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RESEARCH ARTICLE

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Phylogenetic relationships in the genus *Cheracebus* (Callicebinae, Pitheciidae)

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Abstract

Cheracebus is a new genus of New World primate of the family Pitheciidae, subfamily Callicebinae. Until recently, *Cheracebus* was classified as the *torquatus* species group of the genus *Callicebus*. The genus *Cheracebus* has six species: *C. lucifer*, *C. lugens*, *C. regulus*, *C. medemi*, *C. torquatus*, and *C. purinus*, which are all endemic to the Amazon biome. Before the present study, there had been no conclusive interpretation of the phylogenetic relationships among most of the *Cheracebus* species. The present study tests the monophyly of the genus and investigates the relationships among the different *Cheracebus* species, based on DNA sequencing of 16 mitochondrial and nuclear markers. The phylogenetic analyses were based on Maximum Likelihood, Bayesian Inference, and multispecies coalescent approaches. The divergence times and genetic distances between the *Cheracebus* taxa were also estimated. The analyses confirmed the monophyly of the genus and a well-supported topology, with the following arrangement: ((*C. torquatus*, *C. lugens*), (*C. lucifer* (*C. purinus*, *C. regulus*))). A well-differentiated clade was also identified within part of the geographic range of *C. lugens*, which warrants further investigation to confirm its taxonomic status.

KEYWORDS

New World monkeys, phylogeny, taxonomy, titi monkeys

1 | INTRODUCTION

The titi monkeys are small- to medium-sized (adult bodyweight 1–2 kg) New World primates of the family Pitheciidae. The monophyly of this group was not recognized until the beginning of the 20th century, and the species have been allocated to a number of different genera, including *Callithrix* and *Saguinus* (see Hershkovitz, 1963). Thomas (1903) placed all the titis described up to that time in the genus *Callicebus*. Hershkovitz (1963) recognized two species, *Callicebus moloch*, with seven subspecies, and *Callicebus torquatus*, with three subspecies. Subsequently, following the analysis of a much larger number of specimens and geographic localities, Hershkovitz (1988, 1990) updated the diversity of the genus to 13 species and a total of 25 taxa. These species were arranged in four species groups based on their morphological similarities and geographic ranges (Table 1).

Kobayashi and Langguth (1999) accepted the species group approach of Hershkovitz (1988, 1990), but proposed an arrangement with five groups. This arrangement was followed by van Roosmalen, van Roosmalen, and Mittermeier (2002), who also considered all the

TABLE 1 Hypotheses for classification of titi monkeys

Hershkovitz (1988, 1990)	Kobayashi and Langguth (1999)	van Roosmalen et al. (2002)	Groves (2005)	Byrne et al. (2016)
			Subgenus Callicebus	
Callicebus donacophilus group	*Callicebus donacophilus group*	*Callicebus donacophilus group*	*Callicebus group*	*Genus Plecturocebus*
C. d. donacophilus	*C. modestus*	*C. modestus*	*C. donacophilus*	*P. modestus*
C. d. pallescens	*C. d. donacophilus*	*C. donacophilus*	*C. pallescens*	*P. donacophilus*
C. oenanthe	*C. d. pallescens*	*C. pallescens*	*C. oenanthe*	*P. pallescens*
C. olallae	*C. olallae*	*C. oenanthe*	*C. olallae*	*P. oenanthe*
		C. olallae		*P. olallae*
				P. moloch
Callicebus moloch group	*Callicebus moloch group*	*Callicebus moloch group*	*Callicebus moloch group*	*P. cinerascens*
C. moloch	*C. moloch*	*C. moloch*	*C. moloch*	*P. brunneus*
C. cinerascens	*C. cinerascens*	*C. cinerascens*	*C. cinerascens*	*P. hoffmannsi*
C. cupreus cupreus	*C. brunneus*	*C. brunneus*	*C. brunneus*	*P. baptista*
C. c. discolor	*C. hoffmannsi hoffmannsi*	*C. hoffmannsi*	*C. hoffmannsi*	*P. bernhardi*
C. c. ornatos	*C. h. baptista*	*C. baptista*	*C. baptista*	*P. cupreus*
C. caligatus		*C. bernhardi*	*C. bernhardi*	*P. caligatus*
C. brunneus				*P. discolor*
C. hoffmannsi hoffmannsi	*Callicebus cupreus group*	*Callicebus cupreus group*	*Callicebus cupreus group*	*P. ornatos*
C. h. baptista	*C. c. cupreus*	*C. cupreus*	*C. cupreus*	*P. dubius*
C. dubius	*C. c. discolor*	*C. caligatus*	*C. caligatus*	*P. stephennashi*
C. personatus personatus	*C. ornatos*	*C. discolor*	*C. discolor*	*P. aureipalatii*
C. p. melanochir		*C. ornatos*	*C. ornatos*	*P. toppini*
C. p. nigrifrons		*C. dubius*	*C. dubius*	*P. urubambensis*
C. p. barbarabrownae		*C. stephennashi*	*C. stephennashi*	*P. miltoni*
Callicebus modestus group			*Callicebus modestus group*	*P. vieirai*
C. modestus			*C. modestus*	*P. caquetensis*
	Callicebus personatus group	*Callicebus personatus group*	*Callicebus personatus group*	*Genus Callicebus*
	C. personatus	*C. personatus*	*C. personatus*	*C. personatus*
	C. melanochir	*C. melanochir*	*C. melanochir*	*C. melanochir*
	C. nigrifrons	*C. nigrifrons*	*C. nigrifrons*	*C. nigrifrons*
	C. barbarabrownae	*C. barbarabrownae*	*C. barbarabrownae*	*C. barbarabrownae*
	C. coimbrai	*C. coimbrai*	*C. coimbrai*	*C. coimbrai*
			Subgenus Torquatus	
Callicebus torquatus group	*Callicebus torquatus group*	*Callicebus torquatus group*	*Callicebus torquatus group*	*Genus Cheracebus*
C. t. torquatus	*C. t. torquatus*	*C. torquatus*	*C. torquatus*	*C. torquatus*
C. t. lugens	*C. t. lugens*	*C. lugens*	*C. lugens*	*C. lugens*
C. t. lucifer	*C. t. lucifer*	*C. lucifer*	*C. lucifer*	*C. lucifer*
C. t. purinus	*C. t. purinus*	*C. purinus*	*C. purinus*	*C. purinus*
C. t. regulus	*C. t. regulus*	*C. regulus*	*C. regulus*	*C. regulus*
C. t. medemi	*C. t. medemi*	*C. medemi*	*C. medemi*	*C. medemi*

