Uncertainty regarding species delimitation, geographic distribution, and the evolutionary history of south-central Amazonian titi monkey species (*Plecturocebus*, Pitheciidae)

Authors: Hazel Byrne[1*], Rodrigo Costa-Araújo[2,3], Izeni P. Farias[3], Maria N. F. da Silva[4], Mariluce Messias[5], Tomas Hrbek[3,6], Jean P. Boubli[7]

[1]Department of Anthropology, University of Utah, Salt Lake City, Utah, USA. E-mail: byrne.hazel1@gmail.com
[2]Mastozoology collection, Museu Paraense Emílio Goeldi, Belém, Brazil. E-mail: rodrigotbio@gmail.com
[3]Laboratory of Evolution and Animal Genetics, Universidade Federal do Amazonas, Manaus, Brazil. E-mail: izeni@evoamazon.com
[4]Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus, Brazil. E-mail: marianazareth.inpa@gmail.com
[5]Department of Ecology, Universidade Federal de Rondônia (UNIR), Porto Velho, Brazil. E-mail: messias.malu@unir.br
[6]Department of Biology, Trinity University, San Antonio, Texas, USA. E-mail: tomas@evoamazon.com
[7]School of Science, Engineering and the Environment, University of Salford, Manchester, UK. E-mail: j.p.boubli@salford.ac.uk

*__Corresponding author__: Hazel Byrne. Department of Anthropology, University of Utah, Salt Lake City, Utah, USA. Phone: +1 323-504-1546. E-mail: byrne.hazel1@gmail.com

Author Contributions

HB and RCA originally conceived the ideas. HB performed the laboratory work and molecular analyses. RCA raised funds, carried out field expeditions, prepared specimens, and performed the morphological analysis. HB and RCA interpreted the molecular and morphological results. MM carried out field expeditions and contributed specimens for the molecular analyses. MNFS assisted in collecting field and morphological data. HB and RCA led the writing. TH, JPB, and IF raised funds and contributed to the design of the project and writing of the manuscript. All authors approved the final version of the article.

Acknowledgements

R Costa-Araújo was funded and supported by CNPq (140039/2018-1; 316321/2020-6), CAPES (001; 88881.189052/2018-01), Conservation Leadership Programme (F02304217), Global Wildlife Conservation's Margot Marsh Primate Action Fund (6002856), CAPES/PRO-AMAZONIA/AUXPE (3261/2013) and Idea Wild, and is especially grateful for field support from the team of Secretaria Estadual de Meio Ambiente do Amazonas at Manaus and Apuí, the team of ONF Fazenda São Nicolau, José de Sousa e Silva Jr, Raimundo Rodrigues da Silva, Gustavo Canale, Fabiano R. de Melo, Catitu and Ivan Baptista da Silva. This study was financed, in part, by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001. We thank Fabio Rohe for the use of his samples in the molecular analyses.

Uncertainty regarding species delimitation, geographic distribution, and the evolutionary history of south-central Amazonian titi monkey species (*Plecturocebus*, Pitheciidae)

Abstract

Platyrrhine primate taxonomy is a rapidly evolving area of research. The recent description of the Parecis titi monkey, *Plecturocebus parecis*, has raised substantial questions regarding the taxonomy, distribution, and evolutionary history of titi taxa from south-central Amazonia. There is only a single documented record of *P. parecis*, which is the type locality, with uncertainty regarding species monophyly. Moreover, there are questions surrounding the distribution and pelage patterns of the poorly studied *P. cinerascens* and *P. parecis*, which further highlight the uncertainty regarding the taxonomic validity of this new species. Here, we investigate the taxonomy, distribution, and evolutionary history of these lineages through new field work and assessment of pelage pigmentation patterns from 25 localities, as well as maximum likelihood and Bayesian phylogenetic reconstructions based on two mitochondrial and 11 nuclear loci for 19 and 10 specimens of *Plecturocebus*, respectively. Our mitochondrial results recover a paraphyletic arrangement for the four *P. parecis* type specimens which show three distinct haplotypes, with the holotype showing a close affinity to *P. bernhardi*. Our morphological analysis reveals a north-south clinal bleaching gradient through the Aripuanã-Sucundurí/Juruena interfluve from an all-greyish morphotype associated with *P. cinerascens*, through intermediary morphotypes with increasingly whitish hairs on the beard, hands, feet, and tail, to the most whitish morphotype described as *P. parecis*. Based on these findings, we present hypotheses to explain the taxonomy, distribution, and evolutionary history of *P. cinerascens* and *P. parecis*, and discuss the significance of introgression among titi taxa from southern Amazonia given the lack of study systems for natural hybridisation in platyrrhine primates.

Keywords

Plecturocebus parecis, Plecturocebus cinerascens, Plecturocebus bernhardi, morphological cline, phylogenetic conflict, introgression

Introduction

The last decade has witnessed a resurgent interest in taxonomical studies due, in part, to the increased application of molecular genetics in phylogenetic and systematic studies. As a result, knowledge of primate systematics and evolution is advancing rapidly, and integrative taxonomy—based on multiple lines of evidence, for example, molecular genetic and phenotypic data—has proven paramount to unveil the diversity of platyrrhine species. Recent studies are thus changing primate systematics (e.g., Byrne *et al*. 2016; Di Fiore *et al.* 2015; Lynch- Alfaro *et al*. 2012) and describing new species (e.g., Boubli *et al*. 2019; Costa-Araújo *et al*. 2019, 2021), with important consequences for species conservation and setting the stage for further research on ecology, behaviour, and physiology.

The taxonomy and evolutionary history of titi monkeys from southern Amazonia, genus *Plecturocebus* (Callicebinae; Pitheciidae), is a rapidly evolving and contentious area of study. A case in point, the recently described Parecis titi monkey, *Plecturocebus parecis* Gusmão *et al.* 2019, has added further uncertainty to the taxonomy, distribution, and evolutionary history of the poorly-studied *Plecturocebus* species from south-central Amazonia. *Plecturocebus parecis* was described as distinctive from *Plecturocebus cinerascens* based on limited data on patterns of pelage pigmentation, distribution, and phylogenetic relationships of both species, consequentially also impacting the taxonomic stability of *Plecturocebus bernhardi* and *Plecturocebus miltoni*.

Plecturocebus parecis is represented by only four type specimens collected at a single locality (Rondon II hydroelectric dam, Rondônia State, Brazil), which is the sole distribution record documented for this taxon (see Gusmão *et al.* 2019). The other 13 localities attributed to *P. parecis* in the states of Rondônia and Mato Grosso are based on sightings and they lack specimen collection, pictures, and pelage descriptions. Moreover, a large proportion of the range of *P. parecis* formerly constituted part of the range of *P. cinerascens* and overlaps with the distributions of *P. bernhardi* and *P. miltoni* (Byrne *et al.* 2018; Gusmão *et al.* 2019). Furthermore, *P. parecis* was described as morphologically distinctive from *P. cinerascens* in the greyish-white chin, sideburns, throat, tail-tip, and hands, based on pelage data from four specimens (Gusmão *et al.* 2019). It remains unclear, however, whether this pattern should be considered intraspecific variation in pelage colouration of *P. cinerascens* or a distinct species morphotype because the pelage pigmentation patterns and the distribution of *P. cinerascens* species are poorly characterised. The description of *P. cinerascens* was based on the pelage pigmentation of a single specimen of unknown provenance (Spix 1823) and there are few additional specimens and documented records available to assess the taxonomy and distribution of this species.

The four type specimens of *P. parecis* were already known to researchers prior to the publication of Gusmão *et al*. (2019) and included in previous molecular phylogenetic studies. These studies did not provide evidence for the distinction of these specimens as a new species since the *P. parecis* type specimens nested within or formed a very close sister lineage to *P. cinerascens* (Byrne *et al.* 2016; Byrne 2017; Carneiro *et al.* 2016). The phylogenetic reconstruction of Gusmão *et al*. (2019) did not include samples of all *P. parecis* type specimens, or of additional voucher specimens, and they presented results that conflict with some previous studies showing the *P. parecis* holotype (UFRO 354) nested with, and labelled as, *P. bernhardi,* and the two paratypes in a separate clade (see also mislabelling of UFRO 354 in Carneiro *et al.* 2016). In addition to the uncertainties relating to taxonomy and species relationships, the phylogeny of Gusmão *et al*. (2019) raises questions of potential hybrid zones across the range of *P. parecis*. The finding that the *P. parecis* holotype has a distinctive phenotype (Gusmão *et al.* 2019) but shows discordant patterns between mitochondrial and nuclear loci (Byrne 2017) is suggestive of ancient or contemporary admixture between *P. parecis*, or *P. cinerascens,* and *P. bernhardi* (Maddison 1997).

These issues substantiate uncertainty regarding the validity of *P. parecis*, as well as the taxonomy and evolutionary history of *P. cinerascens* and *P. bernhardi*, all three occurring in south-central Amazonia. Knowledge of pelage variation, distribution, and phylogenetic relationships of *P. parecis* and *P. cinerascens* is lacking. Additionally, there is no conspicuous geographic barrier that could prevent or limit gene flow among these three taxa (along with *P. miltoni*) according to the current knowledge on their distribution (Byrne *et al.* 2018). In the following subsections, we provide a brief overview of the taxonomic history and distribution of *P. cinerascens* and *P. parecis,* highlighting some of the outstanding issues with the taxonomy, and then summarise the study aims.

An overview of the taxonomy and distribution of *P. cinerascens* and *P. parecis*

The historical scarcity of specimen collections of *P. cinerascens* has hampered an accurate identification of the species' limits and geographic distribution. *Plecturocebus cinerascens* was described by Spix (1823) based on a single specimen of unknown provenance as possessing an overall greyish agouti pelage, tawny hairs on the dorsum, greyish black tail, and whitish hairs on the beard (Figure 1). Since then, three revisions have addressed *P. cinerascens* taxonomy based on pelage pigmentation patterns of 10 specimens from three localities (Hershkovitz

1990), one specimen from one locality (Van Roosmalen *et al.* 2002), or three specimens from two localities (Gusmão *et al.* 2019). Interestingly, previous taxonomic studies of *P. cinerascens* indicate agreement with Spix's (1823) species description, however, they did not mention a whitish beard in any of the specimens examined. This is problematic because beard colouration is an important diagnostic character in titi monkeys (e.g., Boubli *et al.* 2019), raising uncertainty regarding the typical phenotype of *P. cinerascens* and the typological identification of specimens in previous studies.

