired funding and samples, extracted and sequenced samples, and edited the manuscript. A.H.vH. collected samples from the Spix type specimen. I.R.-G., L.F.K.K., and T.M.-B. extracted and sequenced modern mitogenomes. C.R. led the extraction and sequencing of mitogenomes, acquired funding and samples, interpreted the findings, and participated in writing of the manuscript. All authors read and approved the final version of the manuscript.

ACKNOWLEDGEMENTS

Our most sincere thanks to the Bavarian State Collection of Zoology, Munich, Germany, for granting access to Spix's *Cebuella pygmaea* type specimen for our DNA work. We are indebted to Christiane Schwarz for her dedication and excellent laboratory work at DPZ. Our sincere thanks to G. Garbino for letting us examine his photo archives of pygmy marmoset voucher specimens from museums across the world. L.P., L.C.O., and S.T. thank the staff at the Field Museum of Natural History (FMNH), American Museum of Natural History (AMNH), and University of Michigan Museum of Zoology (UMMZ) for granting them permission to access their collections, and to the FMNH staff for collecting osteocrust samples and to Priscilla and Paige Watson and Merelyn Dolins for their generous hospitality in New York City while L.P. worked at the AMNH. We thank Delaney Cargo, Anna del Valle Peris, Dayanna López, Erika Alzamora, Amalia de la Torre, Ramiro SanMiguel, and Pablo Yépez for their help in the collection of Ecuadorian samples. The Tiputini Biodiversity Station, the Secoya community of San Pablo, and the SanMiguel family greatly facilitated the field work in Ecuador. We thank Nina Q. Espinosa de los Monteros Silva, Cassandra Huerta, Patricia Billete, and Stavi Tennenbaum for their help producing the molecular data from Ecuador and Peru. Finally, L.P., L.C.O., and S.T. thank the Chicago Zoological Society's Chicago Board of Trade Endangered Species and USFQ Chancellor and Collaboration Grants for their help funding research on samples from Peru and Ecuador. J.P.B. thanks M. van Roosmalen for lively discussions on the ecology and biogeography of Amazonian primates that inspired this study.

REFERENCES

Boubli JP, Byrne H, da Silva MNF, Silva-Júnior J, Costa Araújo R, Bertuol F, et al. 2019. On a new species of titi monkey (Primates: *Plecturocebus* Byrne et al., 2016), from Alta Floresta, southern Amazon, Brazil. *Molecular Phylogenetics and Evolution*, **132**: 117−137.

Boubli JP, da Silva MNF, Rylands AB, Nash SD, Bertuol F, Nunes M, et al. 2018. How many pygmy marmoset (*Cebuella* Gray, 1870) species are there? A taxonomic re-appraisal based on new molecular evidence. *Molecular Phylogenetics and Evolution*, **120**: 170−182.

Bouckaert R, Vaughan TG, Barido-Sottani J, Duchêne S, Fourment M, Gavryushkina A, et al. 2019. BEAST 2.5: an advanced software platform for Bayesian evolutionary analysis. *PLoS Computational Biology*, **15**(4): e1006650.

Bouckaert RR, Drummond AJ. 2017. bModelTest: bayesian phylogenetic site model averaging and model comparison. *BMC Evolutionary Biology*, **17**(1): 42.

Burrell AS, Disotell TR, Bergey CM. 2015. The use of museum specimens with high-throughput DNA sequencers. *Journal of Human Evolution*, **79**: 35−44.

Byrne H, Costa-Araújo R, Farias IP, Silva MNF, Messias M, Hrbek T, et al. 2021. Uncertainty regarding species delimitation, geographic distribution, and evolutionary history of titi monkey species (*Plecturocebus*, Pitheciidae) from southwestern Amazonia. *International Journal of Primatology*: in press

Chan KO, Hutter CR, Wood Jr PL, Grismer LL, Das I, Brown RM. 2020. Gene flow creates a mirage of cryptic species in a Southeast Asian spotted stream frog complex. *Molecular Ecology*, **29**(20): 3970−3987.

da Cruz Lima E. 1945. Mammals of Amazonia. 1. General introduction and primates. Museu Paraense Emilio Goeldi de Historia Natural e Etnografia.

Dabney J, Knapp M, Glocke I, Gansauge MT, Weihmann A, Nickel B, et al. 2013. Complete mitochondrial genome sequence of a Middle Pleistocene cave bear reconstructed from ultrashort DNA fragments. *Proceedings of the National Academy of Sciences of the United States of America*, **110**(39): 15758−15763.