subspecies to be valid species. Groves (2005) subsequently proposed the division of *Callicebus* into two subgenera, one of which, *Torquatus*, included the species of the *torquatus* group, with all the other species being allocated to the subgenus *Callicebus*. This arrangement was followed by Silva Júnior (2013). Recently, Byrne et al. (2016) proposed the division of *Callicebus* into three genera, based primarily on divergence times, including two new genera, given the lack of available nomina. The two new genera were designated *Plecturocebus* (composed of the species of the *donacophilus*, *cupreus*, and *moloch* species groups) and *Cheracebus* (composed of the species of the *torquatus* group). The species of the *personatus* group remained in the genus *Callicebus*. The classification proposed by Byrne et al. (2016) was adopted in the present study.

A variety of taxonomic arrangements have been proposed for the titi monkeys since the middle of the 20th century, although the same six taxa comprised the *torquatus* species group of Hershkovitz (1988, 1990), the *Torquatus* subgenus of Groves (2005), and the genus *Cheracebus* of Byrne et al. (2016). These taxa are denominated here as *Cheracebus torquatus* (Hoffmannsegg, 1807), *Cheracebus purinus* (Thomas, 1927), *Cheracebus lucifer* (Thomas, 1914), *Cheracebus lugens* (Humboldt, 1811), *Cheracebus regulus* (Thomas, 1927), and *Cheracebus medemi* (Hershkovitz, 1963). The one exception has been

the proposal of Kobayashi (1995), based on a geometric morphometric analysis, which placed *C. purinus* in the *personatus* species group, the current genus *Callicebus*.

Cheracebus is endemic to the Amazon region, and the species are assumed to have an allopatric distribution, with species ranges separated by major rivers (Figure 1). The exact limits between the ranges of some species are still unclear, primarily due to the sampling deficiencies of many areas, as in the case of *C. lucifer* and *C. medemi*, both of which occur between the Japurá/Solimões and Caquetá/Aguarico rivers, and are not separated by any obvious physical barrier. There are also a number of discrepancies on the distributions of *C. torquatus* and *C. lugens*. Hershkovitz (1990) suggested that a sympatric zone exists between these two species, while van Roosmalen et al. (2002) concluded that *C. lugens* occupies an extensive area to the north of the Branco River, including the basins of the Branco and Orinoco rivers, and a number of other, smaller rivers, whereas *C. torquatus* is restricted to the area between the Japurá and Negro rivers. However, Casado, Bonvicino, and Seuanez (2006) proposed that *C. lugens* occurs on both margins of the Negro River, in agreement with Hershkovitz (1990).

The present study tested the monophyly of the genus *Cheracebus* and proposes a first phylogeny of the species of the genus based on DNA sequencing of mitochondrial and nuclear markers.

2 | MATERIALS AND METHODS

2.1 | Samples, extraction, amplification, and sequencing of the DNA

Samples of blood and muscle tissue were obtained from 26 pitheciid specimens, including 17 representatives of five of the six *Cheracebus* species (one *C. torquatus*, six *C. lugens*, three *C. purinus*, three *C. lucifer*, four *C. regulus*, three *Plecturocebus*, three *Callicebus*, one *Chiropotes*, one *Cacajao*, and one *Pithecia*). No samples of *Cheracebus medemi* could be obtained for analysis in the present study. The samples (Table 2 and Figure 1) were identified based on the morphological traits of the specimens, which were compared with the published descriptions of the respective species. The samples were provided by five Brazilian institutions, the National Institute of Amazonian Research (INPA) and the Federal University of Amazonas (UFAM) in