It is unsurprising that the species limits of *P. cinerascens* are currently poorly defined given its taxonomy is based on pelage pigmentation patterns of few specimens from sparse localities, and the incongruencies in previous studies regarding an important diagnostic character, which is also one of the main diagnostic characters states of *P. parecis.* Unfortunately, such issues are not uncommon in titi monkey taxonomy (e.g., Byrne *et al.* 2020).

Figure 1. Painting of the holotype of *P. cinerascens* (Spix 1823).

The geographic distribution of *P. cinerascens* is also poorly known. Firstly, from the localities provided in previous taxonomic revisions, only six relate to specimen collections and two of these are unreliable due to toponymic and mapping issues (e.g., “Aripuanã”—the river or town?; “Otohô”—the headwaters of the Ji-Paraná or the Guaporé River? [Hershkovitz 1990, pp. 53–54; Van Roosmalen *et al.* 2002]). Second, although a number of localities have been assigned to *P. cinerascens* in field studies (Ferrari *et al.* 2000; Van Roosmalen *et al.* 2002; Noronha *et al.* 2007; Sampaio *et al.* 2012; Gusmão and da Costa 2014; Gusmão *et al.* 2019), most of them are based on sightings rather than on a more thorough examination, for example, as allowed with collected specimens. Assigning species identity to a poorly known taxon based on limited field observations can be problematic; it is not possible to properly observe, in full detail, states of pelage pigmentation characters during a single titi monkey sighting due to light conditions under the canopy, their small body size, and the large number of variable pelage characters. Finally, in the specific case of *P. cinerascens*, previous field reports followed the preliminary and somewhat misleading diagnosis of *P. cinerascens* by Hershkovitz (1990) and Van Roosmalen *et al*. (2002) to identify this species in the field. The majority of field reports on *P. cinerascens* also did not provide a description of phenotype or pictures of specimens, hampering the evaluation of pelage pigmentation and the validation of species assignments for those localities. Consequently, only a few occurrence records are currently available to reliably study the geographic distribution and pelage pigmentation of *P. cinerascens*.

The same issues are also evident in the taxonomy and distribution of *P. parecis*. This species was described based on pelage pigmentation patterns and phylogenetic relationships of three to four type specimens from a single locality. One of the diagnostic characters for *P. cinerascens*—whitish beard (Spix 1823)—is also one of the main diagnostic characters of *P. parecis* (Gusmão *et al.* 2019). Although Gusmão *et al*. (2019) provided 14 occurrence records for *P. parecis*, 13 are based on sightings without information on the phenotype of these specimens. Notably, the living individuals in the photographs in the article are also from the type locality. This impedes the ability to properly distinguish *P. cinerascens* and *P. parecis* based on pelage pigmentation patterns and complicates our understanding of their geographic distribution and the variation between and within both taxa.

Study aims

Here we investigate the taxonomy, distribution, and evolutionary history of *P. cinerascens* and *P. parecis*. Our objective is to clarify the diversity, distribution, and taxonomy, and discuss the processes potentially involved in the speciation/diversification of titi monkeys in south-central Amazonia. We generated new sequences of the mitochondrial loci cytochrome *b* (CYTB) and cytochrome c oxidase subunit I (COI) for specimens included in the *P. parecis* description, as well as additional specimens of *P. bernhardi* and *P. cinerascens,* and inferred their phylogenetic relationships. We also generated a nuclear phylogeny based on 11 loci to assess the phylogenetic signal in the nuclear genome for *P. parecis* specimens. We carried out new field expeditions in south-central Amazonia to obtain specimens and occurrence records, and to clarify pelage pigmentation patterns and the geographic distribution of *P. cinerascens* and *P. parecis*. Our analyses cover localities attributed to *P. cinerascens* and *P. parecis*, as well as new localities without existing records within their proposed region of occurrence

Methods

Mitochondrial phylogeny

The molecular data used by Carneiro *et al*. (2016) and Gusmão *et al*. (2019) are not available on a public repository. As such, we generated new sequences for the mitochondrial loci, COI and CYTB, for the *P. parecis* holotype (UFRO 354), one paratype (UFRO 195), three *P. cinerascens* (FR 31, FR 50, FR 123), and two *P. bernhardi* (FR 26, CCM 173) to infer phylogenetic relationships (Table 1, Figure 2). We collected muscle tissue samples for these seven individuals from museum voucher specimens and extracted DNA using the Qiagen DNeasy Blood & Tissue Kit according to manufacturer's protocol, generating 13 new sequences for COI (6), and CYTB (7) (for primer information, see Byrne *et al*. 2016, 2018). We carried out the PCR reactions in a total volume of 50 µL, containing approximately 30 ng of genomic DNA, 4 µL of dNTPs (200µM each), 5 µL 10X PCR buffer (100 mM Tris-HCL, 500 mM KCL, 15 mM Mg2+), 1 µL of each forward and reverse primer (0.2 µM), and 0.25 µL of TaKaRa *Taq* DNA polymerase (1 Unit). We performed the amplification cycles under the following conditions; initial denaturation at 95 °C for 5 min; 35 cycles of denaturing at 94 °C for 1 min, primer annealing for 1 min (see temperature for each primer in Byrne *et al.* 2016), and extension at 72 °C for 1 min; and a final extension at 72 °C for 5 min. We analysed PCR products on 1.5% agarose gels, which were then Sanger sequenced commercially. We generated consensus sequences from forward and reverse reads using Geneious R7.1 (Biomatters) and deposited newly generated sequences on GenBank under the accession numbers MW680778 to MW680783 and MW684391 to MW684397 (Supplementary Table S1).

We included published sequence data for twelve other individuals originally sampled in Byrne *et al*. (2016) including one *P. cinerascens* (UFRO 499), the other two *P. parecis* paratypes (UFRO 352, UFRO 355), and four *P. bernhardi* (UFRO 413, 42960, 42961, 42964) (Table 1, Supplementary Table S1). We extracted additional sequences for COI and CYTB from a whole mitochondrial genome sequence of one *P. donacophilus* available on GenBank in order to root the phylogeny (Supplementary Table S1). All specimens were represented at both loci except *P. cinerascens* FR 31 and *P. bernhardi* 42961, which are missing data for COI.

For the mitochondrial data set, we aligned the complete CYTB CDS (1140 bp) and 660 bp of the COI locus using the MUSCLE algorithm in Geneious R7.1 for all 20 samples and subsequently concatenated both loci. We used the GTR + G (gamma) substitution model for each COI + CYTB codon position partition. We reconstructed a maximum-likelihood tree using RAxML v. 8.1 (Stamatakis 2014) and estimated node support using the rapid-bootstrapping algorithm (−f a -x option) for 1,000 non-parametric bootstrap replicates (Stamatakis *et al.* 2008). We reconstructed a Bayesian tree using MrBayes 3.2.6 (Ronquist *et al.* 2012) and checked MCMC (Markov Chain Monte Carlo) convergence after two independent four-chain runs of 10 million generations after a burn-in of 10%. We calculated the number of base pairs changes and percentage identity for CYTB, COI, and across both loci between all individuals in Geneious R7.1.

Nuclear phylogeny

We generated sequence data for the *P. parecis* holotype (UFRO 354) for 11 of the nuclear loci used in Byrne *et al.* (2016) to show that it has a nuclear genome similar to other *P. parecis* (UFRO 352 and UFRO 355) as found using genome wide RADseq data (Byrne 2017). We generated nuclear sequences for UFRO 354 at 11 loci (ABCA1, ADORA3, DENND5A. DMRT1, FAM123B, FES, FOXP1, MAPKAP1, NPAS3.2, RPGRIP1, and ZFX) using the protocol outlined in the previous section (for annealing temperatures and primer information see Byrne *et al.* 2016). We generated consensus sequences from forward and reverse reads using Geneious R7.1 and deposited newly generated sequences on GenBank under the accession numbers MW684380 to MW684390 (Supplementary Table S1).

We added nine individuals sampled in Byrne *et al.* (2016), all of which were included in our mitochondrial phylogeny, including two other *P. parecis* (UFRO 352, UFRO 355), one *P. cinerascens* (UFRO 499), and four *P. bernhardi* (UFRO 413, 42960, 42961, 4294) (see Supplementary Table S1). We also included a *P. donacophilus* sampled in Perelman *et al.* (2011) as an outgroup. We did not have enough nuclear sequences for the fourth *P. parecis* (UFRO 195) to include it in this data set. We aligned each locus using the MUSCLE algorithm in Geneious R7.1 for all 11 samples and subsequently concatenated the 11 loci. We used the GTR + G (gamma) substitution model with a single partition given the low amount of information contained in each of the nuclear loci for these closely related species. We reconstructed maximum-likelihood and Bayesian trees as outlined for the mitochondrial data set. Our sole aim for the nuclear phylogeny was to confirm that UFRO 354 clusters with other *P. parecis*, as previously found (Byrne 2017).