Farias IP, Santos WG, Gordo M, Hrbek T. 2015. Effects of forest fragmentation on genetic diversity of the critically endangered primate, the pied tamarin (*Saguinus bicolor*): implications for conservation. *Journal of*

Heredity, **106**(Suppl1): 512−521.

Garbino GST, Casali DM, Nascimento FO, Serrano-Villavicencio JE. 2019. Taxonomy of the pygmy marmoset (*Cebuella* Gray, 1866): geographic variation, species delimitation, and nomenclatural notes. *Mammalian Biology*, **95**: 135−142.

Garcia-Erill G, Kjaer MM, Albrechtsen A, Siegismund HR, Heller R. 2021. Vicariance followed by secondary gene flow in a young gazelle species complex. *Molecular Ecology*, **30**(2): 528−544.

Green RE, Krause J, Ptak SE, Briggs AW, Ronan MT, Simons JF, et al. 2006. Analysis of one million base pairs of Neanderthal DNA. *Nature*, **444**(7117): 330−336.

Groves CP. 2001. Primate Taxonomy. Washington DC: Smithsonian Institution Press.

Groves CP. 2005. Order primates. *In*: Wilson DE, Reeder DM. Mammal Species of the World: A Taxonomic and Geographic Reference. Baltimore, MD: Johns Hopkins University Press.

Haven Wiley R. 2010. Alfonso Olalla and his family: the ornithological exploration of Amazonian Peru. *Bulletin of the American Museum of Natural History*, **2010**(343): 1−68.

Hershkovitz P. 1977. Living New World Monkeys (Platyrrhini) with An Introduction to Primates, Vol. 1. Chicago: University of Chicago Press.

Higuchi R, Bowman B, Freiberger M, Ryder OA, Wilson AC. 1984. DNA sequences from the quagga, an extinct member of the horse family. *Nature*, **312**(5991): 282−284.

Katoh K, Standley DM. 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution*, **30**(4): 772−780.

Kay RF. 2015. Biogeography in deep time–what do phylogenetics, geology, and paleoclimate tell us about early platyrrhine evolution?. *Molecular Phylogenetics and Evolution*, **82**: 358−374.

Lamichhaney S, Han F, Webster MT, Grant BR, Grant PR, Andersson L. 2020. Female-biased gene flow between two species of Darwin's finches. *Nature Ecology & Evolution*, **4**(7): 979−986.

Lönnberg E. 1940. Notes on marmosets. *Arkiv für Zoologi*, **32A**(10): 1−22.

Marsh LK. 2014. A taxonomic revision of the saki monkeys, *Pithecia* Desmarest, 1804. *Neotropical Primates*, **21**(1): 1−165.

Mittermeier RA, Wilson DE, Rylands AB. 2013. Handbook of the Mammals of the World: Primates. Barcelona: Lynx Edicions.

Moritz C. 1994. Defining 'Evolutionarily Significant Units' for conservation. *Trends in Ecology & Evolution*, **9**(10): 373−375.

Naka LN, Bechtoldt CL, Henriques LMP, Brumfield RT. 2012. The role of physical barriers in the location of avian suture zones in the Guiana Shield, northern Amazonia. *The American Naturalist*, **179**(4): E115−E132.

Napier PH. 1976. Catalogue of Primates in the British Museum (Natural History). Part I: Families Callithricidae and Cebidae. London: British Museum (Natural History).

Noonan JP, Coop G, Kudaravalli S, Smith D, Krause J, Alessi J, et al. 2006. Sequencing and analysis of Neanderthal genomic DNA. *Science*, **314**(5802): 1113−1118.

Orlando L, Metcalf JL, Alberdi MT, Telles-Antunes M, Bonjean D, Otte M, et al. 2009. Revising the recent evolutionary history of equids using ancient DNA. *Proceedings of the National Academy of Sciences of the United States of America*, **106**(51): 21754−21759.

Pääbo S. 1985. Molecular cloning of Ancient Egyptian mummy DNA. *Nature*, **314**(6012): 644−645.

Perri AR, Mitchell KJ, Mouton A, Álvarez-Carretero S, Hulme-Beaman A, Haile J, et al. 2021. Dire wolves were the last of an ancient New World canid lineage. *Nature*, **591**(7848): 87−91.