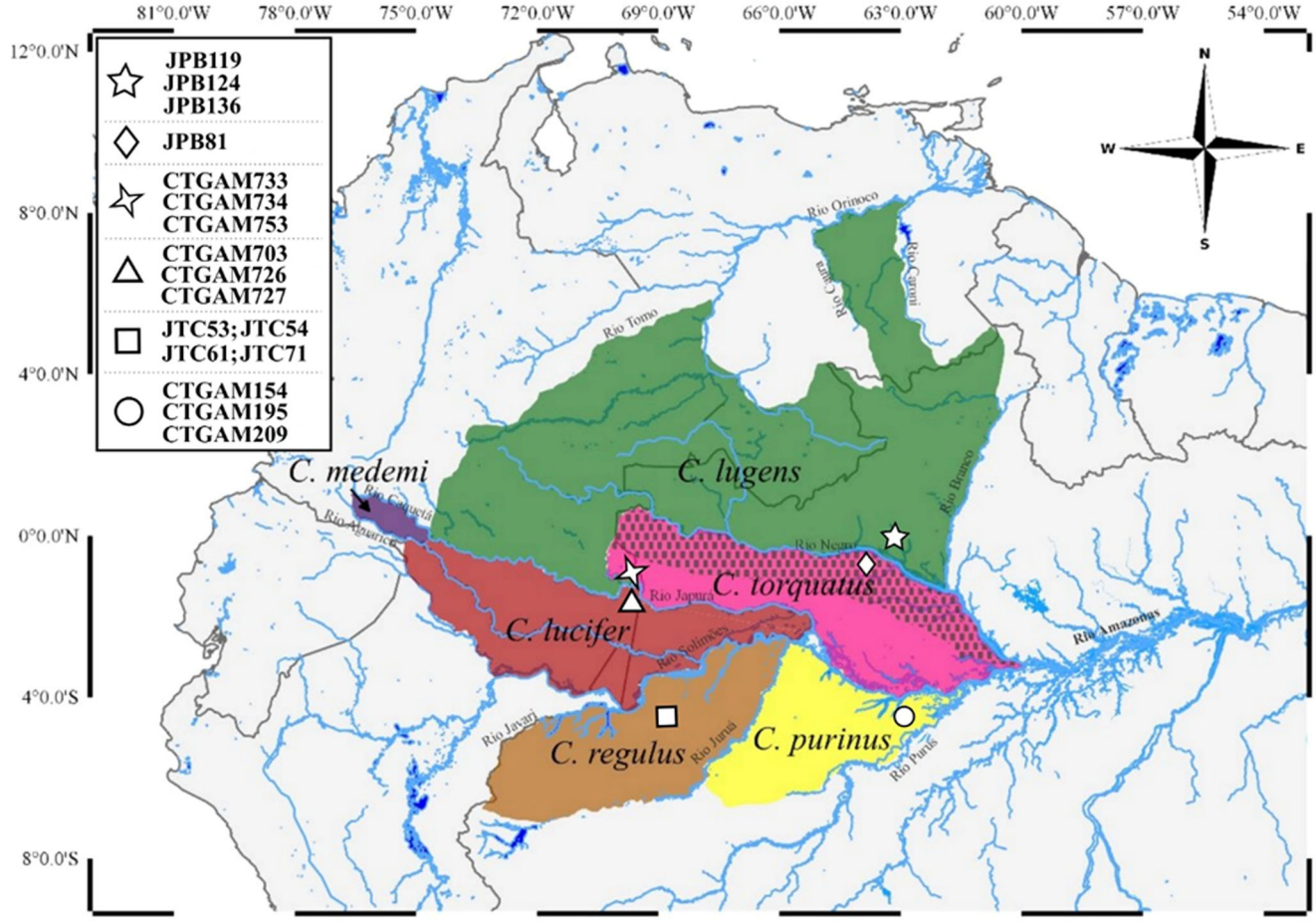

FIGURE 1 Distribution map of *Cheracebus* species (Hershkovitz, 1990; van Roosmalen et al., 2002). Dotted region represents a possible zone of sympathy between *C. lugens* and *C. torquatus* species

TABLE 2 Samples used in the present study and their respective codes, origins, and locations

Specie	Code	Origin	Locality
Cheracebus torquatus	JPB81	INPA	Mandiquie, right bank of Negro River, Amazonas, Brazil
Cheracebus lugens	JPB119	INPA	Marari, left bank of Negro River, Amazonas, Brazil
C. lugens	JPB124	INPA	Igarapé Anta, left bank of Negro River, Amazonas, Brazil
C. lugens	JPB136	INPA	Igarapé Cuieiras, left bank of Negro River, Amazonas, Brazil
C. lugens	CTGAM733	UFAM	Left bank of Japurá River, Amazonas, Brazil
C. lugens	CTGAM734	UFAM	Left bank of Japurá River, Amazonas, Brazil
C. lugens	CTGAM753	UFAM	Left bank of Japurá River, Amazonas, Brazil
Cheracebus purinus	CTGAM154	UFAM	Rebio Abufari, left bank of Purus River, Amazonas, Brazil
C. purinus	CTGAM195	UFAM	Rebio Abufari, left bank of Purus River, Amazonas, Brazil
C. purinus	CTGAM209	UFAM	Rebio Abufari, left bank of Purus River, Amazonas, Brazil
Cheracebus lucifer	CTGAM703	UFAM	Right bank of Japurá River, Amazonas, Brazil
C. lucifer	CTGAM726	UFAM	Right bank of Japurá River, Amazonas, Brazil
C. lucifer	CTGAM727	UFAM	Right bank of Japurá River, Amazonas, Brazil
Cheracebus regulus	JT053	IDSM	Right bank of Jutaí River, Amazonas, Brazil
C. regulus	JT054	IDSM	Right bank of Jutaí River, Amazonas, Brazil
C. regulus	JT061	IDSM	Right bank of Jutaí River, Amazonas, Brazil
C. regulus	JT071	IDSM	Right bank of Jutaí River, Amazonas, Brazil
Plecturocebus moloch	Cmo 1690	UFPA	Left bank of Tocantins River, Amazonas, Brazil
Plecturocebus brunneus	Cbr 2220	UFPA	Right bank of Jamari River, Rondonia, Brazil
Plecturocebus cupreus	Ccu 4986	UFPA	Left bank of Madeira River, Amazonas, Brazil
Callicebus melanochir	Melan 2329	CNRJ	Eunápolis, Bahia, Brazil
Callicebus personatus	Perso 2466	CNRJ	Aracruz, Espirito Santo, Brazil
Callicebus nigrifrons	04	PUC	Minas Gerais, Brazil
Chiropotes utahicki	Cs970	UFPA	Left bank of Tocantins River, Pará, Brazil
Cacajao ayresi	CTGAM5666	UFAM	Aracá River, left bank of Negro River, Amazonas, Brazil
Pithecia pithecia	Pit 22	UFPA	Left bank of Jari River, Amapá, Brazil

Manaus, the Mamirauá Institute of Sustainable Development (IDSM) in Tefé, the Rio de Janeiro Primatology Center (CPRJ), the Pontifical Catholic University of Minas Gerais (PUC) in Belo Horizonte, and the Federal University of Pará (UFPA) in Belém.