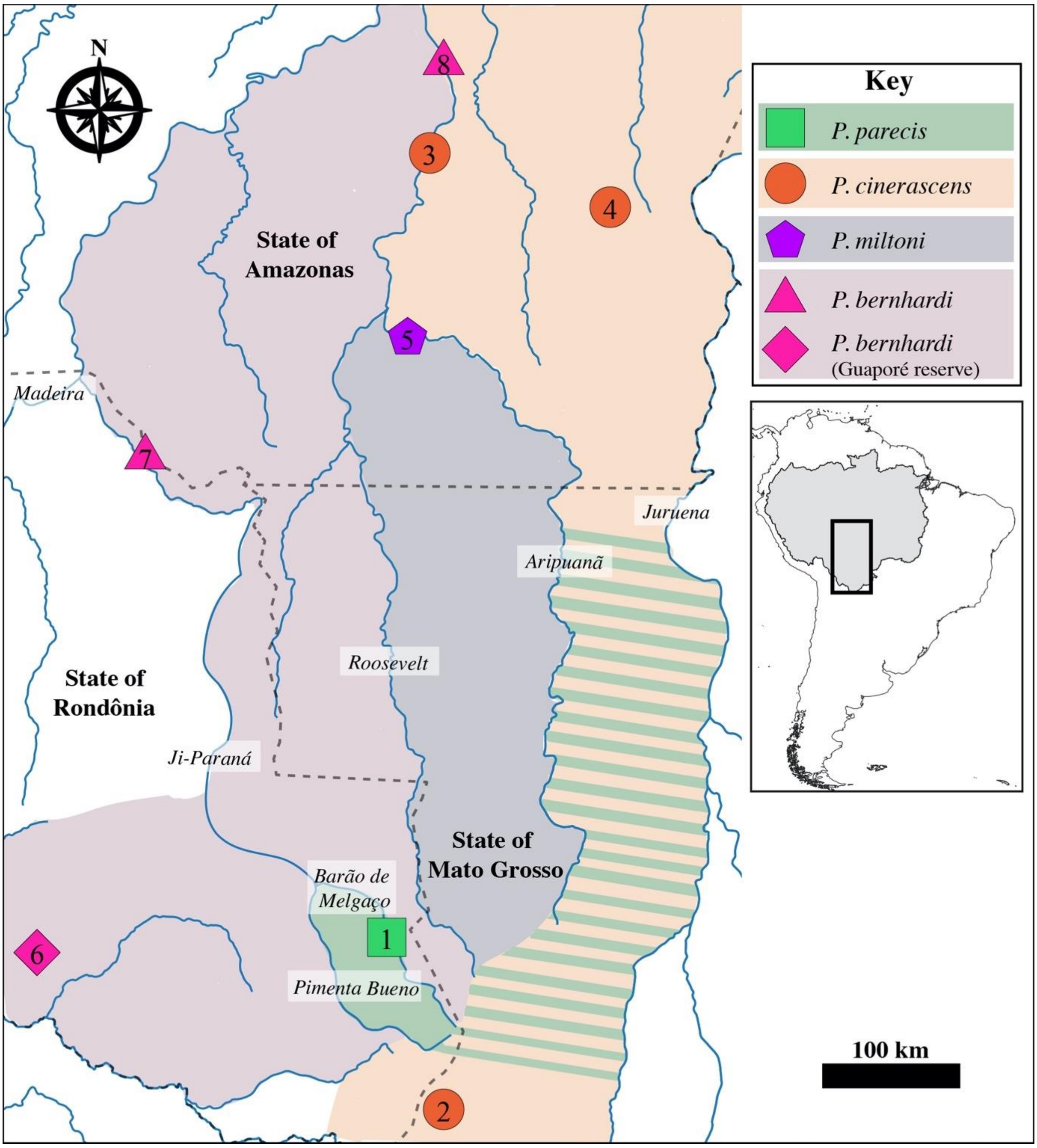

Figure 2. Collection localities for the *P. parecis*, *P. cinerascens*, *P. bernhardi,* and *P. miltoni* samples included in phylogenetic analyses. Numbers correspond to geographic origin in Table 1. Dashed lines represent state borders. Rivers of interest are labelled. Coloured shading corresponds to hypothetical geographic distributions for these species as per the key, however, these distributions are highly uncertain.

Field work and morphological assessment

We carried out new field expeditions across southern Amazonia to obtain field records, tissue samples, and specimens, permitting robust assessment of the taxonomy, distribution, and evolutionary history of *Plecturocebus* taxa for this and other studies. The expeditions targeted regions with little existing information on *Plecturocebus* taxa, each including around 25 days of surveys carried out by RCA and one local field assistant using pre-existent trails. For each titi monkey sighting and collection, the exact geographic coordinates were recorded with Garmin GPSMAP 64x device, and the date, hour, and type of habitat were noted. We prepared and stored specimens in the collections of the Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus, Brazil, and the Museu Paraense Emílio Goeldi, Belém, Brazil. Three newly collected specimens from these expeditions contributed to the morphological assessment for this study (RCA 100, RCA 66, and RCA 92; Table 2).

For these three newly collected specimens, as well as three existing specimens in INPA that were not collected specifically for this study (FR 31, FR 50, and FR 123), we assessed pelage colouration patterns of beard, cheeks, crown, dorsum, hands, feet, and tail through direct examination following previous taxonomic studies and species descriptions of *Plecturocebus* (Boubli *et al.* 2019; Gualda-Barros *et al.* 2012; Gusmão *et al.* 2019). The pelage pigmentation data we obtained for these six titi monkey specimens, which come from five localities covering an area of 350 km along the mid Aripuanã-Sucundurí/Juruena interfluve, were then compared to the patterns described for *P. parecis* and *P. cinerascens.* We also reviewed the literature on taxonomy and distribution of titis between the Aripuanã and Tapajós rivers, and included in our morphological analysis another 20 localities of assured geographical provenance from studies that provided pelage descriptions or pictures for comparison, covering the entire Aripuanã-Sucundurí/Juruena interfluve to the headwaters of the Ji-Paraná River, and including the type locality of *P. parecis* (Table 2).

Ethical note: Our sampling method was adopted only after thorough consideration of all possible alternatives. It was deemed necessary in order to overcome the scarcity of specimens, samples, and distribution records in museums and literature, and allow valid phylogenetic, morphological, distributional, taxonomic, and evolutionary assessments of *P. parecis*, *P. cinerascens*, and *P. bernhardi*. Only the minimum number of specimens necessary for valid research results were collected. Our methodology follows the guidelines for field studies with primates in Amazonia from the Instituto Chico Mendes de Conservação da Biodiversidade

(Vidal 2012), the Brazilian institution responsible for the regulation of biodiversity studies, which also provided permits (SISBio 32095, 10832, 13507). One of the main outcomes of taxonomic research is to identify significant evolutionary lineages (species and other taxa) upon which all further research and conservation efforts are contingent. This is especially imperative in the Amazonian arc of deforestation which entirely encompasses the Amazon/Cerrado transitional forests as well as southern Amazon forests and northern Cerrado wooded savanna. This region is perilously close to the point of environmental collapse (Lovejoy and Nobre 2018).

Results

Phylogenetic analyses

Our mitochondrial data set includes all four type specimens of *P. parecis*, all of which were collected at the Rondon II hydroelectric dam and, interestingly, they show three different patterns: (1) UFRO 195 is very similar to *P. cinerascens* specimens, for example, showing 99.81% sequence identity (two base pair changes) to FR 31 and 99.74% sequence identity (three bp changes) to FR 123 in CYTB, and 99.85% sequence identity (one bp changes) to FR 123 in COI; (2) UFRO 355 and UFRO 352 are identical in both COI and CYTB, and they show 99.39% to 99.54% sequence identity (five to seven bp changes) to *P. cinerascens* specimens as well as 99.39% sequence identity (seven bp changes) to UFRO 195 in CYTB; and (3) the holotype of *P. parecis*, UFRO 354, shows 99.84% sequence identity (1 bp change) for COI and 99.39% sequence identity (7 bp changes) for CYTB with *P. bernhardi* specimens (42960, 42961, 42964) from south of the Ji-Paraná River in the Guaporé Biological Reserve to the west of São Francisco do Guaporé, Rondônia (Table 3; Supplementary Table S2).

These results are reflected in the Bayesian and maximum-likelihood mitochondrial phylogenies, which show an identical topology with *P. parecis* specimens occurring in three different clades (Figure 3; Supplementary Figure S1). UFRO 195 is within a clade containing all *P. cinerascens* specimens with UFRO 355 and UFRO 352 as sister to this clade, while UFRO 354 is sister to a clade containing the three *P. bernhardi* specimens from the Guaporé Biological Reserve and nested within the broader *P. bernhardi* clade. Internode branch lengths between the UFRO 355 + 352 and the *P. cinerascens* + UFRO 195 clades are short, reflecting the overall low degree of genetic divergence between these specimens (Table 3; Supplementary Table S2).

The maximum-likelihood nuclear phylogeny (Figure 4) shows the *P. parecis* holotype clustering with two other *P. parecis* specimens from the same collection locality (UFRO 352, UFRO 355), which together form a clade that is very closely related (short internode branch length) to *P. cinerascens* (UFRO 499). This placement of UFRO354 is largely in agreement with its phenotype but in conflict with the affinity of its mitochondrial genome to *P. bernhardi* (Figure 3). Nonetheless, the Bayesian nuclear phylogeny (Supplementary Figure S2) shows the three *P. parecis* specimens forming a polytomy with *P. cinerascens* (UFRO 499).