Porter LM, de la Torre S, Pérez-Peña P, Cortés-Ortiz L. 2021. Taxonomic diversity of *Cebuella* in the western Amazon: molecular, morphological and pelage diversity of museum and free-ranging specimens. *American Journal of Physical Anthropology*, **175**(1): 251−267.

Prüfer K, Racimo F, Patterson N, Jay F, Sankararaman S, Sawyer S, et al. 2014. The complete genome sequence of a Neanderthal from the Altai Mountains. *Nature*, **505**(7481): 43−49.

Rambaut A, Ho SYW, Drummond AJ, Shapiro B. 2009. Accommodating the effect of ancient DNA damage on inferences of demographic histories. *Molecular Biology and Evolution*, **26**(2): 245−248.

Rohland N, Siedel H, Hofreiter M. 2004. Nondestructive DNA extraction method for mitochondrial DNA analyses of museum specimens. *BioTechniques*, **36**(5): 814−821.

Roos C, Helgen KM, Miguez RP, Thant NML, Lwin N, Lin AK, et al. 2020. Mitogenomic phylogeny of the Asian colobine genus *Trachypithecus* with special focus on *Trachypithecus phayrei* (Blyth, 1847) and description of a new species. *Zoological Research*, **41**(6): 656−669.

Rylands AB, Coimbra-Filho AF, Mittermeier RA. 2009. The systematics and distributions of the marmosets (*Callithrix*, *Callibella*, *Cebuella*, and *Mico*) and callimico (*Callimico*) (Callitrichidae, Primates). *In*: Ford SM, Porter LM, Davis LC. The Smallest Anthropoids: The Marmoset/Callimico Radiation. Boston, MA: Springer, 25–61.

Sambrook J, Fritsch EF, Maniatis T. 1989. In vitro amplification of DNA by the polymerase chain reaction. *In*: Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1431–1435.

Scheel DM, Slater GJ, Kolokotronis SO, Potter CW, Rotstein DS, Tsangaras K, et al. 2014. Biogeography and taxonomy of extinct and endangered monk seals illuminated by ancient DNA and skull morphology. *ZooKeys*, **409**: 1−33.

Seiffert ER. 2006. Revised age estimates for the later Paleogene mammal faunas of Egypt and Oman. *Proceedings of the National Academy of Sciences of the United States of America*, **103**(13): 5000−5005.

Shepherd LD, Tennyson AJD, Lambert DM. 2013. Using ancient DNA to enhance museum collections: a case study of rare Kiwi (*Apteryx* spp.) specimens. *Journal of the Royal Society of New Zealand*, **43**(3): 119−127.

Soini P. 1988. The pygmy marmoset, Genus *Cebuella*. *In*: Mittermeier RA, Rylands AB, Coimbra-Filho AF, da Fonseca GAB. Ecology and Behavior of Neotropical Primates. Vol. 2. Washington, DC: World Wildlife Fund, 79–129.

Stamatakis A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics*, **30**(9): 1312−1313.

Swofford DL. 2002. PAUP*: Phylogenetic Analysis Using Parsimony, Version 4.0 b10. Sunderland, MA: Sinauer Associates.

van Roosmalen MGM, van Roosmalen T. 1997. An eastern extension of the geographical range of the pygmy marmoset. *Cebuella pygmaea*. *Neotropical Primates*, **5**: 3−6.

Verry AJF, Scarsbrook L, Scofield RP, Tennyson AJD, Weston KA, Robertson BC, et al. 2019. Who, where, what, wren? Using ancient DNA to examine the veracity of museum specimen data: a case study of the New Zealand rock wren (*Xenicus gilviventris*). *Frontiers in Ecology and Evolution*, **7**: 496.

von Spix JB. 1823. Simiarum et Vespertilionum Brasiliensium Species Novae. Munich: F. S. Hübschmann.

von Spix JB, von Martius KFP. 1824. Travels in Brazil, in the Years 1817-1820: Undertaken by Command of His Majesty the King of Bavaria. London: Longman, Hurst, Rees, Orme, Brown, and Green.

Weir JT, Faccio MS, Pulido-Santacruz P, Barrera-Guzmán AO, Aleixo A. 2015. Hybridization in headwater regions, and the role of rivers as drivers of speciation in Amazonian birds. *Evolution*, **69**(7): 1823−1834.

Werle E, Schneider C, Renner M, Völker M, Fiehn W. 1994. Convenient single-step, one tube purification of PCR products for direct sequencing. *Nucleic Acids Research*, **22**(20): 4354−4355.