2.2 | Ethics statement

All stages of the experiments and fieldwork were carried out in accordance with Brazilian laws about primate research as well as the rules established by the American Society of Primatologists in relation to the ethical treatment of primates. Research permits were granted by Brazilian authorities (FUNAI and IBAMA/ICMBio), and by institutional IACUC committees. The licenses to fieldwork and collection of tissue samples were provided by IBAMA (License No. 005/2005—CGFAU/LIC) and ICMBio (40217-1 and 5135-1).

Total genomic DNA was extracted using Promega's Wizard Genomic kit, according to the manufacturer's protocol, and 16 molecular markers were amplified by polymerase chain reaction (PCR; Table 3). These markers included three fragments of the mitochondrial DNA—*Cytochrome oxidase subunit I* (COI), *Cytochrome b* (Cytb), and the ribosomal 16S gene (16S)—and 13 nuclear markers, RAG1, SIM, ZFX, and 10 *Alu* elements together with their flanking regions. The PCRs were standardized to a final volume of 15 µl; containing ~30 ng of genomic DNA; 2.4 µl of dNTPs (1.25 mM); 1.5 µl of 10× buffer (200 mM Tris-HCl, 500 mM KCl); 1 µl of $MgCl_2$ (25 mM); 1 µl of each primer (0.2 µM); and 1 U of Taq DNA polymerase. With the exception of the primer annealing temperatures, all other steps of the amplification protocol were identical for all the markers. The thermocycler was programmed for the following schedule: initial denaturation at 9°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 40 s, and extension at 72°C for 40 s, followed by a final

TABLE 3 Molecular markers used in this study, with their annealing temperatures and references

Molecular markers	Primer forward	Primer reverse	Annealing temperature (°C)	Reference
Mitochondrial				
16S	5′-TGGACTATGAGTTGAGCAGAC-3′	5′-TATGCTAATTACTCTTCTTGGGC-3′	58	Palumbi, Martin, and Romano (1991)
COI	5′-TCCATTACCAGGCCAGCTAG-3′	5′-GAACTTGCTGGCTTTCATATC-3′	45	Ward, Zemlak, Innes, Last, and Hebert (2005)
CYTb	5′-GCACCTACCCACGAAAAGAA-3′	5′-ACATTGCCTCTGCAAATTGA-3′	60	Carneiro et al. (2016)
Nuclear				
Pith_Alu1D_24	5′-AAGCCATAACTCCATTACCAAA-3′	5′-AGATTCTGGTCCCAAGTCCA-3′	60	Batzer (2005)
Pith_Alu1D_26	5′-GTTTCATGAGGGCAGAACCT-3′	5′-TCTGCACTTTGCAGCTGTTT-3′	60	Batzer (2005)
Pith_Alu1D_27	5′-AACCACATTTTGACTGTATGCTG-3′	5′-CCCTTCAATGACTCCCTTCA-3′	57	Batzer (2005)
Pith_Alu1D_30	5′-CATGGGACATGCACTTTTTG-3′	5′-AACAYCTTYCATCAACCTYTGAA-3′	61	Batzer (2005)
Titi_1DF2_39	5′-AACAGAGTTGGCCGTTCATCT-3′	5′-GTCCTGTTCAAGTCAGCTACGTTG-3′	54	Batzer (2005)
Pith_Alu1D_84	5′-CTGCTACGTCAGACGTCGTAC-3′	5′-CTGCTAGCACAAGCTAGTCGA-3′	62	Batzer (2005)
Pitheciidae2	5′-CAGCCAAAGGAGTGCTTCAC-3′	5′-CTAAATGGTGYCCCATAAGG-3′	58	Osterholz, Walter, and Roos (2009)
Pitheciidae3	5′-CGGGGGCCTGATTACTAAAA-3′	5′-ACCAAAYATAGGCCTCRAATT-3′	53	Osterholz et al. (2009)
Pitheciidae4	5′-GCTGGACTATTCCTTGCCATC-3′	5′-CAGGCATCCTGTTTGGAATTA-3′	56	Osterholz et al. (2009)
DENND5A1	5′-CCAGAGTTATCATGGCCAATC-3′	5′-GTACCAAGCAAGAAGCTGGG-3′	62	Perelman et al. (2011)
SIM1	5′-GACCTACCGCAGAAAATTCG-3′	5′-CTGGGGCTCATCATTCATTC-3′	60	Perelman et al. (2011)
ZFX	5′-TGGAATGAAATCCCTCAAATA-3′	5′-ATGTCCATCAGGGCCAATAAT-3′	52	Perelman et al. (2011)
RAG1	5′-GCTTTGATGGACATGGAAGAAGACAT-3′	5′-GAGCCATCCCTCTCAATAATTTCAGG-3′	47	Teeling et al. (2000)

extension at 72°C for 5 min. The PCR products were purified with polyethylene glycol (PEG) and ethanol. The sequence reactions were performed using the Big Dye kit (Applied Biosystems), and the samples were resolved on an ABI 3500xL automatic sequencer (Applied Biosystems). The access numbers on GenBank of the sequences produced in the present study are available in Table S1.