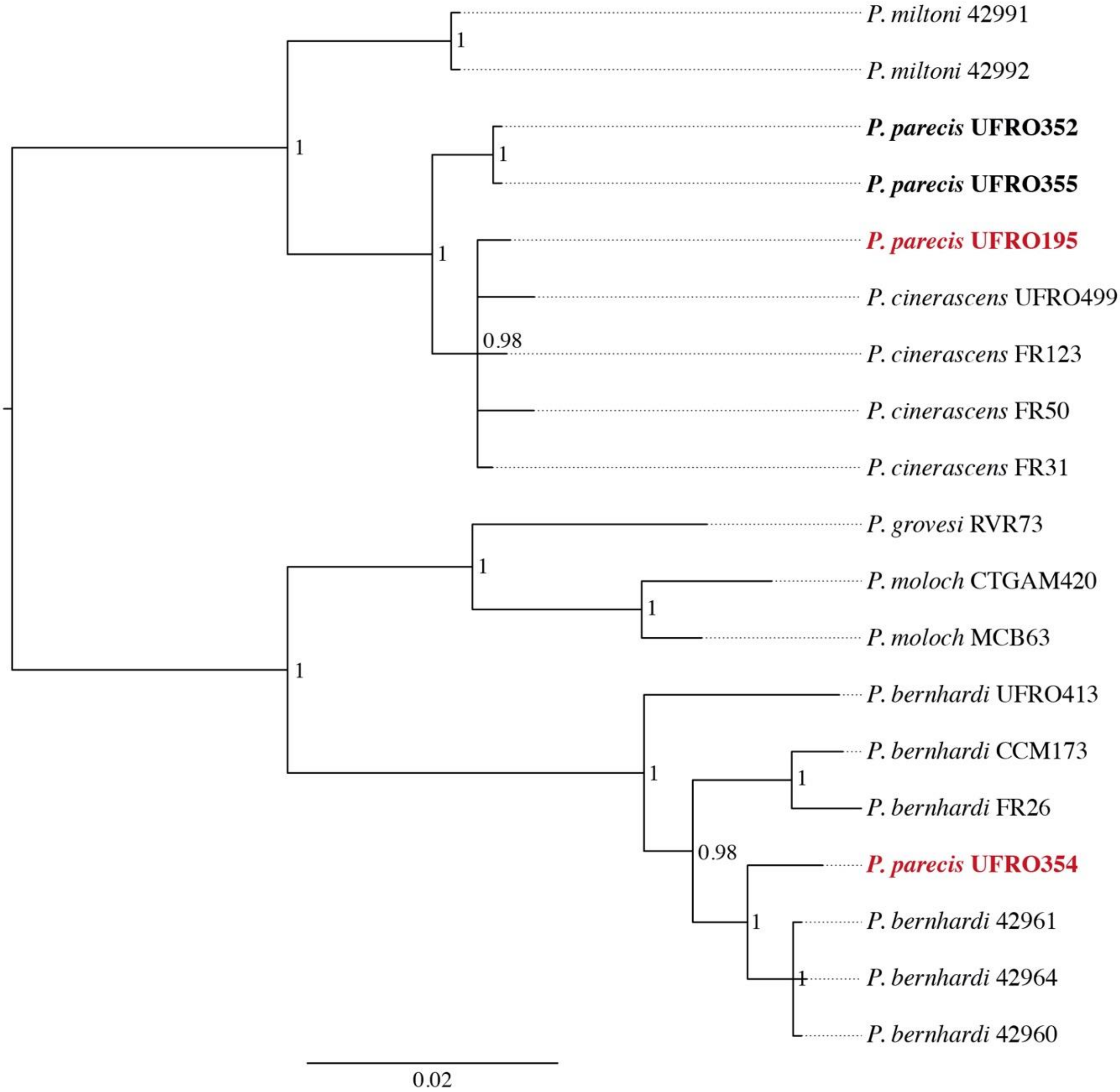

Figure 3. Bayesian mitochondrial phylogeny for some *Plecturocebus* species showing the occurrence of *P. parecis* specimens in three different clades. Samples highlighted in bold show the expected species relationship based off the description of *P. parecis* as sister to *P. cinerascens* (Gusmão *et al.* 2019), while samples in red show that *P. parecis* is paraphyletic.

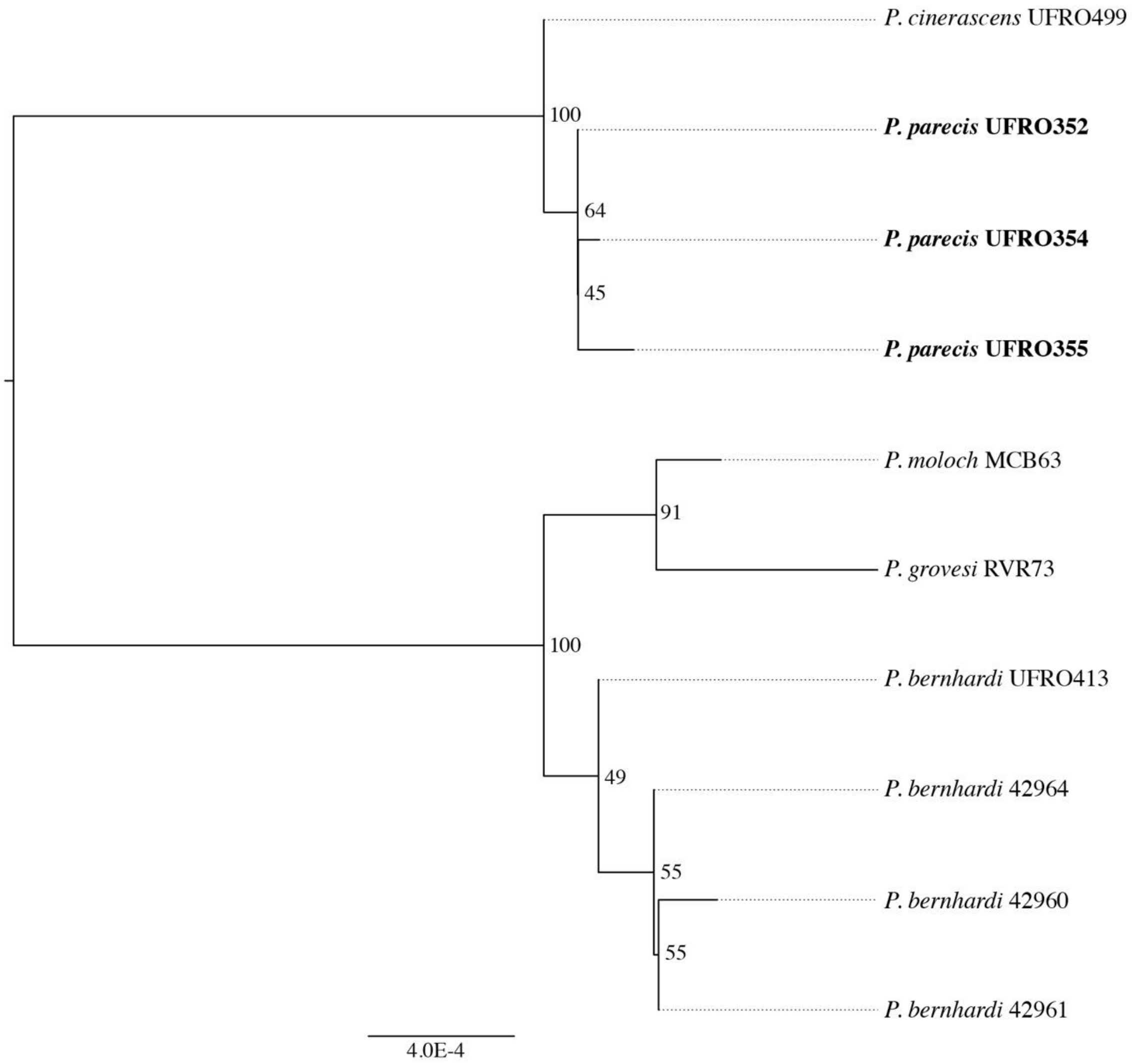

Figure 4. Maximum-likelihood nuclear phylogeny for some *Plecturocebus* species showing the *P. parecis* holotype (UFRO 354) clustering with other *P. parecis*, which are all shown in bold.

Morphological and geographical distribution analysis

Our analysis of pelage pigmentation data included titi monkeys from 25 localities covering the entire Aripuanã-Sucundurí/Juruena interfluve to the headwaters of the Ji-Paraná River, and including the type locality of *P. parecis* (Table 2). We found that the diagnostic characters of *P. parecis*— whitish beard, hands, feet, and tail—appear, to some extent, on the holotype of *P. cinerascens* and/or other intermediary morphotypes here recorded. The illustration of the *P. cinerascens* holotype clearly shows a whitish beard (see Figure 1) and each intermediary morphotype presents this along with some other diagnostic characters of *P. parecis* (Figure 5). More specifically, the pelage patterns in these four diagnostic characters follow evident clinal variation along a north-south (N-S) bleaching gradient, with geographic overlap with records of *P. cinerascens*, intermediary morphotypes, and *P. parecis* (Figure 6): from the all-greyish *P. cinerascens* at the northernmost localities in the Aripuanã-Sucunduri/Juruena interfluve (Figure 5 A), to the whitish bearded morphotype of the *P. cinerascens* holotype (Figure 5 B)––which is of unknown provenance but was probably obtained around the mid Aripuanã River––through three intermediary morphotypes possessing increasing amounts of whitish hairs (Figure 5 C, D, E), towards *P. parecis* which possesses completely whitish hairs on beard, hands, feet, and tail (Figure 5 F; see also Figure 6).

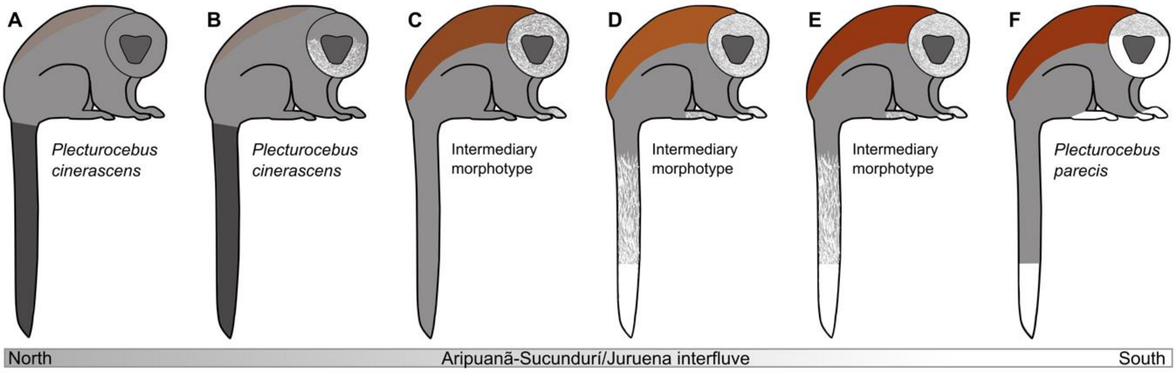

Figure 5. North-south clinal variation in the pelage pigmentation patterns of *P. cinerascens*, intermediary morphotypes, and *P. parecis* along the Aripuanã-Sucundurí/Juruena interfluve, based on the examination of specimens stored in museums and documented field records: (A) *P. cinerascens* morphotype according to Hershkovitz (1990) and Van Roosmalen *et al*. (2002); (B) *P. cinerascens* morphotype according to the species description by Spix (1823); (C), (D), and (E) intermediary morphotypes presented here between both *P. cinerascens* morphotypes and *P. parecis* morphotype (F).

The pelage pigmentation also grades N-S in the extent and colour of variegate hairs of the dorsum. In addition to the greyish hairs, there are specimens bearing few tawny hairs on a reduced part of the dorsum at the northernmost localities of the Aripuanã-Sucunduri/Juruena interfluve (Figures 5 and 6 A, B), whereas specimens along most of this interfluve show brown-reddish hairs covering the entire dorsum (Figures 5 and 6 C, D, E, F), including the type specimens of *P. parecis*. Two localities south of the records of *P. parecis* do not follow the clinal pattern, with individuals in these localities resembling the northernmost morphotype that is considered the “typical” all-greyish *P. cinerascens* phenotype (*sensu* Hershkovitz 1990; Van Roosmalen *et al.* 2002) without the whitish beard, contrary to the phenotype described and depicted by Spix (1823). All prior field surveys reporting on *P. cinerascens* did not follow the original species description and, therefore, are of limited use in understanding geographic variation in this species. We did not consider field surveys without specimen descriptions or pictures in our analysis.