2.3 | Alignment of the sequences, evolutionary models, phylogenetic analyses, and divergence times

The DNA sequences were aligned in ClustalW (Thompson, Higgins, & Gibson, 1994) and edited manually in BioEdit v. 7.2.5 (Hall, 1999). The outgroup was composed of samples of the five remaining pitheciid genera, *Callicebus*, *Plecturocebus*, *Pithecia*, *Cacajao*, and *Chiropotes*. PartitionFinder v.2 (Lanfear, Frandsen, Wright, Senfeld, & Calcott, 2016) was used to identify the best data partitioning scheme and evolutionary models. We used the greedy algorithm (Lanfear, Calcott, Simon, & Guindon, 2012), the Bayesian information criterion (BIC), and protein-coding regions were partitioned by position of the bases in the codons. Analyses were performed for all concatenated markers, only nuclear regions, mitochondrial regions, and each individual molecular marker. The data partitioning schemes and their respective evolutionary models can be viewed in Table S2.

The phylogenetic analyses were based on the Maximum Likelihood (ML), Bayesian Inference (BI), and coalescent approaches. The ML analysis was run in RAxML v.8 (Stamatakis, 2014). The ML trees were found by 1,000 searches followed by 1,000 bootstrap pseudoreplicates. The BI was run in MrBayes v.3.2.1 (Ronquist & Huelsenbeck, 2003) with two independent Markov chain Monte Carlo (MCMC) runs, one cold and three hot, with 500,000 generations, and trees and parameters sampled every 5,000 generations. The first 20% of the runs were discarded as burn-in. The species tree with a multispecies coalescent model was estimated with ASTRAL III (Zang, Rabiee, Sayyari, & Mirarab, 2018). ASTRAL uses non-rooted gene trees as the input file. We use the trees of the individual loci estimated in RaxML.

The percentage of genetic divergence between taxa was estimated with MEGA v.6 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). We perform genetic distance analyzes for all concatenated molecular markers, and for mitochondrial and nuclear data separately. We use K2P for all analyzes of genetic distance.

Divergence times were estimated in BEAST v.1.8.3 (Drummond, Suchard, Xie, & Rambaut, 2012), using two calibration points: (a) the *Cacajao–Chiropotes* separation, estimated at 6.7 ± 2.3 million years ago (Ma; Kiesling, Soojin, Xu, Sperone, & Wildman, 2015); (b) a pitheciine fossil, *Nuciruptor rubricae* (Meldrum & Kay, 1997) dated to 12.4–12.8 Ma, used in the node that groups *Pithecia*, *Chiropotes*, and *Cacajao*. Evolutionary models were assigned to each molecular marker, following PartitionFinder. An uncorrelated relaxed clock was applied to the branch lengths, and a Yule model was applied as the prior for the tree. The analyses were based on three independent runs, and the log parameters and trees were summarized in LogCombiner

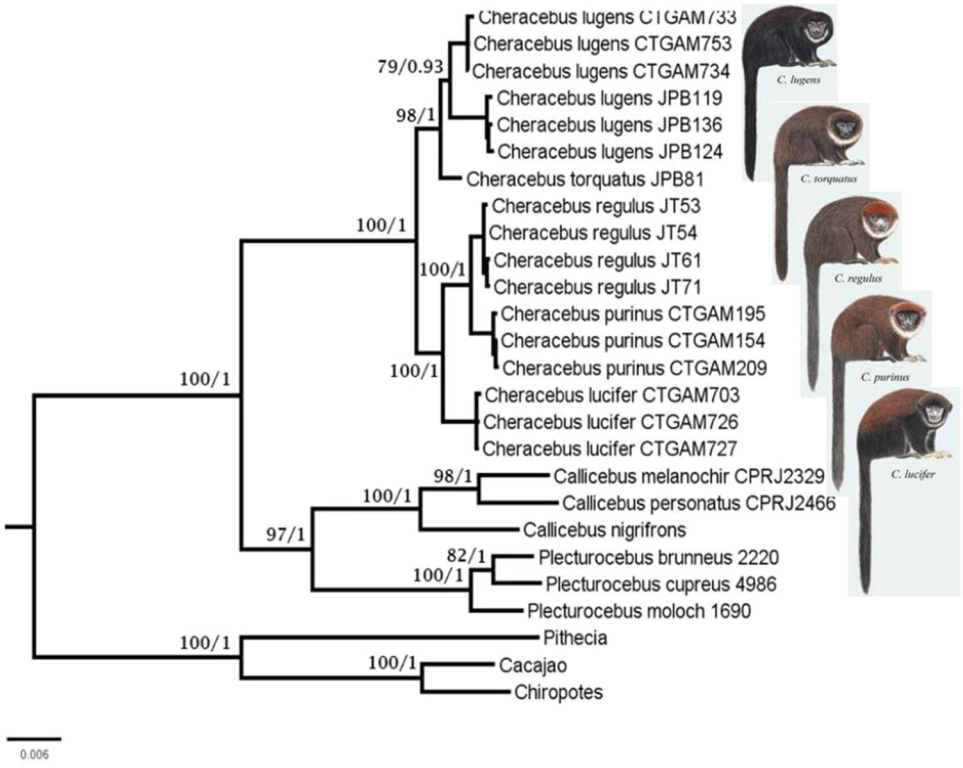

FIGURE 2 Distribution map of the *Cheracebus* genus species with the respective sample collection locations used in the present study. Details about the codes are available in Table 2

v.1.8.3 and TreeAnnotator v.1.8.3 (Drummond et al., 2013), respectively. The convergence of the runs was evaluated in Tracer v.1.6 (Rambaut, Suchard, Xie, & Drummond, 2014), and an effective sample size (ESS) of over 200 was considered to be satisfactory.

3 | RESULTS

The 16 concatenated markers (nuclear and mitochondrial) provided a database of 9,427 bps, 2,181 bps from the mitochondrial sequences, and 7,246 bps from the nuclear sequences. Overall, approximately 16% of the data are missing due to problems encountered in the amplification of the markers in all the samples.

The ML and BI had the same topology, both with maximum support values (bootstraps or posterior probabilities) for most of the nodes (Figure 2). This analysis separates the titis into three main clades, as suggested by Byrne et al. (2016), with *Cheracebus* as the sister taxon of the clade composed of *Callicebus* and *Plecturocebus*.

Two well-supported clades were also identified within the genus *Cheracebus*, one which included *C. lugens* and *C. torquatus*, and the other formed by *C. regulus*, *C. purinus*, and *C. lucifer*. In this latter clade, *C. lucifer* was recuperated as the sister species of the clade formed by *C. regulus* and *C. purinus*. All species were reciprocally monophyletic, and all the relationships within the genus *Cheracebus* were strongly supported. The phylogenetic analysis under the multispecies coalescent model (Figure 3) recovered the

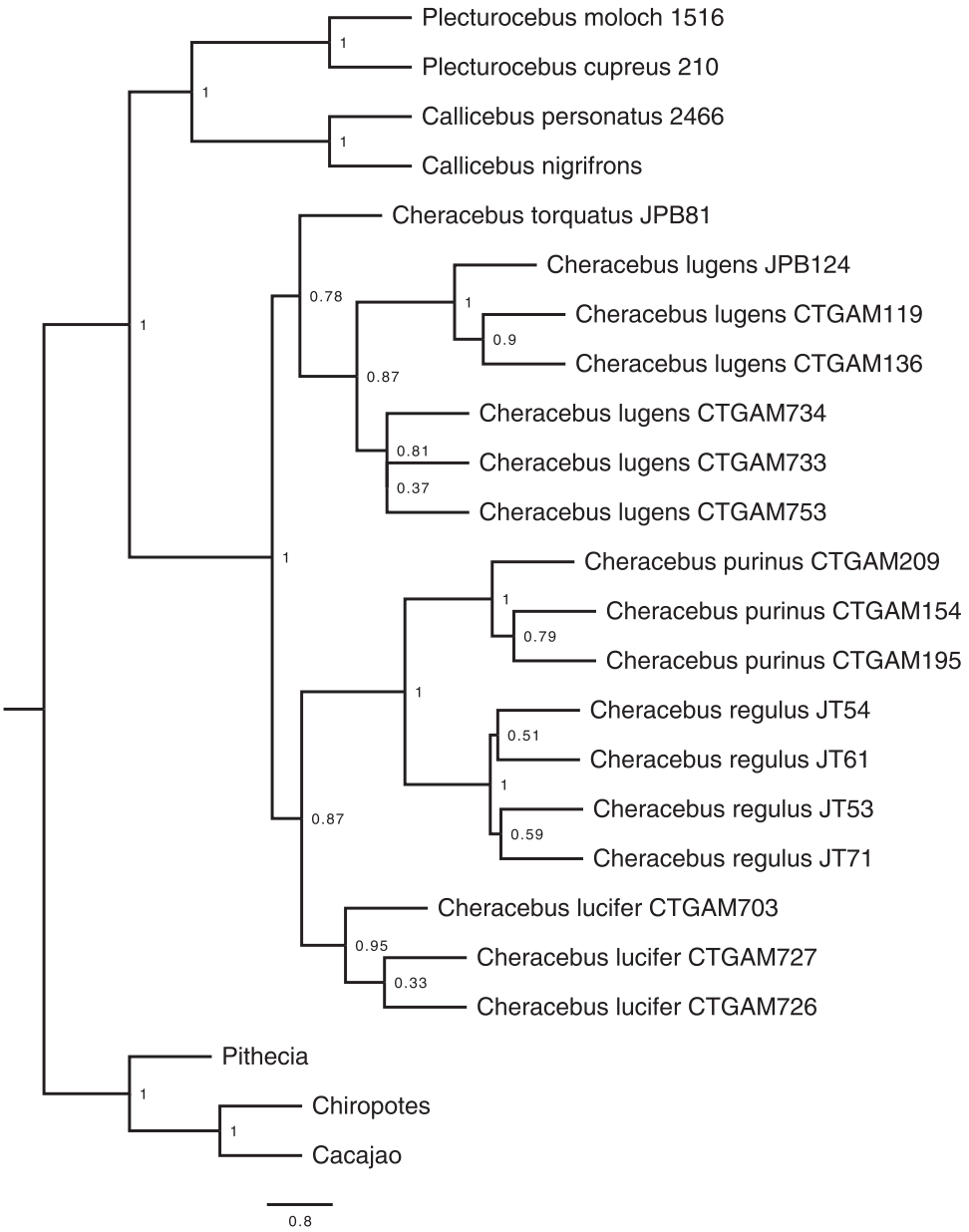

FIGURE 3 Phylogenetic relationships of taxa of the Pitheciidae family. Numbers near nodes refer to bootstrap (left) and posterior probability (right) values

same topology as probabilistic methods (ML and IB), also with most of the nodes strongly supported. We obtained incongruence in the phylogenetic position of *C. torquatus* when analyzing the mitochondrial and nuclear data separately (Figure S1). Only mitochondrial data group *C. torquatus* within of *C. lugens*, with 60% of bootstrap, making *C. lugens* paraphyletic. In contrast, only nuclear markers position *C. torquatus* as sister to other species of the genus *Cheracebus*.