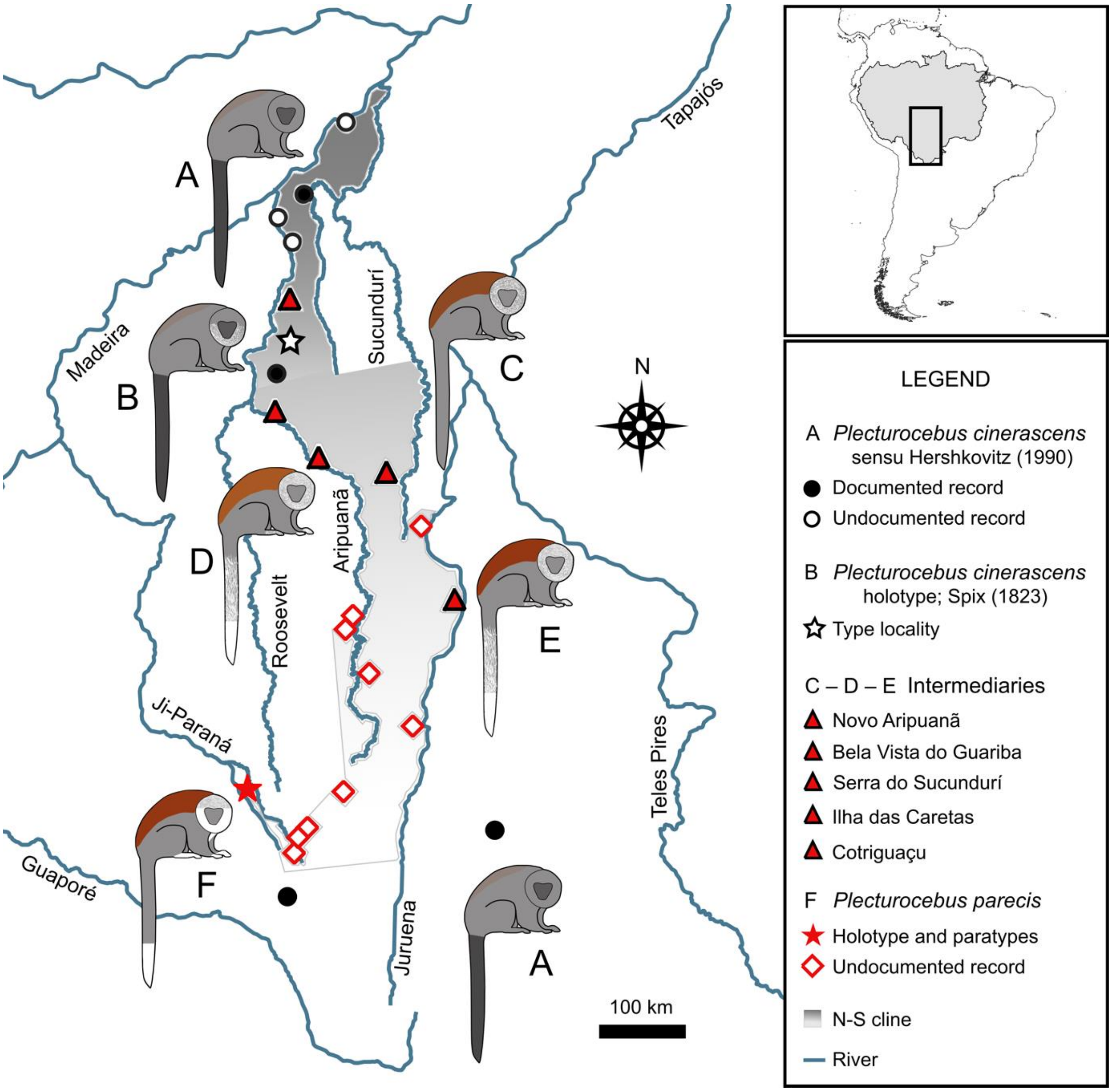

Figure 6. Geographic distribution of titi monkey morphotypes along the Aripuanã-Sucundurí/Juruena interfluve, showing a north-south clinal bleaching gradient from *P. cinerascens* to *P. parecis*.

Discussion

Taxonomy, distribution, and phylogenetic relationships of *<u>P. cinerascens</u>* *and* *<u>P. parecis</u>*

The results of our new molecular, morphological, and distribution analyses lend insight into the taxonomy and evolutionary history of *P. cinerascens*, *P. parecis,* and *P. bernhardi.* The mitochondrial loci (and therefore likely the mitochondrial genome) of the three paratypes of *P. parecis* are very similar to each other and to those of *P. cinerascens* specimens, with less than 0.5% divergence, consistent with their similarity in pelage pigmentation patterns. We retrieved a clade with two *P. parecis* paratypes (UFRO 352, UFRO 355) sister to a *P. cinerascens* clade containing the third *P. parecis* paratype (UFRO 195), which was not included in the molecular phylogenies of Gusmão *et al*. (2019). The paraphyletic arrangement found in the mitochondrial gene tree could be a result of incomplete lineage sorting or introgression following secondary contact between two distinct species or, alternatively, it could lend support to *P. parecis* being a junior synonym of *P. cinerascens* (Maddison 1997; Funk and Omland 2003; McKay and Zink 2010; Zinner *et al.* 2011).

We found N-S, greyish to whitish clinal variation in *P. cinerascens* and *P. parecis* along the Aripuanã-Sucundurí/Juruena interfluve. At the extreme north of this interfluve, the pelage of titis is all-greyish and matches the *P. cinerascens* morphotype *sensu* Hershkovitz (1990) and Van Roosmalen *et al*. (2002). At the mid Aripuanã River, near its confluence with the Roosevelt River, titis have whitish hairs on beard and hands, and are very similar to the description and painting of the *P. cinerascens* holotype from Spix (1823). South of this area to the mid Juruena River (Cotriguaçu), overlapping with records of *P. parecis*, there are two other morphotypes with more whitish hairs on the beard, hands, feet, and tail. There are no specimens collected between Cotriguaçu and the type locality of *P. parecis* but further pelage variation may be found over such large area, which is covered by localities attributed to *P. parecis* by Gusmão *et al*. (2019). Available evidence suggests that the *P. parecis* morphotype—characterised by completely whitish beard, hands, feet, tail tip, chest, and neck—is restricted to the species type locality at the headwaters of Ji-Paraná River. Specimens collected (Gusmão *et al.* 2019) or photographed (Sampaio *et al.* 2012) at two localities south of the records of *P. parecis* seem to present the overall greyish morphotype of the northernmost localities of *P. cinerascens,* and do not match the geographic pattern of pelage variation.

The clinal variation in pelage pigmentation of *P. cinerascens* and *P. parecis* detected here is unsurprising given the lack of physical barriers that could prevent dispersal and gene flow between these lineages along the Aripuanã-Sucundurí/Juruena interfluve. Diagnostic characters of *P. parecis,* such as whitish beard, hands, feet, and tail, can be found in titis along most of the Aripuanã-Sucunduri/Juruena interfluve to variable extents, including the *P. cinerascens* holotype. In fact, the holotype of *P. cinerascens* (Spix 1823) appears to have an intermediary morphotype (whitish beard) between the all-greyish titis historically associated with *P. cinerascens* (Hershkovitz 1990; Van Roosmalen *et al.* 2002), which are primarily found at the extreme north of the interfluve, and the whitish *P. parecis* morphotype (Gusmão *et al.* 2019), which is potentially restricted to the headwaters of the Ji-Paraná River. This suggests that the *P. cinerascens* holotype was collected in the central area of the interfluve, so we propose to restrict the type locality of *P. cinerascens* to the left margin of the mid Aripuanã river (-6.94, -60.26). Interestingly, specimens from the northernmost and southernmost localities are all-greyish and differ from *P. parecis* and all intermediary morphotypes, including *P. cinerascens sensu* Spix (1823), as here discussed.

Based on the existence of a N-S, greyish to whitish cline in the pelage pigmentation of titis along Aripuanã-Sucundurí/Juruena interfluve, the mitochondrial paraphyly of *P. parecis*, the high similarity of nuclear and mitochondrial loci of both taxa, and the geographical distribution of the morphotypes as here discussed, several hypotheses can plausibly explain the taxonomy and distribution of *P. cinerascens* and *P. parecis*, including the following:

1. <u>A single polymorphic species with a wide distribution</u>: *Plecturocebus cinerascens* is the only titi species in the Aripuanã-Sucunduri/Juruena interfluve, supported by the clear lack of abrupt changes in pelage pigmentation patterns and geographical barriers to gene flow in this region. In this case, *P. cinerascens* encompasses all morphological variation and localities discussed here, and *P. parecis* is a junior synonym. It possibly forms a hybrid zone with *P. bernhardi* in a small part of its range (the area around the type locality of *P. parecis*).
2. <u>Two species with restricted distributions and a large hybrid zone</u>: *Plecturocebus parecis* is a valid species with a range restricted to the type locality and adjacencies at the south of the Aripuanã-Sucunduri/Juruena interfluve, and with a history of introgression with *P. bernhardi*. The all-greyish morphotype (*P. cinerascens sensu* Hershkovitz 1990) is a distinct species restricted to the northernmost (and perhaps southernmost) portion of this interfluve. The *P. cinerascens* holotype (Spix 1823) and other intermediary whitish morphotypes are hybrids of these two lineages found in a wide zone of gene flow at the centre of the Aripuanã-Sucunduri/Juruena interfluve. This pattern could be explained by peripatric divergence of *P. parecis*.
3. <u>One polymorphic widely distributed species and one cryptic species with a restricted distribution</u>: *Plecturocebus cinerascens* represents a polymorphic species that encompasses the all-greyish morphotypes and intermediate morphotypes, while *P. parecis* is a valid cryptic species that shows a similar whitish morphotype to some *P.*

cinerascens specimens and a history of introgression with *P. bernhardi.* This could be explained by cryptic speciation of *P. parecis* from a *P. cinerascens* population on the Parecis highlands.

4. <u>One widely distributed polymorphic species and one lineage of hybrid origin with restricted distribution:</u> Introgression with *P. bernhardi* underlies the distinct mitochondrial genome of the *P. parecis* holotype and divergence in nuclear data between *P. parecis* specimens and *P. cinerascens*. *P. cinerascens* is defined as in the hypothesis above, while *P. parecis* is a valid taxon in an early phase of speciation driven by the hybridisation between a population of *P. cinerascens* around the type locality of *P. parecis* with *P. bernhardi.*

Further samples are required to test the hypotheses here proposed and to delimit the species, clarify the geographic distribution and molecular affinity of all morphotypes, and identify the extent of potential contact/hybrid zones. Although available evidence indicates that there are cohesive populations of all-greyish titis in the northern portion of the cline, the presence of this morphotype may be widespread also at the extreme south of the cline but the real scenario is obscured by the scarcity of field efforts in this region. Further field data are likely to highlight regions that contain populations with mixed morphotypes, as well as still undescribed intermediate morphotypes across the Aripuanã-Sucundurí/Juruena interfluve. Additional sampling is particularly essential around the *P. parecis* type locality (Rondon II hydroelectric dam) and the collection locality of *P. bernhardi* specimens in Rondônia State (Guaporé Biological Reserve), as well around the localities of all-greyish *P. cinerascens* at the southern portion of the Aripuanã-Sucundurí/Juruena interfluve (Cabixi River, Rondônia; Cravari River, Mato Grosso).

Introgression from <u>P. bernhardi</u> into <u>P. parecis</u>

Our analysis of mitochondrial data revealed that the holotype of *P. parecis* (UFRO 354) has ~ 0.5% divergence from *P. bernhardi* specimens (42960, 42961, 42964) from the Guaporé Biological Reserve across the CYTB and COI together. The nuclear phylogenies, in contrast, show the *P. parecis* holotype clustering with two *P. parecis* paratypes from the same collection locality (UFRO 352, UFRO 355) in the maximum-likelihood tree and in a polytomy with *P. cinerascens* in the Bayesian tree. This suggests post-divergence gene flow between *P. bernhardi* and *P. parecis*/*P. cinerascens*. We consider incomplete lineage sorting less likely to explain this pattern considering the clades containing *P. cinerascens*/*parecis* and *P. bernhardi* diverged around 2 to 2.2 Mya, putatively representing the deepest divergence within the *Plecturocebus moloch* group (Byrne 2017; Byrne *et al.* 2018), in combination with the high similarity of the *P. bernhardi* and UFRO 354 mitochondrial sequences which suggests a more common ancestor of their mitochondrial genomes than the divergence of these species.

Our results suggest that the holotype of *P. parecis* (UFRO 354) was mislabelled in the molecular phylogenies of Gusmão *et al*. (2019) and Carneiro *et al*. (2016) as a result of its mitochondrial affinity with *P. bernhardi*. Although both studies included a small amount of nuclear data in the form of *alu* sequences, mitochondrial and nuclear data were not analysed separately, which can be problematic. There are, for example, more parsimony informative (PI) sites for titi monkeys in the two mitochondrial loci than in the 20 nuclear loci in the data sets of Byrne *et al*. (2016). It seems that the phylogenetic signal from the mitochondrial sequences in Carneiro *et al*. (2016) and Gusmão *et al*. (2019) overwhelmed the information contained in the small number of nuclear sequences owing to the higher mutation rate and significantly greater number of informative sites, particularly at such short phylogenetic distances. This is shown by the supported clustering of UFRO 354 with *P. bernhardi* specimens in the molecular phylogenies of both studies.

The discovery that the *P. parecis* holotype (UFRO 354) has a mitochondrial genome very similar to *P. bernhardi*, but with distinct pelage pigmentation patterns, is of broader interest beyond the implications for the taxonomic validity of *P. parecis* and its relationship to *P. cinerascens*. The Rondon II dam (*P. parecis* type locality) is at the edge of the southern tip of the range proposed for *P. bernhardi* in its description by Van Roosmalen *et al*. (2002), which was delineated based on the Roosevelt/Aripuanã and Ji-Paraná rivers (and later updated by Byrne *et al.* 2018). There are several differentiated lineages within *P. bernhardi* with the individuals (42960, 42961, 42964) collected south of the Ji-Paraná River in the region of the Guaporé Biological Reserve, Rondônia, consistently forming a relatively distinct clade in the molecular phylogenies generated to date (e.g., Byrne *et al.* 2016; Carneiro *et al.* 2016; Byrne 2017) (Figure 2). The Guaporé Biological Reserve is a considerable distance from all other *P. bernhardi* specimens we have molecular data for currently, but it is the closest to the Rondon II dam where the *P. parecis* type specimens were collected (Figure 2). It is likely that the *P. bernhardi* individuals collected at the Guaporé Biological Reserve are relatively closely related to the *P. bernhardi* donor lineage from which the *P. parecis* holotype's mitochondrial genome originated. Other individuals that have been identified as *P. bernhardi* have been recorded even nearer to the Rondon II dam than the Guaporé Biological Reserve (e.g., see the recorded sightings in Figure 4 in Gusmão *et al.* 2019).

Our results indicate that some *P. parecis* individuals show a high similarity in their mitochondrial genome to the morphologically distinct *P. bernhardi*, as well as *P. cinerascens*. These patterns could be the result of hybrid speciation, ancient admixture, contemporary/on-going gene flow in more recently developed hybrid zones as a result of secondary contact, or incomplete lineage sorting (in the case of *P. cinerascens* and *P. parecis*). We are particularly interested in investigating putative introgressive hybridisation between *P. parecis* and *P. bernhardi* in the region contained by the Ji-Paraná tributaries, the Comemoração and Pimenta Bueno rivers, for example, assessing whether there are introgressed *P. bernhardi* alleles in the nuclear genomes of the *P. parecis* individuals collected at the Rondon II dam. In such a scenario, it would also be essential to establish more information about the geographic extent of gene flow and introgressed alleles with more extensive sampling. Understanding such evolutionary processes is also important to generate more stable and informative taxonomic assessments for the morphotypes here described and their distributions along the Aripuanã-Sucunduri/Juruena, as well as for the *P. bernhardi* lineage from the Guaporé Biological Reserve.

Hybridisation among platyrrhine primates has been primarily studied in howler monkeys (*Alouatta*), with the first genetic evidence reported for *Alouatta palliata* and *A. pigra* at a hybrid zone in Mexico (Cortés-Ortiz *et al.* 2007). There are few well-documented cases of natural hybridisation among other platyrrhine primate clades and many proposed examples of interspecific hybridisation based on phenotypic evidence involve particularly closely related lineages, for example, among the Western clade of *Plecturocebus moloch* group taxa (e.g., Hershkovitz 1988; Serrano-Villavicencio *et al.* 2017). Nonetheless, a similar pattern to what we found here, in terms of distribution, morphology, and phylogenetics, was recently reported for species of *Mico* (Costa-Araújo *et al.* 2019). Given the scarcity of study systems, the putative evidence of introgressive hybridisation between *P. parecis* and *P. bernhardi* is significant not only to Callicebinae, but also for the study of hybridisation among platyrrhine primates. Titi monkeys are monogamous, pair-bonding primates (Norconk 2011) and present a particularly interesting case to assess the dynamics of introgressive hybridisation in an uncommon mating system.

Conclusions

In this study, through a combination of evidence derived from new field expeditions, assessment of pelage pigmentation patterns, and phylogenetic analyses, we clarified the taxonomy, distribution, and evolutionary history of *P. parecis*, *P. cinerascens*, and *P. bernhardi*. Our mitochondrial phylogeny recovered a paraphyletic arrangement for the four *P.*

parecis type specimens which show three distinct haplotypes, with the mitochondrial loci of the holotype specimen showing a close affinity to *P. bernhardi*, and a paratype grouping with *P. cinerascens*. The nuclear phylogenies show the holotype specimen clustering with the other *P. parecis* in a separate clade in the maximum-likelihood tree or in a polytomy with *P. cinerascens* in the Bayesian tree. Paraphyly in the mitochondrial phylogeny and incongruence with and within the nuclear topologies may be a result of incomplete lineage sorting (between *P. cinerascens* and *P. parecis*) and introgressive hybridisation (particularly between *P. bernhardi* and *P. parecis/cinerascens*). Our morphological analysis reveals a N-S clinal bleaching gradient through the Aripuanã-Sucundurí/Juruena interfluve from an all-greyish morphotype associated with *P. cinerascens*, through intermediary morphotypes with increasingly whitish hairs on the beard, hands, feet, and tail, to the whitish morphotype described as *P. parecis*. Further molecular, morphological, and distribution data are required to test the hypotheses proposed here to elucidate the true diversity, distribution, and evolution of titi monkeys in south-central Amazonia. Complex diversification patterns and diverse species assemblages have also been found for other taxa in this region, including many different lineages of birds (e.g., Fernandes 2013; Thom and Aleixo 2015) and some primates (Lynch Alfaro *et al.* 2015). These patterns have most often been associated with a complicated history of geological and river system evolution (Latrubesse 2002), which also concurs with the intricate biogeographic history reconstructed for titi monkeys in this region (Byrne *et al.* 2018).

References

Boubli, J. P., Byrne, H., da Silva, M., Silva-Júnior, J., Costa Araújo, R., Bertuol, F., Gonçalves, J., de Melo, F. R., Rylands, A. B., Mittermeier, R. A., Silva, F. E., Nash, S. D., Canale, G., Alencar, R. M., Rossi, R. V., Carneiro, J., Sampaio, I., Farias, I. P., Schneider, H., & Hrbek, T. (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.

Byrne, H. (2017). Evolutionary History and Taxonomy of the Titi Monkeys. Ph.D. thesis, University of Salford, Manchester, UK.

Byrne, H., Lynch Alfaro, J. W., Sampaio, I., Farias, I., Schneider, H., Hrbek, T., & Boubli, J. P. (2018). Titi monkey biogeography: Parallel Pleistocene spread by *Plecturocebus* and *Cheracebus* into a post- Pebas Western Amazon. *Zoologica Scripta*, 47(5), 499–517.

Byrne, H., Rylands, A. B., Carneiro, J. C., Lynch Alfaro, J. W., Bertuol, F., da Silva, M. N. F., Messias, M., Groves, C. P., Mittermeier, R. A., Farias, I., Hrbek, T., Schneider, H., Sampaio, I., & Boubli, J. P. (2016). Phylogenetic relationships of the New World titi monkeys (*Callicebus*): First appraisal of taxonomy based on molecular evidence. *Frontiers in Zoology*, 13(10), 1–25.

Byrne, H., Rylands, A. B., Nash, S., & Boubli, J. P. (2020). On the taxonomic history and true identity of the collared titi, *Cheracebus torquatus* (Hoffmannsegg, 1807) (Platyrrhini, Callicebinae*). Primate Conservation,* (34), 13–52.