All the concatenated molecular markers have genetic distances of approximately 13% separating the three titi genera, *Cheracebus*, *Plecturocebus*, and *Callicebus* (Table 4), whereas the mean genetic distance between *Cheracebus* species was 2.45%. The distances ranged from 0.9% between *C. regulus* and *C. purinus* to 4% between *C. lugens* and *C. purinus*. The *C. lugens* specimens from opposite margins of the Negro River were separated by a genetic distance of 1.47%, a value similar to that recorded between the two species (*C. lugens* and *C. torquatus*) in this clade. We also analyze genetic distances separately using only mitochondrial and nuclear data. Mitochondrial data had an average genetic distance 5.17 times greater than nuclear data (Tables S3 and S4).

The estimates of divergence times indicated that the present-day pitheciids began to diversify approximately 19.22 Ma, with a 95% highest posterior densities (HPD) range of 15.95–22.49 Ma (Figure 4). It is interesting to note that the estimated timing of the first diversification within the pitheciines (13.58 Ma; 95% HPD: 11.83–15.33 Ma) is virtually the same as that of the first diversification within the callicebines, given that the three lineages of the current genera *Cheracebus*, *Plecturocebus*, and *Callicebus* were already separated by 13.15 (95% HPD: 10.13–17.69 Ma). The current *Cheracebus* species diversified only during the Pliocene, at around 3.92 Ma (95% HPD: 2.97–4.87 Ma). *Cheracebus regulus* and *C. purinus* are the species that diverged most recently, of only 1.93 Ma (95% HPD: 1.38–2.48 Ma).

4 | DISCUSSION

Until recently, the titi monkeys were classified into five species groups within the genus *Callicebus*, although Byrne et al. (2016) proposed a new arrangement, in which the taxon was divided into three genera, *Cheracebus*, *Plecturocebus*, and *Callicebus*. The results of the analyses presented here provide further, conclusive support for this arrangement. The genetic distances between these lineages are comparable with those found between the other pitheciid genera, and appear to be consistent with the timing of the separation of the three genera, in the mid-Miocene (~10 Ma). In fact, the morphological differences among the three callicebines are smaller than those among the three pitheciines. Even so, the DNA sequences support the recognition of the six pitheciid genera conclusively.

Despite the lack of *C. medemi* samples, all the *Cheracebus* species were recovered as monophyletic groups in the present analysis, which is consistent with morphological data (Groves, 2005; Hershkovitz, 1988, 1990; Kobayashi & Langguth, 1999; van Roosmalen et al., 2002). The data on the phylogenetic relationships among the *Cheracebus* species point to an initial dichotomy between the *C. lugens*/*C. torquatus* and *C. lucifer*/*C. purinus*/*C. regulus* clades, which are found exclusively on opposite margins of the Amazon River. *Cheracebus lugens* and *C. torquatus* occur on the northern margin of the Amazon (Solimões) River, while the other clade is found on the southern margin.

The present estimates of divergence times indicate that these two clades separated at approximately 3.9 Ma. The current drainage system of the Amazon basin may have formed around 3 Ma (Ribas et al., 2012), although Hoorn et al. (2010) proposed a date of approximately 7 Ma. Whether or not the formation of the Amazon River caused the separation of the two *Cheracebus* clades, it was almost certainly in place by at least 3 Ma, and would have contributed to their genetic isolation.

TABLE 4 Genetic distance between species of the genus *Cheracebus* and taxa of the family Pitheciidae

		1	2	3	4	5	6	7	8	9	10
1	*Cheracebus lugens*[a]										
2	*C. lugens*[b]	1.47									
3	*Cheracebus torquatus*	1.67	1.73								
4	*Cheracebus regulus*	2.80	3.27	2.67							
5	*Cheracebus purinus*	3.39	4.00	3.38	0.97						
6	*Cheracebus lucifer*	3.59	3.79	3.18	2.01	2.92					
7	*Plecturocebus*	13.7	13.3	12.6	13.1	13.9	13.2				
8	*Callicebus*	12.6	12.4	12.3	12.7	13.3	12.9	13.0			
9	*Chiropotes*	22.4	22.3	21.6	22.1	22.6	22.7	21.8	22.4		
10	*Cacajao*	21.1	20.9	20.3	20.8	21.3	21.4	22.0	21.1	12.7	
11	*Pithecia*	27.6	27.4	25.3	25.2	24.9	26.7	25.7	25.9	17.9	16.2

[a]Left bank of the Negro River.
[b]Right bank of the Negro River.