Carneiro, J. C., Silva Júnior, J. S., Sampaio, I., Pissinatti, A., Hrbek, T., Messias, M., Röhe, F., Farias, I., Boubli, J. P., & Schneider, H. (2016). Phylogeny of the titi monkeys of the *Callicebus moloch* group (Pitheciidae, Primates). *American Journal of Primatology*, 78(9), 904–913.

Cortés-Ortiz, L., Duda, T. F., Canales-Espinosa, D., García-Orduña, F., Rodríguez-Luna, E., & Bermingham, E. (2007). Hybridization in large-bodied New World primates. *Genetics*, 176(4), 2421–2425.

Costa-Araújo, R., Melo, F. R., Canale, G. R., Hernández-Rangel, S. M., Messias, M. R., Rossi, R. V., Silva, F. E., da Silva, M. N. F., Nash, S. D., Boubli, J. P., Farias, I. P., & Hrbek, T. (2019). The Munduruku marmoset: a new monkey species from southern Amazonia. *PeerJ*, 7, e7019.

Costa-Araújo, R., Silva-Jr., J. S., Boubli, J., Rossi, R. V., Canale, G. R., Melo, F. R., Bertuol, F., Silva, F. E., Silva, D. A., Nash, S. D., Sampaio, I., Farias, I. P., & Hrbek, T. (2021). An integrative analysis uncovers a new, pseudo- cryptic species of Amazonian marmoset (Primates: Callitrichidae: *Mico*) from the arc of deforestation. *Scientific Reports*, in press, 10.1038/s41598-021-93943-w

Di Fiore, A., Chaves, P. B., Cornejo, F. M., Schmitt, C. A., Shanee, S., Cortés-Ortiz, L., Fagundes, V., Roos, C., & Pacheco, V. (2015). The rise and fall of a genus: Complete mtDNA genomes shed light on the phylogenetic position of yellow-tailed woolly monkeys, *Lagothrix flavicauda*, and on the evolutionary history of the family Atelidae (Primates: Platyrrhini). *Molecular Phylogenetics & Evolution*, 82B, 495–510.

Fernandes, A. M. (2013). Fine-scale endemism of Amazonian birds in a threatened landscape. *Biodiversity & Conservation*, 22, 2683–2694.

Ferrari, S. F., Iwanaga, S., Messias, M. R., Ramos, E. M., Ramos, P. C. S., da Cruz Neto, E. H., & Coutinho, P. E. G. (2000). Titi monkeys (*Callicebus* spp., Atelidae: Platyrrhini) in the Brazilian state of Rondônia. *Primates*, 41(2), 229–234.

Funk, D. J., & Omland, K. E. (2003). Species-level paraphyly and polyphyly: Frequency, causes, and consequences, with insights from animal mitochondrial DNA. *Annual*

Review of Ecology, Evolution, & Systematics, 34(1), 397–423.

Gualda-Barros, J., Nascimento, F. O., & Amaral, M. K. (2012). A new species of *Callicebus* Thomas, 1903 (Primates, Pitheciidae) from the states of Mato Grosso and Pará, Brazil. *Papéis Avulsos de Zoologia (São Paulo),* 52(23), 261−279.

Gusmão, A. C., Messias, M. R., Carneiro, J. C., Schneider, H., de Alencar, T. B., Calouro, A. M., Dalponte, J. C., de Souza Mattos, F., Ferrari, S. F., Buss, G., & de Azevedo, R. B. (2019). A new species of titi monkey, *Plecturocebus* Byrne *et al.* 2016 (Primates, Pitheciidae), from southwestern Amazonia, Brazil. *Primate Conservation*, 33, 1−15.

Gusmão, A. C., & da Costa, T. M. (2014). Registro de *Callicebus cinerascens* (Spix 1823) no Médio Vale Do Guaporé, Rondônia, Brasil. *Neotropical Primates*, 21(2), 210−211.

Hershkovitz, P. (1988). Origin, speciation, and distribution of South American titi monkeys, genus *Callicebus* (Family Cebidae, Platyrrhini). *Proceedings of the Academy of Natural Sciences of Philadelphia*, 140(1), 240–272.

Hershkovitz, P. (1990). Titis, New World monkeys of the genus *Callicebus* (Cebidae, Platyrrhini): A preliminary taxonomic review. *Fieldiana, Zoology New Series,* (55), 1–109.

Latrubesse, E. M. (2002). Evidence of quaternary palaeohydrological changes in middle Amazonia: The Aripuanã-Roosevelt and Jiparaná fans. *Zeitschrift für Geomorphologie,* 129, 61–72.

Lovejoy, T. E. & Nobre, C. (2018). Amazon tipping point. *Science Advances*, 4(2), eaat2340.

Lynch Alfaro, J. W., Boubli, J. P., Paim, F. P., Ribas, C. C., da Silva, M. N. F., Messias, Röhe, F., Mercês, M. P., Silva Júnior, J. S., Silva, C. R., Pinho, G. M., Koshkarian, G., Nguyen, M. T. T., Harada, M. L., Rabelo, R. M., Queiroz, H. L., Alfaro, M. E., & Farias, I. P. (2015). Biogeography of squirrel monkeys (genus *Saimiri*): South-central Amazon origin and rapid pan-Amazonian diversification of a lowland primate. *Molecular Phylogenetics & Evolution*, 82B, 436–454.

Lynch-Alfaro, J. W., Silva-Jr, J. S., & Rylands, A. B. (2012). How different are robust and gracile capuchin monkeys? An argument for the use of *Sapajus* and *Cebus, American Journal of Primatology,* 74, 273–286.

Maddison, W. P. (1997). Gene trees in species trees. *Systematic Biology*, 46(3), 523–536.

McKay, B. D., & Zink, R. M. (2010). The causes of mitochondrial DNA gene tree paraphyly in birds. *Molecular Phylogenetics & Evolution*, 54(2), 647–650

Norconk, M. A. (2011). Sakis, uakaris, and titi monkeys. In Campbell, C. J., Fuentes, A., MacKinnon, K. C., Bearder, S. K., Stumpf, R. M., (Ed.), *Primates in Perspective* (pp. 122−139). Oxford: Oxford University Press.

Noronha, M. D. A., Spironello, W. R., & Ferreira, D. C. (2007). New occurrence records and eastern extension to the range of *Callicebus cinerascens* (Primates, Pitheciidae). *Neotropical Primates*, 14, 137–139.

Perelman, P., Johnson, W. E., Roos, C., Seuánez, H. N., Horvath, J. E., Moreira, M. A., Kessing, B., Pontius, J., Roelke, M., Rumpler, Y., Schneider, M. P. C., Silva, A., O'Brien, S. J., & Pecon-Slattery, J. (2011). A molecular phylogeny of living primates. *PLOS Genetics*, 7(3), e1001342.

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M. A., & Huelsenbeck, J. P. (2012). MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology*, 61(3), 539–542.

Sampaio, R., Dalponte, J. C., Rocha, E. C., Hack, R. O., Gusmão, A. C., Aguiar, K. M., Kuniy, A. A., de Sousa, J., & Silva-Júnior, J. (2012). Novos registros com uma extensão da distribuição geográfica de *Callicebus cinerascens* (Spix 1823). *Mastozoología Neotropical*, 19(1), 159–164.

Serrano-Villavicencio, J. E., Vendramel, R. L., & Garbino, G. S. T. (2017). Species, subspecies, or color morphs? Reconsidering the taxonomy of *Callicebus* Thomas, 1903 in the Purus–Madeira interfluvium. *Primates*, 58(1), 159–167.

Spix J. B. (1823). *Simiarum et vespertiliarum brasiliensis species novae.* F. S. Hübschmann: Munich.

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

Stamatakis, A., Hoover, P., & Rougemont, J. (2008). A rapid bootstrap algorithm for the RAxML Web servers. *Systematic Biology,* 57(5), 758–771.

Thom, G., & Aleixo, A. (2015). Cryptic speciation in the white- shouldered antshrike (*Thamnophilus aethiops,* Aves – Thamnophilidae): The tale of a transcontinental radiation across rivers in lowland Amazonia and the northeastern Atlantic Forest. *Molecular Phylogenetics & Evolution*, 82, 95–110.

Van Roosmalen, M. G. M., Van Roosmalen, T., & Mittermeier, R. A. (2002). A taxonomic review of the titi monkeys, genus *Callicebus* Thomas, 1903, with the description of two new species, *Callicebus bernhardi* and *Callicebus stephennashi*, from Brazilian Amazonia. *Neotropical Primates,* 10(suppl.), 1–52.

Vidal, M. D. (2012). Protocolo para coleta de dados sobre primatas em Unidades de Conservação da Amazônia. ICMBio. Brasília, Distrito Federal.

Zinner, D., Arnold, M. L., & Roos, C. (2011). The strange blood: Natural hybridization in primates. *Evolutionary Anthropology*, 20(3), 96–103.

Table 1 Information for specimens included in our molecular data sets including species, ID, museum collection, latitude and longitude, locality, and corresponding number in Figure 2.