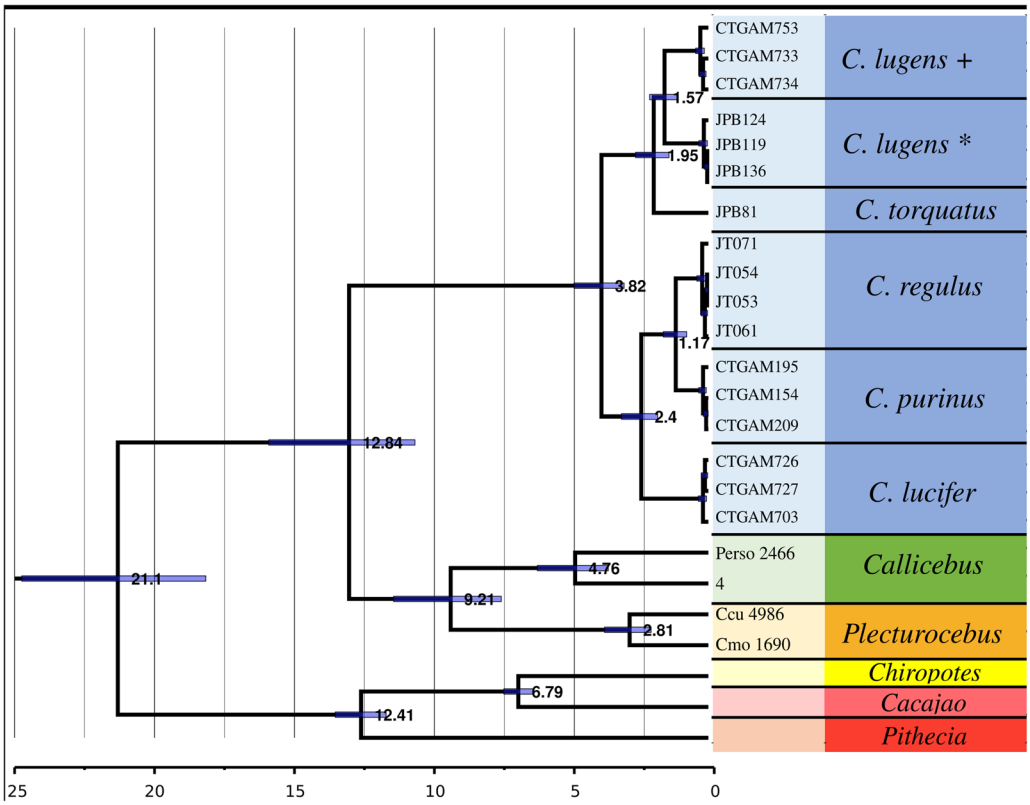

FIGURE 4 Estimated divergence time of Pitheciidae taxa. Each genus has a color: blue: *Cheracebus*, green: *Callicebus*, orange: *Plecturocebus*, yellow: *Chiropotes*, pink: *Cacajao*, and red: *Pithecia*. *Highlights clade of *Cheracebus lugens* on the left bank of the Negro River, while + indicates samples collected on the right bank of this river. Numbers near node represent divergence time

Our phylogenetic analyses with separate mitochondrial and nuclear data were conflicting for the positioning of *C. torquatus*. We believe that such disagreement occurred because the only sample of *C. torquatus* (JPB81) is not present in some of the nuclear marker. Additionally, the separation of *C. torquatus* and *C. lugens* occurred approximately 2 Ma, and recent cladogenesis events are more easily recovered from mitochondrial data than from nuclear genes (Lamarca & Schrago, 2020), due, on average, to the rate of mutation of mitochondrial regions being accelerated.

Cheracebus lugens is the species with the largest geographic distribution of any *Cheracebus* species, although the present analysis identified two clades with a genetic distance of 1.4%, a value greater than that found between some pairs of recognized species, such as *C. regulus* and *C. purinus*, which are separated by a distance of 0.9%. Based on this parameter alone, the data suggest the existence of two valid species within *C. lugens*, although this inference may be premature, given that many species, even well-defined ones, may present intraspecific genetic divergences derived from distinct mutation rates and/or patterns of genetic drift. Furthermore, this genetic distance may be related to the large geographic distance between the samples, and it is possible that the analysis of a broader sample, including additional localities, may reveal a more intermediate genetic distance. Further research will be needed to resolve this question.

5 | CONCLUSIONS

The present study is the first to test the monophyly of the genus *Cheracebus* systematically, and define interspecific phylogenetic relationships based on DNA sequences. The results of the study clearly support the monophyly of *Cheracebus*. However, the phylogenetic position of *C. medemi* remains unclear. This species has a restricted geographic distribution in the Caquetá and Putumayo departments of Colombia. The phylogenetic reconstruction indicated that the initial diversification of the extant species led to the formation of two reciprocaly monophyletic groups on opposite margins of the Amazon River at around 4 Ma. The origin of the clades may thus be associated with the formation of the Amazon drainage system. As the divergence of *Cheracebus* from the other callicebine genera occurred at approximately 13 Ma, this lineage either remained stable (with no speciation) for around 9 Ma or the forms derived from the speciation processes that occurred during this period are now extinct, and may only exist in fossil form. The two clades of *C. lugens* identified in the present study, based on their accentuated genetic distance, indicate the existence of a new, as yet unidentified species of *Cheracebus*. However, confirmation of this hypothesis will require further genetic and morphological samples from the geographic range of *C. lugens*.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

J. C. conceived of the study, participated in the data analyses and drafted the manuscript; I. S. designed the study, provided samples; T. L. carried out the molecular laboratory work and drafted the manuscript, J. S. S. J. provided input on the manuscript, and revised the text; J. B., I. F., T. H., and J. V. provided samples and revised the manuscript; H. S. provided samples, and participated in the data analyses and the final revision of manuscript. All authors have approved the final version of the manuscript for publication.

OPEN RESEARCH BADGES

This article has earned an Open Data badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is available at https://www.ncbi.nlm.nih.gov/.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are openly available. The GenBank access numbers for the strings produced can be found in Table S1.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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