Species	Sample ID	Collection[1]	Lat., long.	Locality	Number in Figure 2
P. parecis	UFRO 195	UNIR	-12.06, -60.67	Rondon II Dam, Pimenta Bueno, Rondônia	1
P. parecis	UFRO 352	UNIR	-12.06, -60.67	Rondon II Dam, Pimenta Bueno, Rondônia	1
P. parecis	UFRO 354	UNIR	-12.06, -60.67	Rondon II Dam, Pimenta Bueno, Rondônia	1
P. parecis	UFRO 355	UNIR	-12.06, -60.67	Rondon II Dam, Pimenta Bueno, Rondônia	1
P. cinerascens	UFRO 499	UNIR	-13.30, -60.26	Cabixi, Rondônia	2
P. cinerascens	FR 31	INPA (5682)	-6.41, -60.36	Novo Aripuanã, R bank of the Rio Aripuanã, Amazonas	3
P. cinerascens	FR 50	INPA	-6.8, -59.06 (estimated)	Sucunduri, Apuí, Amazonas	4
P. cinerascens	FR 123	INPA	-6.41, -60.36	Novo Aripuanã, R bank of the Rio Aripuanã, Amazonas	3
P. miltoni	42991	MPEG	-7.74, -60.52	Novo Aripuanã, L bank of the Rio Aripuanã, Amazonas	5
P. miltoni	42992	MPEG	-7.74, -60.52	Novo Aripuanã, L bank of the Rio Aripuanã, Amazonas	5
P. bernhardi	42960	MPEG	-12.17, -63.19	São Francisco do Guaporé, Guaporé Biological Reserve, Rondônia	6
P. bernhardi	42961	MPEG	-12.17, -63.19	São Francisco do Guaporé, Guaporé Biological Reserve, Rondônia	6
P. bernhardi	42964	MPEG	-12.17, -63.19	São Francisco do Guaporé, Guaporé Biological Reserve, Rondônia	6
P. bernhardi	UFRO 413	UNIR	Unknown	Machadinho D'Oeste, Rondônia	NA (origin too broad)
P. bernhardi	FR 26	INPA (5679)	-5.76, -60.26	Novo Aripuanã, L bank of the Rio Aripuanã, Amazonas	8
P. bernhardi	CCM 173	INPA (4029)	-8.60, -62.41	Rio Mariepauá, R bank tributary of the Rio Madeira, Amazonas	7
P. grovesi	RVR 73	INPA	-9.98, -56.07	Novo Horizonte Community, Alta Floresta, Mato Grosso	NA
P. moloch	CTGAM 420	UFAM	-3.36 -55.21	Belterra, R bank of the Rio Tapajós, Pará	NA
P. moloch	MCB 63	MPEG	-2.45, -51.53	Senador José Porfírio, R bank of the Rio Xingu, Pará	NA

[1] Collection abbreviations: UFAM = Universidade Federal do Amazonas; INPA = Instituto Nacional de Pesquisas da Amazônia; UNIR = Universidade Federal de Rondônia; MPEG = Museu Paraense Emílio Goeldi.

Table 2 Information considered in our morphological assessment including the morphotypes' label according to Figures 5 and 6, species names, sources (with specimen ID and museum name for specimens directly assessed), latitude and longitude, and locality name.

Morphotype	Species	Locality	Lat., long.	Source
A	*P. cinerascens*	Prainha, Amazonas	-7.26, -60.38	Hershkovitz (1990)
	P. cinerascens	Cipotuba	-5.80, -60.21	Van Roosmalen *et al.* (2002)
	P. cinerascens	Prainha, Igarapé da Prainha	-5.75, -60.20	Van Roosmalen *et al.* (2002)
	P. cinerascens	São João, Igarapé Terra Preta	-5.46, -60.36	Van Roosmalen *et al.* (2002)
	P. cinerascens	Right bank of Madeira River, in the vicinity of the town of Novo Aripuanã	-5.11, -60.37	Van Roosmalen *et al.* (2002)
	P. cinerascens	Right bank of Madeira River, left bank of lower Arara River, 40 km east of Novo Aripuanã	-5.20, -60.06	Van Roosmalen *et al.* (2002)
	P. cinerascens	Right bank of Madeira River, in the vicinity of the town of Borba; Amazonas	-4.36, -59.58	Van Roosmalen *et al.* (2002)
	P. cinerascens	Rio Cravari region; Mato Grosso	-12.53, -57.86	Sampaio *et al.* (2012)
	P. cinerascens	Cabixi region (Cabixi River, a tributary of Guaporé River), Rondônia	-13.30, -60.26	Gusmão *et al.* (2019)
B	*P. cinerascens* holotype	Mid Aripuanã River, Apuí, Amazonas (type locality here restricted)	-6.94, -60.26	This paper
C, D, E	Intermediary morphotype	Novo Aripuanã, R bank of the Rio Aripuanã, Amazonas	-6.41, -60.36	This paper FR 31, 123 (INPA)
	Intermediary morphotype	Bela Vista do Guariba, Apuí, Amazonas	-7.69, -60.40	This paper RCA 92 (MPEG)
	Intermediary morphotype	Serra do Sucunduri, Apuí, Amazonas	-6.80, -59.06 (estimated)	This paper FR 50 (INPA)
	Intermediary morphotype	Ilha das Caretas, Apuí, Amazonas	-8.23, -59.90	This paper RCA 100 (INPA)
	Intermediary morphotype	Cotriguaçu, Mato Grosso	-9.86, -58.32	This paper RCA 66 (INPA)
F	*P. parecis* holotype	Rondon II Dam, Pimenta Bueno, Rondônia (type locality)	-12.06, -60.67	Gusmão *et al.* (2019)

[1] Collection abbreviations: INPA = Instituto Nacional de Pesquisas da Amazônia; MPEG = Museu Paraense Emílio Goeldi.

Table 3 Number of base pair differences (top) and percentage identity (bottom) between select specimens at the CYTB locus (left) and COI locus (right).

	P. parecis UFRO352		*P. parecis* UFRO355		*P. parecis* UFRO195		*P. cinerascens* UFRO499		*P. cinerascens* FR123		*P. cinerascens* FR50		*P. cinerascens* FR31		*P. bernhardi* UFRO413		*P. bernhardi* CCM173		*P. bernhardi* FR26		*P. parecis* UFRO354		*P. bernhardi* 42960		*P. bernhardi* 42961		*P. bernhardi* 42964	
P. parecis UFRO352			0	0	7	2	7	4	6	3	7	4	5	--	45	32	47	31	49	30	46	29	47	29	47	--	47	29
			100	100	99.39	99.7	99.39	99.39	99.47	99.55	99.39	99.39	99.54	--	96.05	95.15	95.88	95.3	95.7	95.43	95.96	95.28	95.88	95.61	95.88	--	95.88	95.61
P. parecis UFRO355	0	0			7	2	7	4	6	3	7	4	5	--	45	32	47	31	49	30	46	29	47	29	47	--	47	29
	100	100			99.39	99.7	99.39	99.39	99.47	99.55	99.39	99.39	99.54	--	96.05	95.15	95.88	95.3	95.7	95.43	95.96	95.28	95.88	95.61	95.88	--	95.88	95.61
P. parecis UFRO195	7	2	7	2			4	2	3	1	4	2	2	--	47	30	49	29	51	28	48	27	47	27	47	--	47	27
	99.39	99.7	99.39	99.7			99.65	99.7	99.74	99.85	99.65	99.69	99.81	--	95.88	95.45	95.7	95.61	95.53	95.73	95.79	95.61	95.88	95.91	95.88	--	95.88	95.91
P. cinerascens UFRO499	7	4	7	4	4	2			3	3	4	4	2	--	47	32	47	31	49	30	46	29	47	29	47	--	47	29
	99.39	99.39	99.39	99.39	99.65	99.7			99.74	99.55	99.65	99.39	99.81	--	95.88	95.15	95.88	95.3	95.7	95.43	95.96	95.28	95.88	95.61	95.88	--	95.88	95.61
P. cinerascens FR123	6	3	6	3	3	1	3	3			3	2	1	--	46	31	48	30	50	29	47	28	48	28	48	--	48	28
	99.47	99.55	99.47	99.55	99.74	99.85	99.74	99.55			99.74	99.69	99.91	--	95.96	95.3	95.79	95.45	95.61	95.58	95.88	95.45	95.79	95.76	95.79	--	95.79	95.76
P. cinerascens FR50	7	4	7	4	4	2	4	4	3	2			2	--	43	30	45	29	47	28	44	27	45	27	45	--	45	27
	99.39	99.39	99.39	99.39	99.65	99.69	99.65	99.39	99.74	99.69			99.81	--	96.23	95.41	96.05	95.56	95.88	95.71	96.14	95.56	96.05	95.87	96.05	--	96.05	95.87
P. cinerascens FR31	5	--	5	--	2	--	2	--	1	--	2	--			41	--	43	--	45	--	42	--	43	--	43	--	43	--
	99.54	--	99.54	--	99.81	--	99.81	--	99.91	--	99.81	--			96.2	--	96.02	--	95.83	--	96.11	--	96.02	--	96.02	--	96.02	--
P. bernhardi UFRO413	45	32	45	32	47	30	47	32	46	31	43	30	41	--			14	13	15	12	15	10	12	9	12	--	12	9
	96.05	95.15	96.05	95.15	95.88	95.45	95.88	95.15	95.96	95.3	96.23	95.41	96.2	--			98.77	98.03	98.68	98.17	98.68	98.37	98.95	98.64	98.95	--	98.95	98.64
P. bernhardi CCM173	47	31	47	31	49	29	47	31	48	30	45	29	43	--	14	13			7	1	11	6	10	6	10	--	10	6
	95.88	95.3	95.88	95.3	95.7	95.61	95.88	95.3	95.79	95.45	96.05	95.56	96.02	--	98.77	98.03			99.39	99.85	99.04	99.02	99.12	99.09	99.12	--	99.12	99.09
P. bernhardi FR26	49	30	49	30	51	28	49	30	50	29	47	28	45	--	15	12	7	1			14	5	13	5	13	--	13	5
	95.7	95.43	95.7	95.43	95.53	95.73	95.7	95.43	95.61	95.58	95.88	95.71	95.83	--	98.68	98.17	99.39	99.85			98.77	99.18	98.86	99.24	98.86	--	98.86	99.24
P. parecis UFRO354	46	29	46	29	48	27	46	29	47	28	44	27	42	--	15	10	11	6	14	5			7	1	7	--	7	1
	95.96	95.28	95.96	95.28	95.79	95.61	95.96	95.28	95.88	95.45	96.14	95.56	96.11	--	98.68	98.37	99.04	99.02	98.77	99.18			99.39	99.84	99.39	--	99.39	99.84
P. bernhardi 42960	47	29	47	29	47	27	47	29	48	28	45	27	43	--	12	9	10	6	13	5	7	1			0	--	0	0
	95.88	95.61	95.88	95.61	95.88	95.91	95.88	95.61	95.79	95.76	96.05	95.87	96.02	--	98.95	98.64	99.12	99.09	98.86	99.24	99.39	99.84			100	--	100	100
P. bernhardi 42961	47	--	47	--	47	--	47	--	48	--	45	--	43	--	12	--	10	--	13	--	7	--	0	--			0	--
	95.88	--	95.88	--	95.88	--	95.88	--	95.79	--	96.05	--	96.02	--	98.95	--	99.12	--	98.86	--	99.39	--	100	--			100	--
P. bernhardi 42964	47	29	47	29	47	27	47	29	48	28	45	27	43	--	12	9	10	6	13	5	7	1	0	0	0	--		
	95.88	95.61	95.88	95.61	95.88	95.91	95.88	95.61	95.79	95.76	96.05	95.87	96.02	--	98.95	98.64	99.12	99.09	98.86	99.24	99.39	99.84	100	100	100	--		